Supporting Information

(*R*)-2-Phenylpyrrolidine Substituted Imidazopyridazines: a New Class of Potent and Selective Pan-TRK Inhibitors

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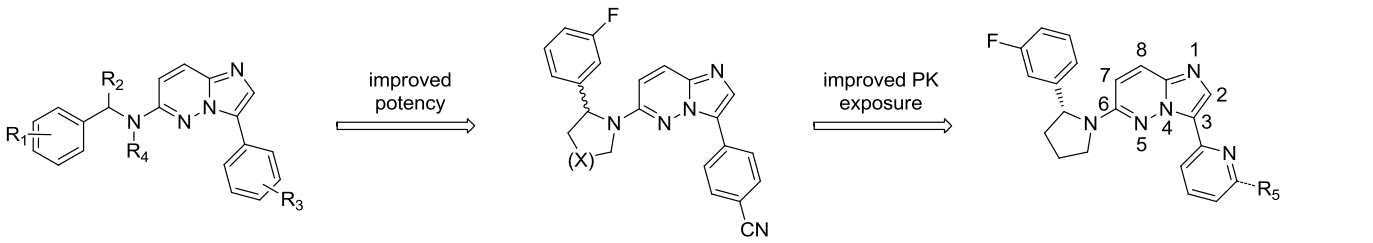
Experimental procedures

Material and Methods

Commercially available starting materials were used as supplied without further purification. Reactions were carried out using dry organic solvents (DCM, ACN, DMF, etc.) unless otherwise noted. Reactions were monitored using thin layer chromatography and an Agilent Technologies 1200 series 6140 Quadruploe LCMS with UV detection at 254 nm in electrospray ionization (ESI) mode. Normal phase chromatography was performed on a CombiFlash Companion (Teledyne Isco) with RediSep normal phase silica gel columns and UV detection at 254 nm unless otherwise noted. Preparative reversed-phase HPLC/MS was performed on an HPLC coupled to a single quadrupole mass spectrometer. The HPLC/MS consisted of a Waters

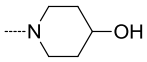
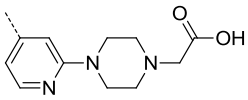
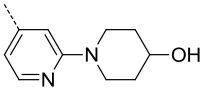
Acquity uPLC system (Waters Corp., Milford, MA) and a Waters 3100 mass spectrometer (Waters Corp., Milford, MA). The diode array detector was configured to collect data between 214 nm and 400 nm at 20 Hz. The HPLC column used was a Acquity UPLC^{TM} HSS T3 C18, 50 x 2.1 mm ID, 1.8 µm, part number 186003538 (Waters Corp). Eluent A was 0.1% (v/v) TFA in water. Eluent B was 0.1% (v/v) TFA in acetonitrile. All NMR spectra were recorded on a Bruker AVANCE-400 spectrometer operating at a frequency of 400.13 MHz for ^{1}H and 100.61 MHz for ^{13}C equipped with a 5mm QNP cryoprobe with Z-gradient. Chemical shifts for ^{1}H and ^{13}C spectra were referenced to residual solvent. MS were obtained on an Agilent Technologies 1200 series 6140 Quadruploe LCMS in electrospray ionization (ESI) mode. HRMS-ESI data were recorded using an Agilent 6520 Accurate-Mass Q-TOF LC/MS system with HPLC-Chip Cube interface and an Agilent 1200 HPLC. All final compounds were isolated analytically pure, >95% purity by HPLC unless otherwise indicated. Elemental combustion analysis was performed by Midwest Microlab, LLC, Indianapolis, IN.

Imidazopyridazines SAR Progression and Numbering Assignment



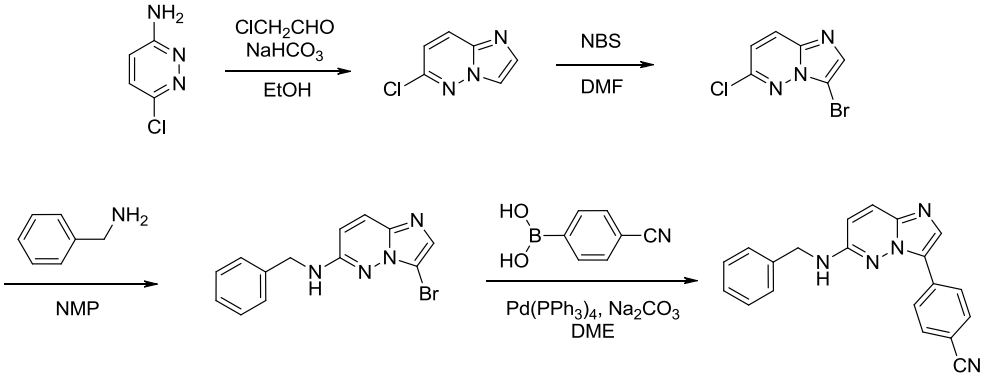
1: R_1 = H; R_2 = H; R_3 = 4-CN; R_4 = H
2: R_1 = H; R_2 = H; R_3 = 3-CN; R_4 = H
3: R_1 = 3-F; R_2 = H; R_3 = 4-CN; R_4 = H
4: R_1 = 2-OMe; R_2 = H; R_3 = 4-CN; R_4 = H
5: R_1 = 3-F; R_2 = H; R_3 = 4-CH2OH; R_4 = H
6: R_1 = 3-F; R_2 = H; R_3 = N-methyltetrahydro-2H-pyran-4-carboxamide; R_4 = H
7: R_1 = 3-F; R_2 = H; R_3 = 4-SO2NH2; R_4 = H
8: R_1 = H; R_2 = H; R_3 = 4-CN; R_4 = Me

9: (racemic) X = -CH_2CH_2-
10: (racemic) X = -OCH_2-
11: (racemic) X = -CH_2-
12: (*S*) X = -CH_2-
13: (*R*) X = -CH_2-

14: (racemic) R_5 = H
15: R_5 = 
16: R_5 = 
17: R_5 = 

General experimental procedure for the synthesis of compounds (1 – 8) is exemplified by compound 1.

Synthesis of compound (1)



6-Chloro-imidazo[1,2-b]pyridazine

A well stirred mixture of 6-chloro-pyridazin-3-ylamine (25.43 g, 196 mmol), $NaHCO_3$ (28 g, 334 mmol) and chloracetaldehyde (55% in water, 126 ml, 880 mmol) in EtOH (600 mL) was heated at reflux for 14 hours. The dark brown reaction mixture was concentrated under vacuum and the resulting residue was dissolved in DCM and filtered through a *Isolute* pad. The solid was washed with DCN and the filtrate was evaporated to provide a dark brown oil. HCl (290 mL of a 2 M aq solution) and water (300 mL) were added to the residue and the resulting slurry was stirred at room temperature for 15 min then filtered through a *Isolute* pad and the filter cake was washed with water. The filtrate was extracted with Et_2O (3 x 200 ml), cooled in an ice bath and carefully neutralized by the addition of solid sodium hydroxide (24 g dissolved in water). The resulting slurry was extracted with Et_2O (6 x 300 mL) then the combined organic fractions were dried over Na_2SO_4, filtered, and evaporated to dryness to yield crude 6-chloro-imidazo[1,2-b]pyridazine (24.05 g, 157 mmol, 80%) as a brown solid, which was used in the next step without further purification. MS *m/z* 155.1 $[M+1]^+$.

3-Bromo-6-chloro-imidazo[1,2-b]pyridazine

A solution of crude 6-chloro-imidazo[1,2-b]pyridazine (24.05 g, 157 mmol) in DMF (240 mL) was cooled to 0 °C, treated with NBS (32.3 g, 172 mmol), then stirred at 0 °C for 30 min and at room temperature for 1 hour. The reaction mixture was poured into water (2.4 L) and the resulting precipitate was stirred for 30 min then filtered. The solid was washed with water and dried under high vacuum at 40 °C for 14 hours to yield crude 3-bromo-6-chloro-imidazo[1,2-b]pyridazine (29.72 g, 128 mmol, 82%) as a beige solid, which was used in the next step without further purification. MS *m/z* 233.9 $[M+1]^+$.

Benzyl-(3-bromo-imidazo[1,2-b]pyridazin-6-yl)-amine

In a sealed flask, a solution of 3-bromo-6-chloro-imidazo[1,2-b]pyridazine (1.0 g, 4.3 mmol) and benzylamine (2.8 ml, 25.9 mmol) in NMP (7.5 mL) was stirred at 150 °C for 14 hours. The reaction mixture was cooled to room temperature and poured into water (150 mL). The resulting precipitate was filtered, washed with water and dried under high vacuum for 14 hours at 40 °C to yield benzyl-(3-bromo-imidazo[1,2-b]pyridazin-6-yl)-amine (1.29 g, 4.28 mmol, 99%) as a beige solid, which was used in the next step without further purification. MS *m/z* 303.1 $[M+1]^+$.

4-(6-(Benzylamino)imidazo[1,2-b]pyridazin-3-yl)benzonitrile (1)

In a sealed tube, a mixture of benzyl-(3-bromo-imidazo[1,2-b]pyridazin-6-yl)-amine (50 mg, 0.17 mmol), 4-cyano-phenylboronic acid (26 mg, 0.20 mmol), tetrakis(triphenylphospine)palladium(0) (9.5 mg, 0.008 mmol) and Na_2CO_3 (2.0 M aq solution, 0.29 mL) in DME (0.50 mL) was heated at 150 °C for 20 min in a microwave oven. The reaction mixture was cooled to room temperature, filtered through a *Isolute* pad and the filter cake was washed with DCM. The filtrate was dried over Na_2SO_4, filtered, and concentrated to dryness. The resulting residue was purified by reverse phase prep-HPLC to give 4-(6-(benzylamino)imidazo[1,2-b]pyridazin-3-yl)benzonitrile (**1**) (19.4 mg, 28%) as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.28 (s, 1H), 8.20 (d, J = 8.3 Hz, 2H), 7.94 – 7.89 (m, 3H), 7.49 – 7.19 (m, 5H), 7.13 – 7.00 (m, 1H), 4.52 (d, J = 5.4 Hz, 2H). MS *m/z* 326.1 $[M+1]^+$.

3-[6-(Benzylamino)imidazo[1,2-b]pyridazin-3-yl]benzonitrile (2)

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.55 (s, 1H), 8.31 (d, J = 8.1 Hz, 1H), 8.27 (s, 1H), 8.15 (br s, 1H), 7.95 (d, J = 9.8 Hz, 1H), 7.82 (d, J = 7.8 Hz, 1H), 7.64 (t, J = 7.9 Hz, 1H), 7.44 (d, J = 7.5

Hz, 2H), 7.36 (t, J = 7.5 Hz, 2H), 7.25 (t, J = 7.5 Hz, 1H), 7.07 (d, J = 9.7 Hz, 1H), 4.53 (d, J = 5.4 Hz, 2H). MS *m/z* 326.1 $[M+1]^+$.

4-(6-{[(3-Fluorophenyl)methyl]amino}imidazo[1,2-b]pyridazin-3-yl)benzonitrile (3)

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.27 (s, 1H), 8.18 (d, J = 8.2 Hz, 2H), 8.12 (s, 1H), 7.95 (d, J = 9.9 Hz, 1H), 7.85 (d, J = 8.2 Hz, 2H), 7.44 (q, J = 7.3 Hz, 1H), 7.27 (dd, J = 15.1, 9.1 Hz, 2H), 7.07 (dd, J = 16.3, 8.9 Hz, 2H), 4.54 (d, J = 5.3 Hz, 2H). MS *m/z* 344.2 $[M+1]^+$.

4-(6-{[(2-Methoxyphenyl)methyl]amino}imidazo[1,2-b]pyridazin-3-yl)benzonitrile (4)

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.32 (d, J = 4.7 Hz, 1H), 8.20 (app d, J = 8.0 Hz, 2H), 7.99 – 7.90 (m, 1H), 7.84 (d, J = 8.0 Hz, 2H), 7.29 (t, J = 8.1 Hz, 2H), 7.13- 7.10 (m, 3H), 6.90 (t, J = 7.3 Hz, 1H), 4.47 (d, J = 5.1 Hz, 2H), 3.86 (s, 3H). MS *m/z* 356.2 $[M+1]^+$.

[4-(6-{[(3-Fluorophenyl)methyl]amino}imidazo[1,2-b]pyridazin-3-yl)phenyl]methanol (5)

^{1}H NMR (400 MHz, DMSO-d_6) δ 7.99 (s, 1H), 7.87- 7.84 (m, 3H), 7.40 (q, J = 7.5 Hz, 1H), 7.33 (d, J = 8.0 Hz, 2H), 7.24 (d, J = 7.6 Hz, 1H), 7.22 – 7.14 (m, 2H), 7.05 (d, J = 10.7 Hz, 1H), 6.97 (d, J = 9.8 Hz, 1H), 4.52 (s, 2H), 4.50 (app d, J = 6.1 Hz, 2H). MS *m/z* 349.2 $[M+1]^+$.

N-[3-(6-{[(3-Fluorophenyl)methyl]amino}imidazo[1,2-b]pyridazin-3-yl)phenyl]oxane-4-carboxamide (6)

^{1}H NMR (400 MHz, DMSO-d_6) δ 9.96 (s, 1H), 8.59 (s, 1H), 7.81 (d, J = 9.4 Hz, 2H), 7.70 (t, J = 5.8 Hz, 1H), 7.61 (d, J = 8.0 Hz, 1H), 7.41 (d, J = 8.3 Hz, 1H), 7.32 (dt, J = 15.7, 7.4 Hz, 2H), 7.23 (app dd, J = 14.4, 9.0 Hz, 2H), 7.04 (t, J = 8.6 Hz, 1H), 6.83 (d, J = 9.6 Hz, 1H), 4.60 (d, J =

5.8 Hz, 2H), 3.97 – 3.80 (m, 4H), 2.61 (tt, J = 9.9, 5.1 Hz, 1H), 1.67 (td, J = 10.8, 9.5, 4.1 Hz, 4H). MS *m/z* 446.1 $[M+1]^+$.

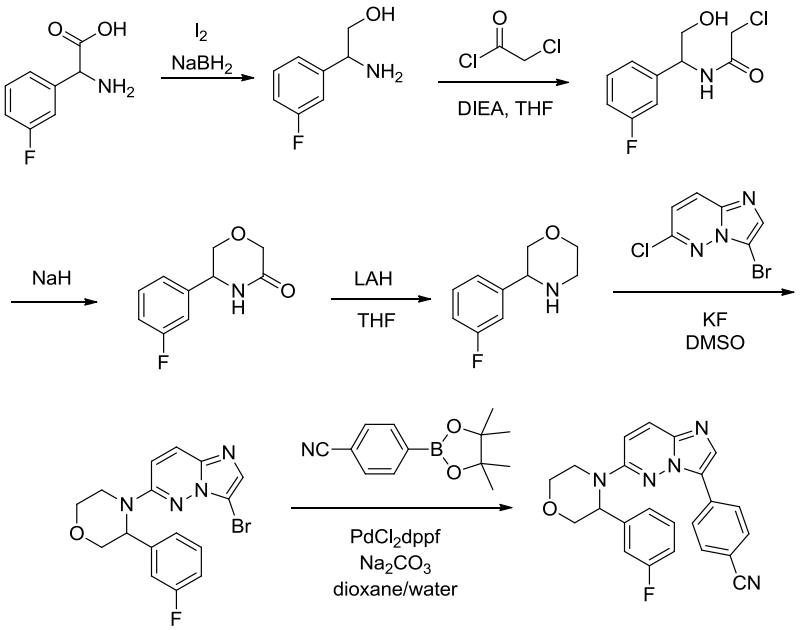
4-(6-{[(3-Fluorophenyl)methyl]amino}imidazo[1,2-b]pyridazin-3-yl)benzene-1-sulfonamide (7)

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.28 (s, 1H), 8.16 (d, J = 6.8 Hz, 1H), 8.12 (d, J = 8.3 Hz, 2H), 7.98 (d, J = 9.4 Hz, 1H), 7.93 (s, 1H), 7.88 – 7.79 (m, 2H), 7.48 – 7.37 (m, 2H), 7.32 – 7.19 (m, 2H), 7.10 (dd, J = 9.7, 7.0 Hz, 2H), 4.55 (d, J = 5.5 Hz, 2H). MS *m/z* 398.1 $[M+1]^+$.

4-{6-[Benzyl(methyl)amino]imidazo[1,2-b]pyridazin-3-yl}benzonitrile (8)

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.39 (s, 1H), 8.30 (d, J = 8.4 Hz, 2H), 8.07 (d, J = 10.0 Hz, 1H), 7.90 (d, J = 8.4 Hz, 2H), 7.41 (d, J = 10.0 Hz, 1H), 7.43 – 7.25 (m, 5H), 4.86 (s, 2H), 3.27 (s, 3H). MS *m/z* 340.2 $[M+1]^+$.

4-(6-(3-(3-Fluorophenyl)morpholino)imidazo[1,2-b]pyridazin-3-yl)benzonitrile (10)



2-Amino-2-(3-fluorophenyl)ethanol

A suspension of $NaBH_4$ (5.37 g, 142 mmol) in THF (200 mL) was added D/L-3-fluorophenylglycine (10 g, 59.2 mmol). The suspension was cooled to 0 ^oC, and then a iodine solution (15 g, 59.2 mmol in THF) was added drop wise. After addition, the resulting suspension was heated at reflux for overnight. The reaction was subsequently cooled to room temperature and quenched with KOH (150 mL, 20% aq solution). The resulting solution was stirred for 4h then extracted with DCM (3 x 150 mL). The combined organic layers were dried over $MgSO_4$, concentrated and purified with silica chromatography (0 - 10 % MeOH/EtOAc) to give 2-amino-2-(3-fluorophenyl)ethanol as a white waxy solid (8.54 g, 93% yield).

2-Chloro-*N*-(1-(3-fluorophenyl)-2-hydroxyethyl)acetamide

A solution of 2-amino-2-(3-fluorophenyl)ethanol (7.57 g, 48.8 mmol) in THF was cooled to 0 ^oC. To this solution was added TEA (8.15 mL, 58.6 mmol) then chloroacetyl chloride (5.08 mL, 58.6 mmol) drop wise. After addition, the resulting solution was stirred at 0 ^oC for 1 hour. The reaction mixture was quenched with water. The organic phase was separated, washed with 0.5 N aq HCl, saturated aq $NaHCO_3$, brine, and dried over $MgSO_4$. The crude organic was concentrated and purified with silica chromatography (50% EtOAc/hexanes) gave 2-chloro-N-(1-(3-fluorophenyl)-2-hydroxyethyl)acetamide as a yellow solid.

5-(3-Fluorophenyl)morpholin-3-one

A suspension of NaH (1.05 g, 26 mmol) in THF (600 mL) at 0 ^oC was added 2-chloro-*N*-(1-(3-fluorophenyl)-2-hydroxyethyl)acetamide (5.5 g, 24 mmol) as a solution in THF (150 mL)

slowly. After addition, the suspension was warmed to room temperature and stirred overnight. The suspension was quenched with water and concentrated. The residue was dissolved in EtOAc, washed with water, brine and dried over $MgSO_4$. Concentration gave 5-(3-fluorophenyl)morpholin-3-one as an off white foam (4.56 g, 49% yield over two steps).

3-(3-Fluorophenyl)morpholine

A solution of 5-(3-fluorophenyl)morpholin-3-one (4.56 g, 23.4 mmol) in THF at 0 ^oC was treated with LAH (4.3 g, 107 mmol) in one portion. The resulting suspension was warmed to room temperature and stirred for overnight. The reaction was subsequently quenched with $Na_2SO_4 \cdot 10H_2O$ and stirred for 4 hours. The mixture was filtered through a Celite pad and the filtrate was concentrated. Purification with silica chromatography (5 - 10 % MeOH/EtOAc) gave 3-(3-fluorophenyl)morpholine as a clear oil (2.12 g, 50 % yield). ^1H NMR (400 MHz, $CDCl_3$): δ 7.32 - 7.25 (m, 1H), 7.18 - 7.12 (m, 2H), 6.97 (dddd, *J* = 9.2, 6.0, 6.0, 1.2 Hz, 1H), 3.93 (dd, *J* = 10.0, 3.2 Hz, 1H), 3.89 - 3.75 (m, 1H), 3.81 (dd, J = 11.2, 3.2 Hz, 1H), 3.64 (td, *J* = 11.2, 2.8 Hz, 1H), 3.35 (dd, *J* = 7.6, 6.4 Hz, 1H), 3.12 (td, *J* = 11.6, 3.6 Hz, 1H), 3.04 - 2.97 (m, 1H). MS: *m/z* 182.1 $[M+1]^+$.

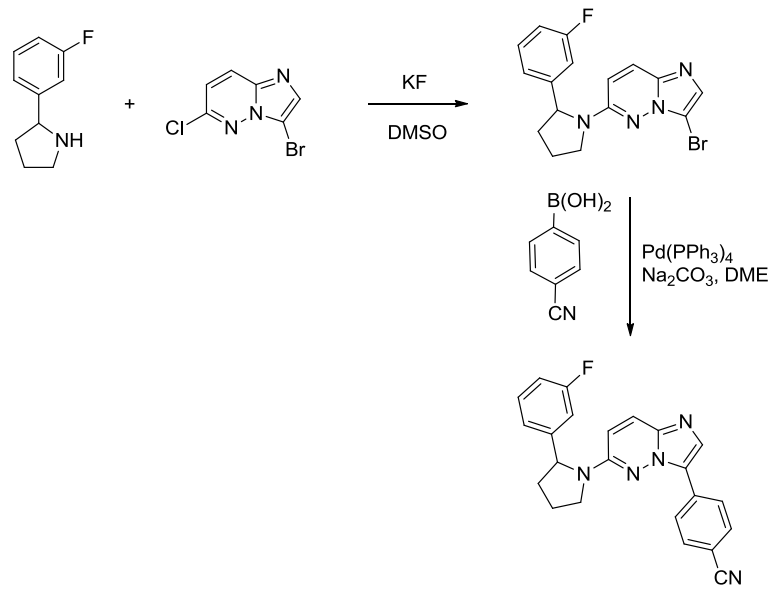
4-(3-Bromoimidazo[1,2-b]pyridazin-6-yl)-3-(3-fluorophenyl)morpholine

A suspension of 3-(3-fluorophenyl)morpholine (1.59 g, 6.9 mmol), 3-bromo-6-chloroimidazo[1,2-b]pyridazine (1.5 g, 8.29 mmol) and KF (1.6 g, 27.6 mmol) in DMSO (6 mL) was heated at 180 ^oC for 18 hours. The resulting solution was cooled to room temperature and purified by HPLC to yield 4-(3-bromoimidazo[1,2-b]pyridazin-6-yl)-3-(3-fluorophenyl)morpholine as a yellow foam (0.64 g, 26% yield).

4-(6-(3-(3-Fluorophenyl)morpholino)imidazo[1,2-b]pyridazin-3-yl)benzonitrile (10)

A suspension of 4-(3-bromoimidazo[1,2-b]pyridazin-6-yl)-3-(3-fluorophenyl)morpholine (25 mg, 0.067 mmol), 4-cyano-phenyl boronic ester (26 mg, 0.13 mmol), [1,1′-bis(diphenylphosphino)ferrocene]dichloropalladium(II) (2.7 mg, 0.0033 mmol) in 1,4-dioxane (3 mL) and Na_2CO_3 (0.5 mL of a 2.0 M aq solution) was bubbled with nitrogen and then heated at 150 ^oC for 20 hours. After cooling to room temperature, the reaction mixture was purified by HPLC to yield 4-(6-(3-(3-fluorophenyl)morpholino)imidazo[1,2-b]pyridazin-3-yl)benzonitrile (**10**) as a white solid. ^1H NMR (400 MHz, MeOD-d_4) δ 8.01 (br s, 1H), 7.96 (d, J = 8.0 Hz, 2H), 7.25 (d, J = 8.0 Hz, 2H), 7.66 (br s, 1H), 7.45 (s, 1H), 7.24 (ddd, J = 6.0, 6.0, 2.0 Hz, 1H), 7.11 (d, J = 7.6 Hz, 1H), 7.07 (ddd, J = 6.9, 4.0, 2.0 Hz, 1H), 6.89 (ddd, J = 6.0, 6.0, 2.4 Hz, 1H), 5.21 - 5.15 (m, 1H), 4.46 (dd, J = 12.4, 2.0 Hz, 1H), 4.05 - 3.92 (m, 3H), 3.76 (ddd, J = 12.0, 12.0, 3.2 Hz, 1H), 3.68-3.60 (m, 1H). MS: *m/z* 400.1 $[M+1]^+$.

Synthesis of compound (11)



3-Bromo-6-(2-(3-fluorophenyl)pyrrolidin-1-yl)imidazo[1,2-b]pyridazine

A solution of 3-bromo-6-chloroimidazo[1,2-b]pyridazine (0.26 g, 1.1 mmol) and (rac)-2-(3-fluorophenyl)pyrrolidine (0.50 g, 3.0 mmol) in DMSO (4 mL) was treated with powderized KF (0.72 g, 12.4 mmol). The resulting heterogeneous mixture was then stirred at 140 °C for 3 hours. The reaction mixture was then poured into stirring water (10 mL) and stirred at room temperature for 30 minutes. This mixture was extracted with EtOAc (3×50 mL). The combined organic layers were washed with brine (2×30 mL), dried over anhydrous Na_2SO_4, filtered and concentrated the solvent to dryness to yield a yellow oil. The crude product was purified using flash chromatography on silica with hexanes/EtOAc (30 min run with 10 to 50% gradient). The final product was obtained as a dense oil, (*rac*)-3-bromo-6-(2-(3-fluorophenyl)pyrrolidin-1-yl)imidazo[1,2-b]pyridazine (0.36 g, 89% yield). ^{1}H NMR (400 MHz, MeOD-d_4) δ 7.48 (d, J = 9.6 Hz, 1H), 7.33 (s, 1H), 7.32 -7.17 (m, 1H), 6.99 (d, J = 7.6 Hz, 1H), 6.92 (d, J = 10 Hz, 1H), 6.86 (dt, J = 8.8, 2.8 Hz, 1H), 6.60 (d, J = 10 Hz, 1H), 5.03 (dd, J = 10, 3.2 Hz, 1H), 3.88-3.82

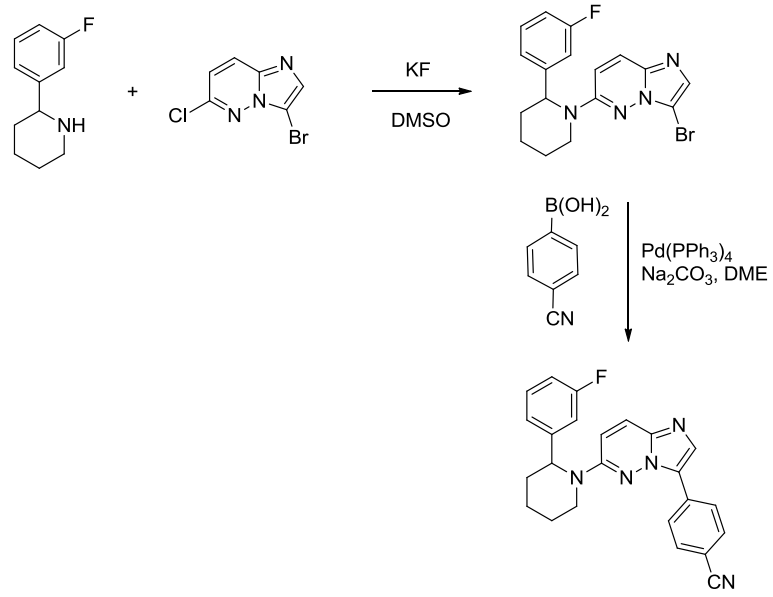
(m, 1H), 3.65 (q, *J* = 7.6 Hz, 1H), 2.46-2.36 (m, 1H), 2.03-1.95 (m, 2H), 1.91-1.85 (m, 1 H). MS: *m/z* 361.1 $[M+1]^+$.

4-(6-(2-(3-Fluorophenyl)pyrrolidin-1-yl)imidazo[1,2-b]pyridazin-3-yl)benzonitrile (11)

To a microwave vessel (2 mL chargeable volume, Biotage company brand, equipped with a 5 mm stir bar) was added (*rac*)-3-bromo-6-(2-3-fluorophenyl)pyrrolidin-1-yl)imidazo[1,2-b]pyridazine (50 mg, 0.14 mmol), tetrakis(triphenylphosphine)palladium(0) (17 mg, 0.015 mmol), and (4-cyanophenyl)boronic acid (37 mg, 0.25 mmol). The solids were then followed by reaction solvents of 1,4-dioxane (2 mL) and water (0.5 mL) to furnish a biphasic solution that was microwave-heated to 150 ^oC for 20 minutes. Upon cooling, the resulting dark reaction suspension was then diluted with EtOAc (50 mL) and water (10 mL). The organic extract was separated, and the aqueous layer was further extracted with EtOAc (2 X 10 mL). The combined organic extracts were Na_2SO_4, filtered (0.2 micron Whatman brand) and concentrated to a residue. The purification of the residue was performed by using reverse phase C-18 chromatography utilizing a 4 min run time and a 10 to 90% water/ACN gradient (0.05% TFA modified). The final product was then lyophilized from the acetonitrile/water fractions to dryness, re-suspended in MeOH (6 mL) and mobilized through a polymer bound SPE-carbonate cartridge to remove any residual TFA and provide the final compound 4-(6-(2-(3-fluorophenyl)pyrrolidin-1-yl)imidazo[1,2-b]pyridazin-3-yl)benzonitrile (**11**), upon removal of MeOH *in vacuo*, as a white solid (37 mg, 0.10 mmol, 69%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.33 (d, *J* = 2.9 Hz, 1H), 8.12 (br s, 1H), 8.02 (d, *J* = 9.9 Hz, 1H), 7.81 (d, *J* = 8.2 Hz, 1H), 7.64 – 7.59 (m, 2H), 7.43 (q, *J* = 7.5 Hz, 1H), 7.26 – 7.11 (m, 2H), 7.10 – 6.95 (m, 2H), 5.22 – 5.10 (m, 1H), 4.01 (dd, *J* = 10.3, 5.3 Hz, 1H), 3.68 (q, *J* = 8.5 Hz, 1H), 2.46 (d, *J* = 10.0 Hz, 1H), 2.04

(t, *J* = 7.1 Hz, 2H), 1.90 (s, 1H). MS *m/z* 384.2 $[M+1]^+$. Analytical method to resolve enantiomers on a ChiralPal AD-H column with solvent A hexane (60%) and IPA (40%) with a flow rate of 1mL/min and ambient temperature afforded (*S*)-4-(6-(2-(3-fluorophenyl)pyrrolidin-1-yl)imidazo[1,2-b]pyridazin-3-yl)benzonitrile (**12**) and (*R*)-4-(6-(2-(3-fluorophenyl)pyrrolidin-1-yl)imidazo[1,2-b]pyridazin-3-yl)benzonitrile (**13**) optically pure.

Synthesis of compound (9)

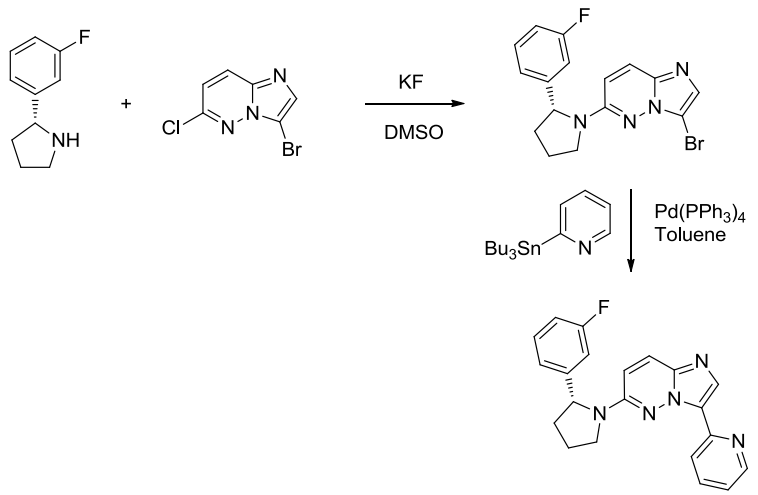


4-(6-(2-(3-Fluorophenyl)piperidin-1-yl)imidazo[1,2-b]pyridazin-3-yl)benzonitrile (9)

The synthesis of compound **9** was accomplished in the same manner as that of compound **11**, with the substitution of commercially available (*rac*) 2-(3-fluorophenyl)piperidine in place of (*rac*) 2-(3-fluorophenyl)pyrrolidine, in the first step. Acquired analytical data as follows, ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.38 (s, 1H), 8.15 (d, *J* = 8.1 Hz, 2H), 8.07 (d, *J* = 10.3 Hz, 1H), 7.89 – 7.81 (m, 2H), 7.56 (d, *J* = 9.8 Hz, 1H), 7.45 – 7.34 (m, 1H), 7.14 (t, *J* = 8.3 Hz, 2H), 7.08

– 6.97 (m, 1H), 4.18 (app d, *J* = 13.4 Hz, 1H), 3.52 – 3.36 (m, 2H), 2.20 (app d, *J* = 13.6 Hz, 1H), 2.07 (app d, *J* = 14.5 Hz, 1H), 1.80 (br s, 1H), 1.68-1.63 (m, 2H), 1.42 (br s, 1H). MS *m/z* 398.2 $[M+1]^+$.

Synthesis of compound 14



(*R*)-3-bromo-6-(2-(3-fluorophenyl)pyrrolidin-1-yl)imidazo[1,2-b]pyridazine

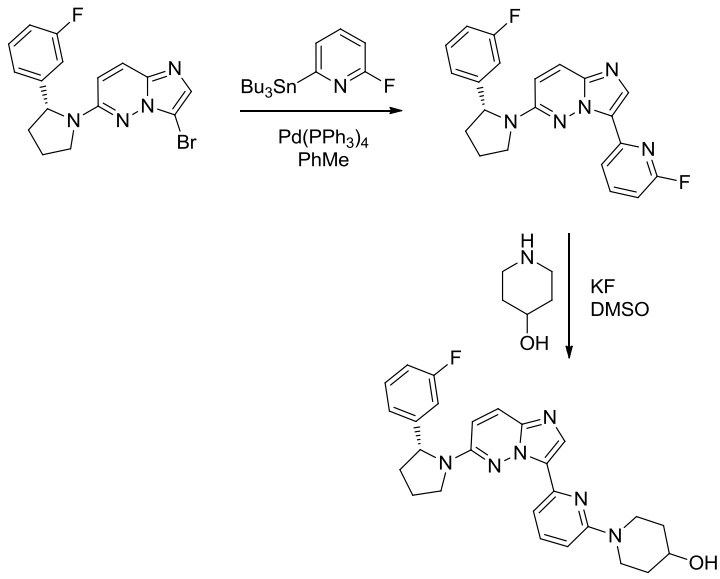
To a solution of 3-bromo-6-chloroimidazo[1,2-b]pyridazine (1.57 g, 6.8 mmol) and (*R*)-2-(3-fluorophenyl)pyrrolidine (1.13 g, 6.8 mmol) in DMSO (80 mL) was added spray dried KF (4.0 g, 6.8 mmol). The resulting heterogeneous mixture was then stirred at 100 °C for 72 hours. The reaction mixture was then poured into stirring water (50 mL) and stirred at room temperature for 30 minutes. This mixture was extracted with EtOAc (4×20 mL). The combined organic layers were washed with brine (2×100 mL), dried over anhydrous Na_2SO_4, filtered and concentrated to dryness to yield a yellow oil. The crude product was then purified using flash chromatography on silica with hexanes/EtOAc gradient to yield (*R*)-3-bromo-6-(2-(3-fluorophenyl)pyrrolidin-1-

yl)imidazo[1,2-b]pyridazine (2.03 g, 5.6 mmol, 85%) . ^{1}H NMR (400 MHz, MeOD-d_4) δ 7.48 (d, *J* = 9.6 Hz, 1H), 7.33 (s, 1H), 7.32 – 7.17 (m, 1H), 6.99 (d, *J* = 7.6 Hz, 1H), 6.92 (d, *J* = 10 Hz, 1H), 6.86 (dt, *J* = 8.8, 2.8 Hz, 1H), 6.60 (d, *J* = 10 Hz, 1H), 5.03 (dd, *J* = 10, 3.2 Hz, 1H), 3.88 – 3.82 (m, 1H), 3.65 (q, *J* = 7.6 Hz, 1H), 2.46 – 2.36 (m, 1H), 2.03 – 1.95 (m, 2H), 1.91 – 1.85 (m, 1 H). MS: *m/z* 361.1 $[M+1]^+$.

(*R*)-6-(2-(3-fluorophenyl)pyrrolidin-1-yl)-3-(pyridin-2-yl)imidazo[1,2-b]pyridazine (14)

2-(Tributylstannyl)pyridine (0.46 g, 1.24 mmol) in toluene (10 mL) was cannula transferred under an argon atmosphere to a suspension of (*R*)-3-bromo-6-(2-3-fluorophenyl)pyrrolidin-1-yl)imidazo[1,2-b]pyridazine (299 mg, 0.83 mmol) and tetrakis(triphenylphospine)palladium(0) (48 mg, 0.042 mmol) in toluene (20 mL). The resulting reaction mixture was heated to 110 ^{o}C for overnight. The reaction progress was monitored by LC/Mass. After the reaction was complete, the reaction vessel was cooled to room temperature and the solvent was reduced to 1/5 of the original volume and diluted with EtOAc and water. The aqueous layer was extracted with EtOAc (2 x 30 mL). The combined organic solvents were washed with saturated NH_4Cl, water and brine, then dried over anhydrous Na_2SO_4, filtered and concentrated to dryness. The purification was performed by silica chromatography using 80% EtOAc in hexane to afford (*R*)-6-(2-(3-fluorophenyl)pyrrolidin-1-yl)-3-(pyridin-2-yl)imidazo[1,2-b]pyridazine (**14**) (218 mg, 73% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.65 (d, *J* = 4.0 Hz, 1H), 8.14 (s br, 2H), 7.80 (s br, 1H), 7.47 – 7.35 (m, 3H), 7.25 – 7.15 (m, 3H), 7.09 – 7.01 (m, 1H), 5.22 (dd, *J* = 8.0, 1.6 Hz, 1H), 4.05 (dt, *J* = 9.2, 5.2Hz, 1H), 3.70 (q, *J* = 8.4 Hz, 1H), 2.52 – 2.42 (m, 1H), 2.11 – 2.02 (m, 2H), 1.95 – 1.87 (m, 1H). MS: *m/z* 360.1 $[M+1]^+$.

Synthesis of compound 15



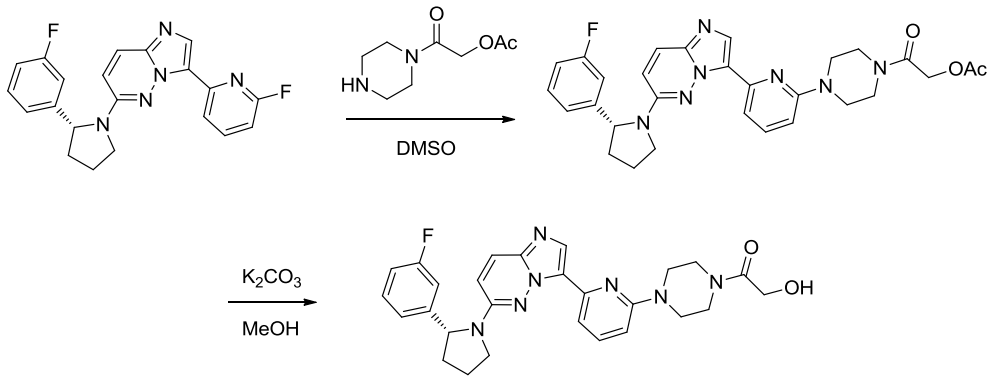
(*R*)-6-(2-(3-fluorophenyl)pyrrolidin-1-yl)-3-(6-fluoropyridin-2-yl)imidazo[1,2-b]pyridazine

A solution of tetrakis(triphenylphospine)palladium(0) (140 mg, 0.121 mmol), 6-fluoro-2(tributylstannyl)pyridine (2900 mg, 7.51 mmol), and (*R*)-3-bromo-6-(2-(3-fluorophenyl)pyrrolidin-1-yl)imidazo[1,2-b]pyridazine (800 mg, 2.21 mmol) in toluene (23 mL) was heated to 110 ^{o}C for 12 hours. The reaction mixture was then cooled to room temperature and purified using silica chromatography (ethyl acetate/hexanes, 0 to 100%, 24 min gradient) to furnish intermedite (*R*)-6-(2-(3-fluorophenyl)pyrrolidin-1-yl)-3-(6-fluoropyridin-2-yl)imidazo[1,2-b]pyridazine (870 mg, 83% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.16 – 8.04 (m, 2H), 7.94 (d, *J* = 9.9 Hz, 1H), 7.92 – 7.88 (m, 1H), 7.45 – 7.35 (m, 1H), 7.21 – 7.16 (m, 2H), 7.09 – 6.98 (m, 2H), 6.93-6.90 (m, 1H), 5.19 (dd, *J* = 8.1, 2.8 Hz, 1H), 4.02 (dt, *J* = 10.7, 5.5 Hz, 1H), 3.75 – 3.64 (m, 1H), 2.11 – 1.96 (m, 3H), 1.89 (dt, *J* = 12.5, 4.0 Hz, 1H). MS *m/z* 378.1 $[M+1]^{+}$.

(*R*)-1-(6-(6-(2-(3-fluorophenyl)pyrrolidin-1-yl)imidazo[1,2-b]pyridazin-3-yl)pyridin-2-yl)piperidin-4-ol (15)

To a solution of (*R*)-6-(2-(3-fluorophenyl)pyrrolidin-1-yl)-3-(6-fluoropyridin-2-yl)imidazo[1,2-b]pyridazine (910 mg, 2.411 mmol) in DMSO (15 mL) was added piperidin-4-ol (732 mg, 7.23 mmol) and KF (1401 mg, 24.11 mmol). The mixture was stirred at 130 °C overnight. The mixture was cooled to room temperature and diluted with EtOAC (100 mL), washed with water (2 X 100 mL) and brine (100 mL). The organic layer was separated, dried over $MgSO_4$, filtered and concentrated *in vacuo*. The crude residue was purified by silica gel column chromatography (DCM/CH_3OH, 0 to 15%, 25 min gradient) to furnish (*R*)-1-(6-(6-(2-(3-fluorophenyl)pyrrolidin-1-yl)imidazo[1,2-b]pyridazin-3-yl)pyridin-2-yl)piperidin-4-ol (**15**) as a white solid (928 mg, 84% yield). ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.04 (s, 1H), 7.87 (d, *J* = 9.8 Hz, 1H), 7.42 (br s, 1H), 7.37 (dd, *J* = 14.1, 7.9 Hz, 2H), 7.19 – 7.09 (m, 2H), 7.03 (t, *J* = 7.7, 1H), 6.80 (br s, 1H), 6.73 (d, *J* = 8.5 Hz, 1H), 5.16 (dd, *J* = 7.9, 2.0 Hz, 1H), 4.67 (d, *J* = 4.3 Hz, 1H), 4.08 (dt, *J* = 13.5, 4.6 Hz, 2H), 4.02 – 3.94 (m, 1H), 3.67 (ddt, *J* = 17.6, 10.0, 6.0 Hz, 2H), 3.08 (app dt, *J* = 13.2, 10.0 Hz, 2H), 2.45 (tdd, *J* = 14.2, 8.7, 4.2 Hz, 1H), 2.06 – 2.00 (m, 2H), 1.90 – 1.84 (m, 1H), 1.82 – 1.75 (m, 2H), 1.38 (tdd, *J* = 14.4, 7.8, 3.8 Hz, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 163.53, 161.12, 157.95, 151.68, 147.22 (d, *J* = 6.1 Hz), 145.56, 137.77, 137.29, 132.57, 130.30 (d, *J* = 9.1 Hz), 127.35, 125.88, 121.58, 113.37 (d, *J* = 21.2 Hz), 112.39 (d, *J* = 22.2 Hz), 109.58, 108.54, 66.42, 61.23, 48.36, 42.48, 35.14, 33.70, 22.65. MS *m/z* 459.2 $[M+1]^+$.

Synthesis of compound 16



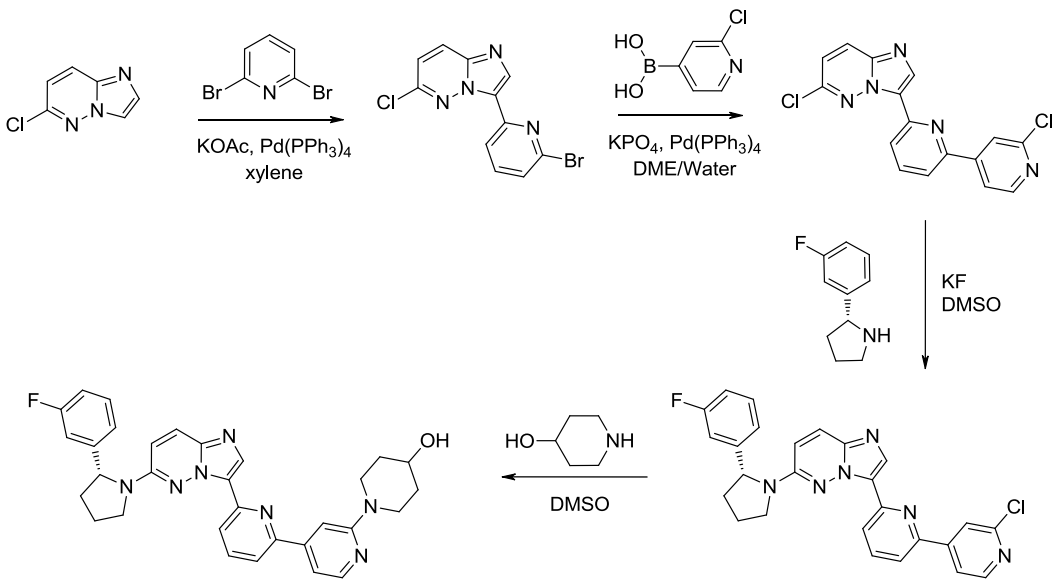
(*R*)-2-(4-(6-(6-(2-(3-fluorophenyl)pyrrolidin-1-yl)imidazo[1,2-b]pyridazin-3-yl)pyridin-2-yl)piperazin-1-yl)-2-oxoethyl acetate

To a solution of (*R*)-6-(2-(3-fluorophenyl)pyrrolidin-1-yl)-3-(6-fluoropyridin-2-yl)imidazo[1,2-b]pyridazine (400 mg, 1.06 mmol) in DMSO (10 mL) was added 2-oxo-2-(piperazin-1-yl)ethyl acetate (790 mg, 4.24 mmol) and the resulting solution was heated to 110 ^{o}C for 2 hours. The reaction was cooled to room temperature and quenched with aqueous saturated NH_4Cl (100 mL). The mixture was extracted with EtOAc (3 x 100 mL). The combined organic layers were dried over Na_2SO_4, filtered and concentrated *in vacuo* to afford a crude residue, which was purified using silica chromatography (40 g column size) with EtOAc/hexane gradient (10 to 100%, 20 min) to furnish (*R*)-2-(4-(6-(6-(2-(3-fluorophenyl)pyrrolidin-1-yl)imidazo[1,2-b]pyridazin-3-yl)pyridin-2-yl)piperazin-1-yl)-2-oxoethyl acetate (311 mg, 54% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.11 (d, *J* = 5.6 Hz, 1H), 7.89 (d, *J* = 9.8 Hz, 1H), 7.50 (s, 1H), 7.37 (dd, *J* = 8.2, 5.8 Hz, 1H), 7.16 (d, *J* = 7.5 Hz, 2H), 7.03 (t, *J* = 8.1 Hz, 1H), 6.90 – 6.70 (m, 2H), 5.17 (d, *J* = 7.8 Hz, 1H), 4.84 – 4.80 (m, 3H), 3.99 (dt, *J* = 10.6, 5.6 Hz, 1H), 3.79 – 3.37 (m, 8H), 2.33 (s, 3H), 2.31 – 1.93 (m, 2H), 1.88 – 1.86 (m, 1H). MS *m/z* 544.2 $[M+1]^{+}$.

(*R*)-1-(4-(6-(6-(2-(3-fluorophenyl)pyrrolidin-1-yl)imidazo[1,2-b]pyridazin-3-yl)pyridin-2-yl)piperazin-1-yl)-2-hydroxyethanone (16)

To a solution of (*R*)-2-(4-(6-(6-(2-(3-fluorophenyl)pyrrolidin-1-yl)imidazo[1,2-b]pyridazin-3-yl)pyridin-2-yl)piperazin-1-yl)-2-oxoethyl acetate (298 mg, 0.548 mol) in MeOH (8 mL) was added solid K_2CO_3 (276 mg, 2.0 mmol) and the resulting solution was heated to 60 ^oC for 5 min. The reaction was cooled to room temperature, concentrated *in vacuo* and purified by silica gel chromatography (40 g column) with EtOAc/hexane gradient (10 to 100%, 20 min) to furnish (*R*)-1-(4-(6-(6-(2-(3-fluorophenyl)pyrrolidin-1-yl)imidazo[1,2-b]pyridazin-3-yl)pyridin-2-yl)piperazin-1-yl)-2-hydroxyethanone (**16**) (206 mg, 75% yield). ^1H NMR (400 MHz, DMSO-d_6) δ 8.10 (s, 1H), 7.87 (d, *J* = 9.8 Hz, 1H), 7.60 – 7.47 (m, 1H), 7.37 (td, *J* = 7.6, 5.7 Hz, 1H), 7.21 – 7.09 (m, 2H), 7.07 – 6.96 (m, 1H), 6.75 (d, *J* = 8.5 Hz, 2H), 5.13 (dd, *J* = 8.4, 2.8 Hz, 1H), 4.64 (d, *J* = 5.8 Hz, 1H), 4.15 (d, *J* = 4.2 Hz, 2H), 3.96 (dt, *J* = 10.5, 5.6 Hz, 1H), 3.72 – 3.44 (m, 8H), 2.44 (dt, *J* = 12.2, 8.6 Hz, 1H), 2.01 (tt, *J* = 10.7, 6.3 Hz, 2H), 1.91 – 1.75 (m, 1H). MS *m/z* 502.2 $[M+1]^+$. ^{13}C NMR (101 Hz, DMSO-d_6) δ 170.14, 163.55, 161.13, 157.86, 151.70, 147.20 (d, *J* = 5.0 Hz), 145.56, 137.97, 137.39, 132.75, 130.30 (d, *J* = 8.1 Hz), 127.16, 125.89, 121.59 (d, *J* = 4.0 Hz), 113.31 (d, *J* = 20.0 Hz, 1H), 112.53 (d, *J* = 22.0 Hz, 1H), 109.69 (d, *J* = 35.0 Hz, 1H), 105.90, 61.24, 60.12, 48.36, 44.32 (d, *J* = 21.0 Hz), 40.99, 35.13, 22.64. Analytically calculated for $C_{27}H_{28}FN_7O_2$ with 0.50 equivalent hydrate: C, 63.52; H, 5.73; N, 19.20. Found: C, 63.43; H, 5.65; N, 19.11.

Synthesis of compound 17



3-(6-Bromopyridin-2-yl)-6-chloroimidazo[1,2-b]pyridazine

To a clean dry three neck round bottom flask was added 6-chloroimidazo[1,2-b]pyridazine (90 g, 0.586 mol), 2,6-dibromopyridine (166.54 g, 0.703 mol), xylene (1.2 L), KOAc (114.86 g, 1.172 mol) and tetrakis(triphenylphospine)palladium(0) (33.84 g, 29.3 mmol). The flask was equipped with condenser and mechanical stirrer. The mixture was heated to reflux and stirred overnight under nitrogen. HPLC showed about 20% of 6-chloroimidazo[1,2-b]pyridazine remained. The mixture was cooled to room temperature. Then EtOAc (1 L) and MeOH (250 mL) was added and the mixture was filtered. The filtrate was concentrated under vacuum to give a pale yellow solid. The solid was partitioned in EtOAc (100 mL) and water (500 mL) then stirred for 1 hour. Filtration gave 3-(6-bromopyridin-2-yl)-6-chloroimidazo[1,2-b]pyridazine which was pure enough for the next step (110g, 61% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.53 - 8.43 (m, 2H), 8.40 (d, J = 9.5 Hz, 1H), 7.99 (t, J = 7.9 Hz, 1H), 7.66 (dd, J = 0.8, 7.9 Hz, 1H), 7.56 (d, J = 9.5 Hz, 1H). MS m/z 309.0 and 311.0 $[M+1]^+$.

6-Chloro-3-(2'-chloro-[2,4'-bipyridin]-6-yl)imidazo[1,2-b]pyridazine

To a solution of 3-(6-bromopyridin-2-yl)-6-chloroimidazo[1,2-b]pyridazine (120.0 g, 390 mmol) in a mixture of water (1.0 L) and DME (500 mL) was added (2-chloropyridin-4-yl)boronic acid (72.0 g, 468 mmol), K_3PO_4 (165 g, 780 mmol), tetrakis(triphenylphospine)palladium(0) (9 g, 7.79 mmol). The flask was equipped with condenser and mechanical stirrer. The mixture was heated to reflux and stirred overnight under nitrogen. HPLC showed complete conversion. The mixture was cooled to room temperature. Filtration gave a pale yellow solid. This cake was washed with EtOAc (250 mL) and dried in vacuum to afford 6-chloro-3-(2'-chloro-[2,4'-bipyridin]-6-yl)imidazo[1,2-b]pyridazine (127 g , 99% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.78 (s, 1H), 8.59 (dd, J = 0.7, 5.2 Hz, 1H), 8.54 (dd, J = 3.3, 5.4 Hz, 1H), 8.41 (d, J = 9.4 Hz, 1H), 8.37 (dd, J = 0.7, 1.5 Hz, 1H), 8.29 (dd, J = 1.5, 5.2 Hz, 1H), 8.24 - 8.20 (m, 2H), 7.56 (d, J = 9.4 Hz, 1H). MS *m/z* 342.1 and 344.1 $[M+1]^+$.

(*R*)-3-(2'-chloro-[2,4'-bipyridin]-6-yl)-6-(2-(3-fluorophenyl)pyrrolidin-1-yl)imidazo[1,2-b]pyridazine

To a dry four-neck round bottom flask (2 L) was added 6-chloro-3-(2'-chloro-[2,4'-bipyridin]-6-yl)imidazo[1,2-b]pyridazine (106 g, 0.310 mol), (*R*)-2-(3-fluorophenyl)pyrrolidine HCl (68.76 g, 0.341 mol), KF (89.6 g, 1.543 mol) and DMSO (1.0 L). The mixture was heated to 120 ^{o}C and stirred overnight. The reaction was cooled to 80 ^{o}C and activated carbon (30 g) was added, then stirred at 80 ^{o}C for 1 hour. The mixture was filtered, washed with DMSO (200 mL) and cooled to room temperature. To the filtrate was added water (1.2 L), then stirred for 2 hours. Filtration gave a yellow solid that was stirred in water (2 x 800 mL), then dried in air at room temperature to give crude (*R*)-3-(2'-chloro-[2,4'-bipyridin]-6-yl)-6-(2-(3-fluorophenyl)pyrrolidin-1-

yl)imidazo[1,2-b]pyridazine (140 g, 90% purity). The crude product was used in the next step without further purification.

(*R*)-1-(6-(6-(2-(3-fluorophenyl)pyrrolidin-1-yl)imidazo[1,2-b]pyridazin-3-yl)-[2,4'-bipyridin]-2'-yl)piperidin-4-ol (17)

To a flask was added (*R*)-3-(2'-chloro-[2,4'-bipyridin]-6-yl)-6-(2-(3-fluorophenyl)pyrrolidin-1-yl)imidazo[1,2-b]pyridazine (130 g, 0.28 mol), DMSO (750 mL) and piperidin-4-ol (223 g, 2.21 mol). The flask was equipped with condenser and mechanical stirrer. The mixture was heated to 125 ^{o}C and stirred overnight under nitrogen. The mixture was cooled to 80 ^{o}C then water (750 mL) was added and the product was precipitated to give (*R*)-1-(6-(6-(2-(3-fluorophenyl)pyrrolidin-1-yl)imidazo[1,2-b]pyridazin-3-yl)-[2,4'-bipyridin]-2'-yl)piperidin-4-ol (**17**) (130 g, 86% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.30 (s, 1H), 8.22 (d, *J* = 5.2 Hz, 1H), 8.10 – 8.24 (bs, 1H), 7.90 – 7.98 (m, 2H), 7.76 – 7.86 (bs, 1H), 7.52 (s, 1H), 7.40 (q, *J* = 7.4 Hz, 1H), 7.34 (d, *J* = 5.2 Hz, 1H), 7.04 (t, *J* = 8 Hz, 1H), 6.80 – 6.94 (bs, 1H), 5.19 (d, *J* = 8 Hz, 1H), 4.71 (d, *J* = 4 Hz, 1H), 4.10 – 4.18 (m, 2H), 3.93 – 4.06 (m, 1H), 3.65 – 3.75 (m, 2H), 3.10 – 3.20 (m, 2H), 1.98 – 2.10 (m, 2H), 1.78 – 1.92 (m, 3H), 1.34 – 1.45 (m, 2H). ^{13}C NMR (400 MHz, DMSO-d_6) δ 163.53, 161.11, 159.53,153.95, 151.76, 148.27 (d, *J* = 34.7 Hz), 147.69, 147.10 (d, *J* = 25.6 Hz), 146.92, 137.61, 133.14 (d, *J* = 23.2 Hz), 130.34 (d, *J* = 32.4 Hz), 126.54, 126.04, 121.56, 119.26, 118.75, 113.40 (d, *J* = 83.6 Hz), 112.41 (d, *J* = 86.0), 110.26, 109.86, 103.75, 66.34, 61.24, 48.37, 42.72, 35.12, 33.71, 22.65. MS *m/z* 536.2 $[M+1]^{+}$. Analytically calculated for $C_{31}H_{30}FN_7O$ with 0.50 equivalent hydrate: C, 68.37; H, 5.74; N, 18.00. Found: C, 68.35; H, 5.58; N, 18.01.

Assay Descriptions

Preparation of compound dilutions

Compounds were dissolved in DMSO (10 mM) and transferred into 1.4 mL flat bottom or V-shaped Matrix tubes carrying a unique 2D matrix chip by individual compound hubs. The numbers of these chips were distinctively linked to the individual compound identification numbers. The stock solutions were stored at –20°C if not used immediately. For the test procedure the vials were defrosted and identified by a scanner whereby a working sheet is generated that guides the subsequent working steps.

Compound dilutions were made in 384 well plates. This format enabled the assay of maximally 28 individual test compounds at 11 concentrations (single points) including 2 reference compounds. The dilution protocol included the production of pre-dilution plates, master plates and assay plates.

Compound plates: 30 µL of individual compound (10 mM) DMSO solution including reference compound were transferred into columns 1 and 13 of a 384 well plate. 20 µL of DMSO were added to the rest of the wells and the compounds were serially diluted (1:3) by transferring 10 µL from a well in column 1 or 13 to the next well in column 2 or 14 respectively and successively with the help of a Minitrack robot.

Assay plates: Identical assay plates were then prepared by adding 50 nL each of compound dilutions of the compound plates into 384-well "assay plates". In the following the compounds were mixed with 50 µL of assays components (cells or enzyme) and tested for their inhibitory activity.

Ba/F3 cell proliferation assay panel

Compounds were tested for their ability to inhibit the proliferation of wt Ba/F3 cells and Ba/F3 cells transformed with constitutively expressed luciferase reporter and BCR-ABL or Tel-KDR or other Tel fusion kinases. Parental Ba/F3 cells were maintained in media containing recombinant mouse IL3 and the kinase transformed Ba/F3 cells were maintained in media without IL-3. 7.5 nL of compounds were spotted to each well of 1536-well assay plates by Liquid handling System Echo 555 (Labcyte). 700 cells were then plated into each well of the assay plates in 7 uL culture media per well and compounds were tested at 0.17 nM to 10 uM in 3-fold serial dilutions. The cells were then incubated for 48 hours at 37 ˚C. 3 uL of Bright-Glo® (Promega) was added to each well and the plates were read using ViewLux (PerkinElmer).

Inhibition of cellular TrkA, TrkB and TrkC dependent proliferation

Compounds were assayed to measure their capacity to selectively inhibit cell proliferation of Ba/F3 cells expressing activated TrkA, TrkB or TrkC through fusion to the dimerization domain of Tel (ETV6) transcription factor as well as Ba/F3 cells co-expressing full length TrkA and mNGF compared with parental Ba/F3 cells**.**

Method for KM-12-luc assay

KM12-luc cells were generated by retroviral infection to stably express the luciferase gene in KM12 cells. Cell proliferation and growth were then monitored by measuring the luciferase level. KM12-luc cells (2,000 per well in a 384-well plate) were incubated with compounds for 48 hours before Bright Glo was added and luminescence signals of plates were read by EnVision Plate Reader.

Inhibition of biochemical TrkA, TrkB and TrkC

TrkA and TrkC biochemical assays were carried out by HTRF method. The TrkA enzyme was purchased from Carna, TrkC enzyme as made in house, both were kinase domain only constructs. The substrate peptide Biotin-(Ahx)-GAEEEIYAAFFA-OH was made by New England Peptide. The reaction mixtures contained 1 μM peptide substrate, 1 μM ATP, and either 1.8 nM TrkA or 34 nM TrkC in the reaction buffer (50mM HEPES pH 7.1, 10mM $MgCl_2$, 2 mM $MnCl_2$, 0.01% BSA, 2.5 mM DTT and 0.1 mM Na_3VO_4) at a final volume of 10 μL. All reactions were carried out at room temperature in white ProxiPlate™ 384-well Plus plates (PerkinElmer) and were quenched with 5 μL of 0.2 M EDTA at 60 min. Five μL of the detection reagents (2.5 ng PT66K and 0.05 μg SAXL per well) were added, the plates were incubated at room temperature for 1 h and then read in EnVision reader. Compounds were diluted into assay mixture (final DMSO 0.5%), and IC_{50} values were determined by 12-point (from 50 to 0.000282 μM) inhibition curves in duplicate under the assay conditions as described above.

TrkB biochemical assay was carried out by caliper microfluidic method. The TrkB enzyme was kinase domain only construct, and was purchased from Invitrogen. The peptide substrate used was FAM-KKKKEEIYFFF-CONH2. The reaction mixtures contained 1 μM peptide substrate, 10 μM ATP, and 2 nM TrkB in a reaction buffer containing 100 mM HEPES, pH 7.5, 5 mM MgCl2, 0.01% Triton X-100, 0.1% BSA, 1 mM DTT, 10 uM Na_3VO_4, and 10 uM Beta-Glycerophosphate. The reactions were carried out at room temperature for 3 hrs, and the products were determined by Caliper EZ-reader. Compounds were diluted into assay mixture

(final DMSO 1%), and IC_{50} values were determined by 12-point (from 50 to 0.000282 μM) inhibition curves in duplicate under the assay conditions as described above.

Method for KM12 in vivo study

3 x 106 KM12 cells were injected subcutaneously into nude mice. When tumor size reached 300-400 mm3, tumors were harvested, with necrotic and non-tumor tissues removed, cut into small pieces of 2-3 mm3, and put into a mixture of serum-free RPMI1640 medium and matrigel at a ratio of 1:1 for implantation in nude rats. 5-6 pieces of tumor tissues with matrigel were implanted subcutaneously into the RNU nude rats that had received a single radiation dose of 400-500 rads 3 days before implantation. Tumors were measured by a caliper 2 times per week once palpable, and tumor volumes were calculated using (L x W x H)/2. Compound **17** was formulated in 0.5% MC/0.5%Tween 80 as suspension. Tumor bearing animals were treated with the vehicle (0.5% MC/0.5%Tween 80) or different doses of compound **17** twice a day for 14 days as indicated.

Pharmacokinetic studies

All animal related procedures at GNF were conducted under an IACUC approved protocol in compliance with Animal Welfare Act regulations and the Guide for the Care and Use of Laboratory Animals.

In the single dose rat PK studies, compounds **15**, **16** and **17** were formulated at 2 mg/ml in 75% PEG300 and 25% D5W (5% dextrose in water) for intravenous and oral dosing. The formulation was filtered using a 0.45 μm syringe filter before dosing. Three male Wistar or Sprague Dawley rats were administered intravenously via a jugular vein catheter at 3 mg/kg with a dose volume of 1.5 mL/kg. In another group, three rats were administered orally via gavage at 10 mg/kg with

a dose volume of 5.0 mL/kg. Six blood samples of 100 µL each were collected serially from each animal via saphenous vein up to 24 h after dosing.

In the single dose mouse brain tissue distribution studies, compounds **16** and **17** were formulated at 2 mg/ml in 75% PEG300 and 25% D5W (5% dextrose in water). Ten male Balb/c mice were administered orally via gavage at 20 mg/kg with a dose volume of 10.0 mL/kg. The terminal blood and brain samples were collected at 1, 3, 7, 10 and 24 h post dose (2 animals per time points)

The plasma and brain concentrations were quantified using a Liquid Chromatography /Mass Spectrometry (LC/MS/MS) assay. Pharmacokinetic parameters were calculated by non-compartmental regression analysis using an in house fitting program. The highest plasma concentrations (C_{max}) were recorded. The area under each concentration-time curve, $AUC_{0-\infty}$ was calculated using the linear trapezoidal rule. Clearance (CL) and the steady-state volume of distribution (V_{ss}) were calculated using the data from the intravenous dose and the following equations:

$$CL = \frac{(Dose)}{(AUC_{0-\infty})}$$

$$V_{ss} = \frac{(Dose) \times (AUMC_{0-\infty})}{(AUC_{0-\infty})^2}$$

The absolute oral bioavailability was estimated as follows:

$$F = \frac{AUC_{0-\infty,p.o.}}{AUC_{0-\infty,i.v.}} \cdot \frac{Dose_{i.v.}}{Dose_{p.o.}}$$

TrkC/A Crystallography

The recombinant kinase domain consisting of residues 530-839 of TrkC (Genbank: NM_002530.3) was expressed in baculovirus SF9 cells and purified using Ni-NTA affinity chromatography. After removing the cleavable N-terminal His tag with TEV protease, the protein was further purified by reverse Ni-NTA and gel filtration chromatography.

Purified TrkC protein was concentrated to ~10 mg/ml and incubated with a 3-fold molar excess of compound **1**. Crystals of the complex could be obtained by sitting drop crystallization at 20 ^{0}C using a 1:1 ratio of protein to crystallization condition (2.5 M NaCl, 0.1 M Na/K phosphate, pH 6.2). Diffraction data to 2.0 Å were collected on Beamline 5.0.3 at the ALS, Berkeley.

For human TrkA, the kinase domain (residues 502-796; Genbank: NM_001007792) was expressed in baculovirus cells and purified according to the TrkC purification protocol. Crystals of apo TrkA were grown from 0.8 M Ammonium Sulfate, 0.1 M Citric acid pH 5.0 and used for soaking of compounds **10** and **14**, respectively. Diffraction data to 2.1 Å (**10**) and 2.0 Å (**14**) were collected on Beamline 5.0.3 at the ALS, Berkeley, and all structures were solved by molecular replacement using an in-house TrkC crystal structure as starting model.

Coordinates for the refined models have been deposited with the RCSB PDB: TrkC/**1**: xxx.pdb; TrkA/**10**: xxx.pdb; TrkA/**14**: xxx.pdb.

Additional Data for GNF-8625 (17)

Table 1. Physicochemical properties

Property	17-Maleate Salt
Melting Point	139 ^{0}C
PKa	5.5
cLogP	3.72
Thermodynamic solubility (μM)	

pH 1	>9335
pH 4.6	3.7
pH 7.4	1.7
Chemical stability in bulk 1 week - RT , 50˚C, 80˚C	99.1, 96.7, 92.3
Chemical stability in suspension at 80˚C / 1 week - pH7.4, pH4, pH1 (solution stock)	96.3, 93.8, 96.0 (96.3)
Chiral stability in bulk 1 week - RT , 80˚C	>99.6, >99.6
Chiral stability in suspension at 80˚C / 1 week - pH7.4, pH4, pH1	>99.6, >99.6, >99.6
HT-permeability (PAMPA)	High
CaCo-2 permeability	Low (passive paracellular)

Table 2. Compound **17** Kinase Selectivity in a Biochemical Kinase Panel (Caliper)

Kinase	% Inhib.[a]	Kinase	% Inhib.[a]	Kinase	% Inhib.[a]	Kinase	% Inhib.[a]
Abl	37	**DYRK1a**	5	**JNK2a2**	8	**PKA**	3
Abl (T315I)	8	**EGFR**	47	**KDR**	46	**PKCa**	15
Akt1	10	**EphA3**	8	**Lck**	39	**PLK1**	-7
AMPK	-3	**EphB3**	29	**Lyn**	55	**RET**	20
AurB	54	**FAK2**	32	**MAPK1**	-9	**ROCK2**	7
AXL	6	**FGFR3**	6	**MAPK13**	3	**RSK1**	1
BMX	18	**Flt3**	25	**MAPK14**	0	**SGK1**	4
BTK	23	**FMS/CSF1R**	22	**MAPKAPK2**	3	**Src**	46
CaMK2a	16	**Fyn**	37	**MET**	-1	**Syk**	41
CDK2/cycA	3	**GSK3b**	-3	**NEK2**	-8	**TTK**	-1
CHK2	3	**IGF1R**	-2	**p70S6K**	8	**ZAP70**	-37
CK1a	9	**IKKb**	8	**PAK2**	6		
cKit	1	**InsR**	-3	**PDGFRa**	9		
c-RAF	17	**IRAK4**	16	**PDK1**	10		
CSK	15	**JAK2**	82	**Pim2**	13		

[a] Values indicate percentage of kinase activity inhibition using compound **17** at 5μM concentration

Cytochrome P450 inhibition

Inhibition of most relevant human Cytochrome P450 3A4 (CYP3A), 2C9 (CYP2C9) and 2D6 (CYP2D6) drug metabolizing enzymes may alter the metabolism of co-administered compounds leading to a change in drug exposure and possible toxicity. The drug-drug interaction (DDI)

potential of the compounds was assayed by measuring the inhibition of the metabolic degradation of known isoform substrates. Briefly, the assay used probe substrates to assess CYP inhibition: tolbutamide or diclofenac (CYP2C9), S-mephenytoin (CYP2C19), dextromethorphan (CYP2D6), midazolam (CYP3A4_I), and testosterone (CYP3A4_II).

The probe substrates phenacetin, tolbutamide, S-mephenytoin, dextromethorphan and testosterone were pooled and the enzyme activity determined using this cocktail and a protein concentration of 0.5 mg/mL with an incubation time of 20 minutes. The CYP inhibition assays done using midazolam or diclofenac as the probe substrates required separate assays, as both CYP3A4 and CYP2C9 required a protein concentration of 0.05 mg/mL. Midazolam required an incubation time of 5 minutes while diclofenac required an incubation of ten minutes. All probe substrates were assayed at a concentration equal to its K_m. The dose-response curves covered a concentration range of 0.95 µM to 10.0 µM. LC/MS/MS analysis was done using an API4000 (MDS Inc., Toronto, Canada) with a CTC Leap autosampler (LEAP Technologies, Carborro, NC) and an Agilent 1100 HPLC pump (Agilent Technologies, Inc., Palo Alto, CA).

GNF-8625 **(17)** did not significantly inhibit CYP2C9, CYP2C19, CYP2D6, CYP3A4_I or CYP3A4_II metabolism, indicating minimal drug-drug potential with compounds cleared predominantly via these metabolic pathways.

Table 3. Inhibition of CYP Isoenzymes

Compound	**CYP2C9**	**CYP2C19**	**CYP2D6**	**CYP3A4_I**	**CYP3A4_II**
17	>10	>10	>8.6	>10	>10

IC_{50} values are expressed in µM

***In vitro* metabolic stabilities in multiple species**

The in vitro metabolic stability in hepatic microsomes from various species can predict the potential for in vivo hepatic metabolism and clearance. GNF-8625 (**17**) metabolic stability was tested *in vitro* in mouse, rat and human hepatic microsomal preparations. The compound was incubated at concentration 1 µM at 37 °C for up to 30 minutes with liver microsomes containing 0.2 mg protein/mL in phosphate buffer. Compound **17** showed a low intrinsic clearance (CLint < 50 µl/min/mg) in human and dog liver microsomes and higher in rodents.

Table 4. Metabolic stability in human, mouse and rat liver microsomes

Compound	**CL(int) Human**	**CL(int) Mouse**	**CL(int) Rat**	**CL (int) Dog**
17	39	53	68	41

CL(int) values are expressed in µl/min/mg

Genetic toxicity: MiniAmes

Compound **17** was tested for bacterial reverse mutation in a miniscreen Ames test, at concentrations of 10, 30 and 100, 300 and 1000 µg/well, using *Salmonella typhimurium* strains TA98 and TA100 -/+S9. Under the testing conditions used and applying standard mutagenicity criteria, it did not show evidence of a mutagenic potential.

Cardiac electrophysiology

In the hERG binding assay, compound **17** showed very weak binding activity with 42% inhibition at 30 µM (IC_{50}>30 µM). In the hERG patch clamp test using stably transfected HEK293 cells, compound **17** showed 28% inhibition of hERG channel activity at 10 µM.

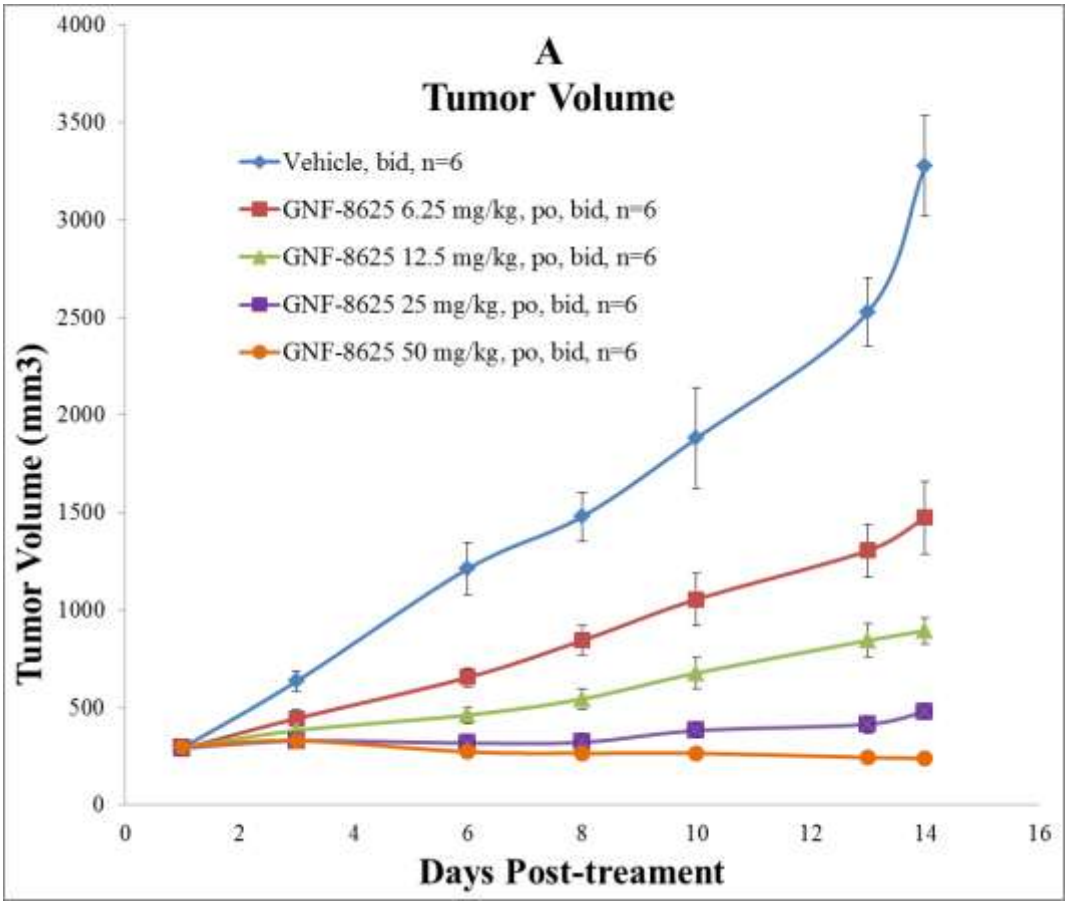
Brain PK

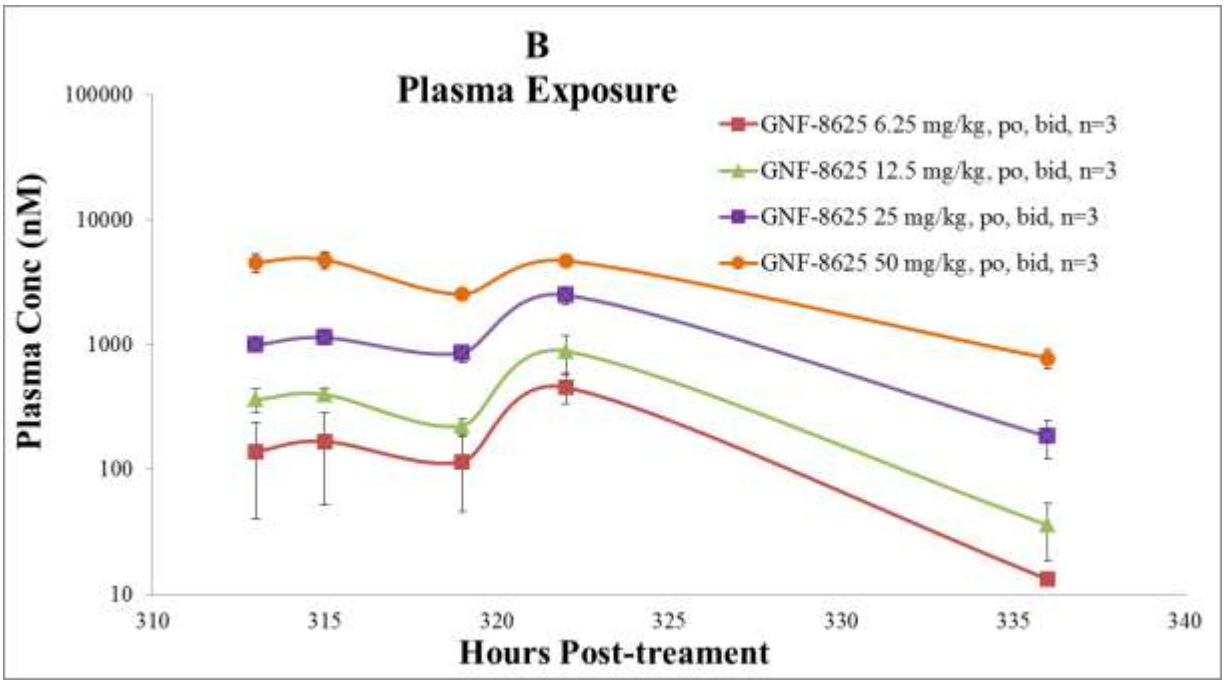
Table 5. *In Vivo* mouse brain tissue distribution.

Brain PK[a]	16	17
Plasma AUC (h*nM)	14300	13736
Plasma C_{max} (nM)	3819	2839
Brain AUC (h*nM)	10958	3149
Brain C_{max} (nM)	2911	498
B/P ratio AUC	0.8	0.2
B/P ratio C_{max}	0.8	0.2

[a]Oral administration at 20 mg/kg using 75% PEG300/25% D5W solution formulation in male Balb/C mouse. Brain and plasma samples were collected at 1, 3, 7, 10 and 24 h post dose; 2 mice/ time point.

KM12 model




B
Plasma Exposure
Plasma Conc (nM)
100000
10000
1000
100
10
310
315
320
325
330
335
340
Hours Post-treament
GNF-8625 6.25 mg/kg, po, bid, n=3
GNF-8625 12.5 mg/kg, po, bid, n=3
GNF-8625 25 mg/kg, po, bid, n=3
GNF-8625 50 mg/kg, po, bid, n=3

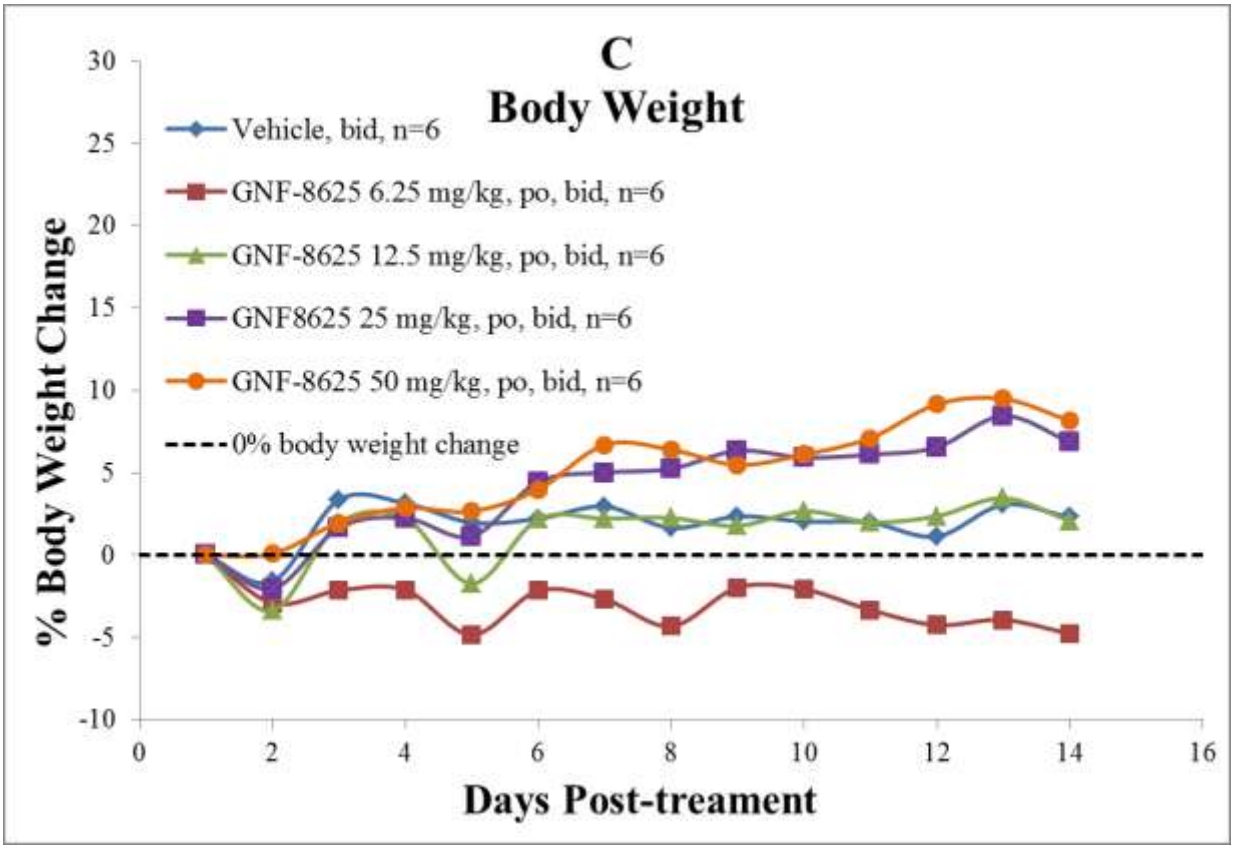

C
Body Weight
% Body Weight Change
30
25
20
15
10
5
0
-5
-10
0
2
4
6
8
10
12
14
16
Days Post-treament
Vehicle, bid, n=6
GNF-8625 6.25 mg/kg, po, bid, n=6
GNF-8625 12.5 mg/kg, po, bid, n=6
GNF8625 25 mg/kg, po, bid, n=6
GNF-8625 50 mg/kg, po, bid, n=6
0% body weight change

Figure 1. KM12 efficacy model in female CRL RNU nude rats with compound 17 (GNF-8625). Animals were transplanted with KM12 tumor tissues and dosing began 10 days post implant. Vehicle and 17 were dosed twice a day (bid) for 14 days. A) Efficacy measured as tumor volume. B) Compound's plasma concentrations. Samples were collected for PK assessment on days 13 to 14. C) Body weight changes on day 14 post treatment.