# **UNC2025**, a potent and orally bioavailable Mer/$Flt_3$ dual inhibitor

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## Supporting Information

## Synthesis of Analogues

### Experimental

Microwave reactions were carried out using a CEM Discover-S reactor with a vertically-focused IR external temperature sensor and an Explorer 72 autosampler. The dynamic mode was used to set up the desired temperature and hold time with the following fixed parameters: PreStirring, 1 min; Pressure, 200 psi; Power, 200 W; PowerMax, off; Stirring, high. Flash chromatography was carried out with pre-packed silica gel disposable columns. Preparative HPLC was performed with the UV detection at 220 or 254 nm. Samples were injected onto a 75 x 30 mm, 5 μM, C18(2) column at room temperature. The flow rate was 30 mL/min. Various linear gradients were used with A being $H_2O$ + 0.5% TFA and B being MeOH. Analytical thin-layer chromatography (TLC) was performed with silica gel 60 $F_{254}$, 0.25 mm pre-coated TLC plates. TLC plates were visualized using $UV_{254}$ and phosphomolybdic acid with charring. All $^1H$ NMR spectra were obtained with a 400 MHz spectrometer using $CDCl_3$ (7.26 ppm), or $CD_3OD$ (2.05 ppm) as an internal reference. Signals are reported as m (multiplet), s (singlet), d (doublet), t (triplet), q (quartet), and bs (broad singlet); and coupling constants are reported in hertz (Hz). $^{13}C$ NMR spectra were obtained with a 100 MHz spectrometer using $CDCl_3$ (77.2 ppm), or $CD_3OD$ (49.0 ppm) as the internal standard. LC/MS was performed using an analytical instrument with the UV detector set to 220 nm, 254 nm, and 280 nm, and a single quadrupole mass spectrometer using electrospray ionization (ESI) source. Samples were injected (2 μL) onto a 4.6 x 50 mm, 1.8 μM, C18 column at room temperature. A linear gradient from 10% to 100% B (MeOH + 0.1% acetic Acid) in 5.0 min was followed by pumping 100% B for another 2 or 4 minutes with A being $H_2O$ + 0.1% acetic acid. The flow rate was 1.0 mL/min. High-resolution (positive ion) mass spectra (HRMS) were acquired using a LCMS-TOF mass spectrometer.

**Synthetic procedures for Scheme 2:**

**5-Bromo-7-(*trans*-4-((tert-butyldimethylsilyl)oxy)cyclohexyl)-2-chloro-7*H*-pyrrolo[2,3-*d*]pyrimidine** (**5**) To a suspension of 5-bromo-2-chloro-7*H*-pyrrolo[2,3-*d*]pyrimidine (**3**, 0.13 g, 0.50 mmol) and *cis*-4-(*tert*-butyldimethylsilyloxy)cyclohexanol (**4**, 0.23g, 1.0 mmol) in toluene (8.0 mL) was added (cyanomethylene)trimethylphosphorane (CMMP; prepared according to Chem. Pharm. Bull. 2003, 51(4), 474-476.) (6.3 mL, 0.16 M in THF, 1.0 mmol). The resulting clear solution was refluxed for 16 h. The reaction mixture was washed with brine and extracted with EtOAc (3X). The combined organic solution was dried ($Na_2SO_4$) and concentrated. The residue was purified by an ISCO silica gel column to afford the title compound **5** as a white solid (0.16 g, 72%). $^1$H NMR (400 MHz, $CD_3OD$) $\delta$ 8.71 (s, 1H), 7.27 (s, 1H), 4.70 (tt, $J$ = 12.2, 3.9 Hz, 1H), 3.69 (tt, $J$ = 10.5, 4.2 Hz, 1H), 2.09–1.99 (m, 3H), 1.86–1.71 (m, 2H), 1.66–1.54 (m, 3H), 0.90 (s, 9H), 0.08 (s, 6H).

**5-Bromo-*N*-butyl-7-(*trans*-4-((tert-butyldimethylsilyl)oxy)cyclohexyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-2-amine** (**6**) (Method A with reaction conditions in Scheme 3) To a solution of 5-bromo-7-(trans-4-(tert-butyldimethylsilyloxy)cyclohexyl)-2-chloro-7*H*-pyrrolo[2,3-*d*]pyrimidine (82 mg, 0.18 mmol) in isopropyl alcohol (2.0 mL) was added *n*-butylamine (33 mg, 0.45 mmol) in a microwave tube. The resulting solution was heated at 150 $^o$C for 1.5 h. After the reaction cooled to room temperature, the solvent and excess amine was evaporated under vacuum to give the title compound as off white solid, which was used in the next step without further purification.

(Method B with reaction conditions in Scheme 3) To a solution of **5** (50.0 g, 112.9 mmol) in anhydrous 2-propanol (150 mL) was added butylamine (55.8 mL, 564.3 mmol) and DIEA (29.5 mL, 169.3 mmol) at room temperature. The resulting mixture was heated at 95 $^o$C for overnight.

The solvent was removed. The residue was dissolved in EtOAc (400 mL), washed with water (3x), dried ($Na_2SO_4$), and concentrated to afford the tile compound **6** (54 g, 99%) as a white solid. $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ 8.31 (s, 1H), 6.74 (s, 1H), 4.99 (s, 1H), 4.43–4.33 (m, 1H), 3.63–3.54 (m, 1H), 3.40–3.31 (m, 2H), 1.98–1.87 (m, 4H), 1.74–1.60 (m, 2H), 1.57–1.44 (m, 4H), 1.41–1.30 (m, 2H), 0.92–0.85 (m, 3H), 0.82 (s, 9H), 0.00 (s, 6H); $^{13}$C NMR (101 MHz, $CDCl_3$) $\delta$ 164.3, 156.5, 154.5, 125.3, 116.0, 92.7, 75.1, 57.0, 46.1, 39.5, 36.4, 35.4, 30.5, 24.8, 22.8, 18.5, 0.0.

***trans*-4-(2-(Butylamino)-5-(4-(morpholinosulfonyl)phenyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-7-yl)cyclohexan-1-ol** (**2**) (General Procedure A) To a solution of **6** (165 mg, 0.34 mmol) in a mixture of dioxane and $H_2O$ (2.5 mL, 4:1, v/v) was added 4-*N*-morpholinylsulfonyl phenylboronic acid (139 mg, 0.51 mmol), $Pd(PPh_3)_4$ (19.7 mg, 0.02 mmol) and $K_2CO_3$ (94.7 mg, 0.68 mmol). The resulting mixture was heated at 150 $^o$C for 15 min. Then it was diluted with EtOAc (10 mL) at room temperature, filtered through a short pad of Celite®, and concentrated. The residue was purified by an ISCO silica gel column to afford the crude product **8.** A solution of **8** in MeOH (10 mL) was added a 4.0N solution of HCl in dioxane (2.0 mL) at room temperature. After stirred at room temperature for 1.0 h, the solvent was removed under the reduced pressure. The residue was purified by HPLC to afford the title compound **2** (**UNC1666**) as a yellow solid (155 mg, 72% yield over 2 steps). Melting point: 215.4-216.2 $^o$C; $^1$H NMR (400 MHz, $CD_3OD$) $\delta$ 8.85 (s, 1H), 8.04 (s, 1H), 7.88 (d, $J$ = 8.3 Hz, 2H), 7.80 (d, $J$ = 8.3 Hz, 2H), 4.66–4.52 (m, 1H), 3.75–3.63 (m, 5H), 3.54 (t, $J$ = 7.1 Hz, 2H), 3.04–2.90 (m, 4H), 2.19–2.00 (m, 6H), 1.76–1.63 (m, 2H), 1.61–1.41 (m, 4H), 1.01 (t, $J$ = 7.4 Hz, 3H); $^{13}$C NMR (101 MHz, $CD_3OD$) $\delta$ 154.8, 151.3, 139.2, 137.0, 133.4, 128.5, 128.1, 126.7, 115.8, 109.7, 68.5, 65.8, 53.9, 46.0, 40.9, 33.7, 30.6, 29.5, 19.6, 12.7; purity > 98%.

***trans*-4-(2-(Butylamino)-5-(4-((4-methylpiperazin-1-yl)sulfonyl)phenyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-7-yl)cyclohexan-1-ol** (**9**) The title compound **9** (59 mg, 35%) was prepared according to the general procedure A from 1-((2-fluoro-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl)sulfonyl)-4-methylpiperazine (179.7 mg, 0.47 mmol) as a yellow solid. $^{1}$H NMR (400 MHz, $CD_3OD$) $\delta$ 8.94 (s, 1H), 8.16 (s, 1H), 7.95–7.86 (m, 1H), 7.77–7.68 (m, 2H), 4.67–4.55 (m, 1H), 4.13–3.92 (bs, 2H), 3.78–3.68 (m, 1H), 3.68–3.49 (m, 4H), 3.30–3.19 (bs, 2H), 3.18–3.02 (bs, 2H), 2.93 (s, 3H), 2.21–2.01 (m, 6H), 1.78–1.66 (m, 2H), 1.60–1.43 (m, 4H), 1.03 (t, $J$ = 7.4 Hz, 3H); $^{13}$C NMR (101 MHz, $CD_3OD$) $\delta$ 160.6, 158.1, 155.0, 151.3, 140.3, 139.4, 131.7, 129.4, 122.4, 122.2, 114.8, 114.6, 109.4, 68.5, 54.0, 52.9, 42.9, 42.3, 41.0, 33.7, 30.6, 29.6, 19.6, 12.7; purity > 95%.

***trans*-4-(2-((2-Cyclopropylethyl)amino)-5-(2-fluoro-4-((4-methylpiperazin-1-yl)sulfonyl) phenyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-7-yl)cyclohexan-1-ol** (**10**) The title compound **10** (118 mg, 52%) was prepared according to the general procedure A from 5-bromo-7-(*trans*-4-((tert-butyldimethylsilyl)oxy)cyclohexyl)-*N*-(2-cyclopropylethyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-2-amine (200 mg, 0.41 mmol) and 1-((3-fluoro-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl)sulfonyl)-4-methylpiperazine (234 mg, 0.61 mmol) as a yellow solid. $^{1}$H NMR (400 MHz, $CD_3OD$) $\delta$ 8.59 (d, $J$ = 1.4 Hz, 1H), 7.85 (s, 1H), 7.81 (d, $J$ = 8.0 Hz, 1H), 7.57–7.52 (m, 2H), 4.54–4.42 (m, 1H), 3.94–3.68 (m, 2H), 3.69–3.53 (m, 2H), 3.54–3.48 (m, 2H), 3.46–3.29 (m, 2H), 3.14–3.00 (m, 1H), 2.77 (s, 5H), 2.08–1.86 (m, 6H), 1.49 (dd, $J$ = 14.3, 7.1 Hz, 2H), 1.46–1.33 (m, 2H), 0.73–0.63 (m, 1H), 0.41–0.34 (m, 2H), 0.02 (dd, $J$ = 4.8, 1.2 Hz, 2H); $^{13}$C NMR (101 MHz, $CD_3OD$) δ 160.1, 157.6, 154.7, 151.3, 139.9, 135.4, 130.2, 125.3, 124.2, 115.7, 115.4, 109.9, 68.5, 54.1, 52.6, 43.2, 42.1, 41.4, 33.7, 33.5, 29.5, 8.0, 3.4; purity > 98%.

***trans*-4-(2-(Butylamino)-5-(4-((4-methylpiperazin-1-yl)methyl)phenyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-7-yl)cyclohexan-1-ol (11)** The title compound **11** (**UNC2025**) (118 mg, 52%) was prepared according to the general procedure A from 1-methyl-4-[4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl]piperazine (98.5 mg, 0.31 mmol) as a yellow solid. $^{1}$H NMR (400 MHz, $CD_3OD$) $\delta$ 8.82 (s, 1H), 7.96 (s, 1H), 7.80 (d, $J$ = 8.4 Hz, 2H), 7.74 (d, $J$ = 8.4 Hz, 2H), 4.66–4.56 (m, 1H), 4.52 (s, 2H), 3.90–3.58 (m, 9H), 3.55 (t, $J$ = 7.1 Hz, 2H), 3.01 (s, 3H), 2.20–1.99 (m, 6H), 1.79–1.66 (m, 2H), 1.59–1.43 (m, 4H), 1.03 (t, $J$ = 7.4 Hz, 3H); $^{13}$C NMR (101 MHz, $CD_3OD$) $\delta$ 154.6, 151.1, 138.7, 134.0, 132.3, 127.3, 127.0, 126.9, 116.6, 109.9, 68.6, 53.9, 40.9, 33.7, 30.6, 29.5, 19.7, 12.8. MS *m/z* 477.30 $[M+H]^+$; purity: >96%.

***trans*-4-(2-((2-Cyclopropylethyl)amino)-5-(4-((4-methylpiperazin-1-yl)methyl)phenyl)-7*H*-pyrrolo[2,3-d]pyrimidin-7-yl)cyclohexan-1-ol (12)** The title compound **12** (141 mg, 58%) was prepared according to the general procedure A from 5-bromo-7-(*trans*-4-((tert-butyldimethylsilyl)oxy)cyclohexyl)-*N*-(2-cyclopropylethyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-2-amine (200 mg, 0.41 mmol) and 1-methyl-4-[4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl]piperazine (193 mg, 0.61 mmol) as a yellow solid. $^{1}$H NMR (400 MHz, $CD_3OD$) $\delta$ 8.58 (s, 1H), 7.47 (d, J = 8.2 Hz, 2H), 7.28–7.21 (m, 3H), 4.48–4.36 (m, 1H), 3.66–3.53 (m, 1H), 3.47–3.37 (m, 4H), 2.53–2.29 (m, 6H), 2.19 (s, 3H), 2.06–1.97 (m, 2H), 1.96–1.81 (m, 4H), 1.50–1.34 (m, 4H), 1.23–1.09 (m, 1H), 0.90–0.63 (m, 2H), 0.42–0.34 (m, 2H), 0.06–-0.03 (m, 2H); $^{13}$C NMR (101 MHz, $CD_3OD$) $\delta$ 154.6, 151.1, 138.7, 134.0, 132.2, 127.2, 127.0, 116.7, 109.9, 68.5, 59.4, 53.9, 49.9, 42.0, 41.4, 33.7, 33.6, 29.5, 8.0, 3.5; MS *m/z* 489.40 $[M+H]^+$; purity: >96%.

**Synthetic procedures for Scheme 3:**

***trans*-4-((5-Bromo-2-chloropyrimidin-4-yl)amino)cyclohexan-1-ol** (**14**) To a solution of 2,4-dichloro-5-bromopyrimidine (**13**, 51.0 g, 223.8 mmol) and *N,N*-diisopropylethylamine (37.6 g, 290.1 mmol) in anhydrous isopropyl alcohol (125 mL) was slowly added *trans*-4-aminocyclohexanol (26.3 g, 228.3 mmol) in anhydrous isopropyl alcohol (75 mL) at 0 $^{o}$C. The resulting reaction mixture was stirred at 0 $^{o}$C for 4 h, then warmed to room temperature and stirred for another 4 h. Then solvent was removed under reduced pressure. The residue was dissolved in EtOAc (150 mL), washed with water (3x) and brine, dried ($Na_2SO_4$), and concentrated. The title compound **14** was obtained as a white solid (51.3 g) after recrystallization from a mixture of EtOAc and Hexane (1:5). The mother liquid was condensed and purified by an ISCO silica gel column to afford 16.46 g of the title compound **14** as a slightly yellow solid. The overall yield is 99%. $^{1}$H NMR (400 MHz, $CDCl_3$) $\delta$ 8.06 (s, 1H), 5.30 (d, $J$ = 7.7 Hz, 1H), 4.02–3.91 (m, 1H), 3.69–3.57 (m, 1H), 2.28 (s, 1H), 2.14–2.04 (m, 2H), 2.04–1.92 (m, 2H), 1.52–1.36 (m, 2H), 1.38–1.19 (m, 2H); $^{13}$C NMR (101 MHz, $CDCl_3$) $\delta$ 159.3, 158.7, 156.2, 102.9, 69.4, 49.3, 33.6, 30.4.

***trans*-4-((2-Chloro-5-((trimethylsilyl)ethynyl)pyrimidin-4-yl)amino)cyclohexanol** (**15**) To a solution of **14** (8.0g, 26.09 mmol) in anhydrous toluene (70 mL) was added ethynyltrimethylsilane (2.56g, 26.09 mmol), copper (I) iodide (99.2 mg, 0.52 mmol), $Pd(PPh_3)_2Cl_2$ (366 mg, 0.52 mmol) and triethylamine (13.2g, 130.5 mmol) at room temperature. After thoroughly degassing, the resulting mixture was heated under nitrogen atmosphere at 60 ºC for 150 min, then quenched with water, and partitioned with EtOAc. The organic layer was dried ($Na_2SO_4$) and concentrated. The residue was purified by an ISCO silica gel column to afford the title compound **15** (4.8g, 57%) as a white solid. $^{1}$H NMR (400 MHz, CDCl3) $\delta$ 7.80 (s, 1H), 5.19 (d, $J$ = 7.8 Hz, 1H), 3.83–3.62 (m, 1H), 3.50–3.33 (m, 1H), 1.98–1.81 (m, 3H), 1.81–1.66 (m,

2H), 1.31–1.15 (m, 2H), 1.13–0.94 (m, 2H), 0.21–-0.26 (m, 9H); $^{13}$C NMR (101 MHz, CDCl3) $\delta$ 162.2, 159.6, 158.1, 106.5, 101.2, 96.0, 69.6, 49.0, 33.7, 30.6, 0.0.

***trans*-4-(2-Chloro-7*H*-pyrrolo[2,3-*d*]pyrimidin-7-yl)cyclohexan-1-ol** (**16**) To a solution of **15** (75.0 mg, 0.23 mmol) in anhydrous THF (10 mL) was added a 1.0 M THF solution of TBAF (0.58 mL) at room temperature. The resulting mixture was heated at 65 $^{o}$C for 5 h, quenched with water (10 mL), and extracted with EtOAc (3X). The combined organic layers were dried ($Na_2SO_4$) and concentrated. The residue was purified by an ISCO silica gel column to afford the title compound **16** (44.7 mg, 77% yield) as a white solid. $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ 8.70 (s, 1H), 7.20 (d, $J$ = 3.7 Hz, 1H), 6.49 (d, $J$ = 3.7 Hz, 1H), 4.66 (tt, $J$ = 12.1, 3.9 Hz, 1H), 3.72 (tt, $J$ = 11.0, 4.2 Hz, 1H), 2.24 (s, 1H), 2.17–1.85 (m, 4H), 1.86–1.68 (m, 2H), 1.62–1.43 (m, 2H); $^{13}$C NMR (101 MHz, $CDCl_3$) $\delta$ 153.1, 151.5, 150.8, 126.6, 117.9, 100.2, 69.5, 52.5, 34.3, 31.0.

**7-(*trans*-4-((*tert*-Butyldimethylsilyl)oxy)cyclohexyl)-2-chloro-7*H*-pyrrolo[2,3-*d*]pyrimidine** (**17**) To a solution of **16** (226 mg, 0.90 mmol) in anhydrous DMF (15 mL) was added imidazole (152 mg, 2.24 mmol) and TBSCl (203 mg, 1.34 mmol) at 0 $^{o}$C. The resulting mixture was stirred at 0 $^{o}$C for 30 min and at room temperature for another 30 min, quenched with water (10 mL), and extracted with $CH_2Cl_2$ (3X). The combined organic layers were dried ($Na_2SO_4$) and concentrated. The residue was purified by an ISCO silica gel column to afford the title compound (291.3 mg, 89%) as a white solid. $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ 8.67 (s, 1H), 7.19 (d, $J$ = 3.7 Hz, 1H), 6.46 (d, $J$ = 3.7 Hz, 1H), 4.66–4.55 (m, 1H), 3.68–3.58 (m, 1H), 2.03–1.86 (m, 4H), 1.81–1.67 (m, 2H), 1.58–1.45 (m, 2H), 0.82 (s, 9H), 0.00 (s, 6H); $^{13}$C NMR (101 MHz, $CDCl_3$) $\delta$ 157.7, 156.1, 155.3, 131.2, 122.4, 104.6, 74.9, 57.2, 39.3, 35.7, 30.4, 22.7, 0.0.

**5-Bromo-7-(*trans*-4-((tert-butyldimethylsilyl)oxy)cyclohexyl)-2-chloro-7*H*-pyrrolo[2,3-*d*]pyrimidine** (**5**): To a solution of **17** (9.5 g, 26.0 mmol) in anhydrous DMF (50 mL) was added

NBS (4.63 g, 26.0 mmol) in several portions at room temperature. The resulting mixture was stirred at room temperature until the reaction was complete (based on TLC), quenched with water (10 mL), and extracted with $CH_2Cl_2$ (3X). The combined organic layers were dried ($Na_2SO_4$) and concentrated. The residue was purified by an ISCO silica gel column to afford the title compound **5** (10 g, 87% yield) as a white solid. $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ 8.61 (s, 1H), 7.21 (s, 1H), 4.67–4.56 (m, 1H), 3.67–3.57 (m, 1H), 2.03–1.90 (m, 4H), 1.80–1.66 (m, 2H), 1.57–1.44 (m, 2H), 0.81 (s, 9H), -0.00 (s, 6H); $^{13}$C NMR (101 MHz, $CDCl_3$) $\delta$ 158.83, 155.3, 154.9, 130.5, 122.0, 93.0, 74.7, 57.7, 39.2, 35.7, 30.4, 22.7, 0.0.

***N*-Butyl-7-(*trans*-4-((tert-butyldimethylsilyl)oxy)cyclohexyl)-5-(4-((4-methylpiperazin-1-yl)methyl)phenyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-2-amine** (**19**) To a solution of **6** (16.6 g, 34.4 mmol), 4-(4-methylpiperazino)methylphenylboronic acid pinacol ester (**18**, 11.2 g, 35.3 mmol) and $K_2CO_3$ (7.2 g, 51.6 mmol) in a mixture of dioxane and $H_2O$ (120 mL, 5:1, v/v) was added $Pd(PPh_3)_4$ (795 mg, 0.69 mmol) at room temperature. The resulting mixture was heated at 90 $^o$C for 2 h, then diluted with EtOAc (300 mL) at room temperature. The mixture was filtered through a short pad of Celite®, extracted with EtOAc (3X), dried ($Na_2SO_4$), and concentrated. The residue was purified by an ISCO silica gel column (eluent: a mixture of EtOAc and hexane, then a mixture of MeOH and $CH_2Cl_2$) to afford the title compound **19** (17.12 g, 84%) as a white solid. $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ 8.69 (s, 1H), 7.42 (d, $J$ = 8.1 Hz, 2H), 7.25 (d, $J$ = 8.1 Hz, 2H), 6.94 (s, 1H), 5.08 (t, $J$ = 5.7 Hz, 1H), 4.50–4.37 (m, 1H), 3.68–3.55 (m, 1H), 3.44–3.33 (m, 4H), 2.49–2.25 (m, 8H), 2.19 (s, 3H), 2.02–1.87 (m, 4H), 1.83–1.69 (m, 2H), 1.58–1.45 (m, 4H), 1.41–1.30 (m, 2H), 0.92–0.84 (m, 3H), 0.82 (s, 9H), -0.00 (s, 6H); $^{13}$C NMR (101 MHz, $CDCl_3$) $\delta$ 163.7, 157.8, 154.8, 140.7, 138.0, 134.4, 130.8, 123.1, 120.2, 114.6, 75.2, 67.4, 59.7, 57.9, 56.8, 50.6, 46.1, 39.6, 36.5, 35.4, 30.5, 24.8, 22.8, 18.6, 0.0.

***trans*-4-(2-(Butylamino)-5-(4-((4-methylpiperazin-1-yl)methyl)phenyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-7-yl)cyclohexan-1-ol (11)** To a solution of **19** (17.12 g, 28.97 mmol) in a mixture of MeOH and $CH_2Cl_2$ (75 mL, 1:4, v/v) was added a 4.0 M solution of HCl in dioxane (43.5 mL) at room temperature. The resulting mixture was stirred at room temperature for 2 h. The solid was collected by passing through a filter paper, and washed with $^{i}$PrOH (2x). The filtrate was purified by a reversed ISCO silica gel column (eluent: a mixture of MeOH and $H_2O$ with 0.1% TFA). The combined fraction was condensed and the residue was dissolved in DCM/MeOH (2:1, v/v), HCl (4N in dioxane, 18 mL) was added, the resulting mixture was stirred for 2h at room temperature to convert the TFA salt to HCl salt. The combined solid was dissolved in a mixture of $H_2O$ and acetonitrile (15 mL, 25:1, v/v), cooled and lyophilized to give the title compound **11** (**UNC2025**) as a fine yellow powder (15.06 g, 89%). $^1$H NMR (400 MHz, $CD_3OD$) $\delta$ 8.82 (s, 1H), 7.96 (s, 1H), 7.80 (d, $J$ = 8.4 Hz, 2H), 7.74 (d, $J$ = 8.4 Hz, 2H), 4.66–4.56 (m, 1H), 4.52 (s, 2H), 3.90–3.58 (m, 9H), 3.55 (t, $J$ = 7.1 Hz, 2H), 3.01 (s, 3H), 2.20–1.99 (m, 6H), 1.79–1.66 (m, 2H), 1.59–1.43 (m, 4H), 1.03 (t, $J$ = 7.4 Hz, 3H); $^{13}$C NMR (101 MHz, $CD_3OD$) $\delta$ 154.6, 151.1, 138.7, 134.0, 132.3, 127.3, 127.0, 126.9, 116.6, 109.9, 68.6, 53.9, 40.9, 33.7, 30.6, 29.5, 19.7, 12.8. MS *m/z* 477.30 $[M+H]^+$; purity: >99%.

***trans*-4-(2-(Butyl(methyl)amino)-5-(4-((4-methylpiperazin-1-yl)methyl)phenyl)-7*H*-pyrrolo[2,3-d]pyrimidin-7-yl)cyclohexanol (20)** The title compound **20** (185 mg, 76%) was prepared according to the general procedure A from 5-bromo-*N*-butyl-7-(*trans*-4-((*tert*-butyldimethylsilyl)oxy)cyclohexyl)-*N*-methyl-7*H*-pyrrolo[2,3-d]pyrimidin-2-amine (184 mg, 0.484 mmol) and 1-methyl-4-[4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl]piperazine (156 mg, 0.494 mmol) as a yellow solid. $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ 8.63 (s, 1H), 7.88 (s,

1H), 7.71 (d, *J* = 8.0 Hz, 2H), 7.63 (d, *J* = 8.0 Hz, 2H), 4.75-4.38 (m, 1H), 4.38 (s, 2H), 3.72-3.54 (m, 10 H), 3.50 (s, 3H), 3.16-3.15 (m, 1H), 2.92 (s, 3H), 2.06–1.97 (m, 6H), 1.67-1.61 (m, 2H), 1.40–1.34 (m, 4H), 0.95 (t, *J* = 8.0 Hz, 3H); MS *m/z* 491.4 $[M+H]^+$; purity: >95%.

## Selectivity Profiling for 11 (UNC2025)

### $IC_{50}$ determination by Carna Biosciences

**Compound preparation:** Test compound was dissolved in and diluted with dimethylsulfoxide (DMSO) to achieve 100-fold higher concentration as specified for the compound. The solution was further diluted 25-fold with assay buffer to make the final test compound solution. Reference compounds for assay control were prepared similarly.

**Assay reagents and procedures**

Off-chip Mobility Shift Assay (MSA)

1) 5 uL of 4× compound solution, 5 uL of 4× Substrate/ATP/Metal solution, and 10 uL of 2× kinase solution were prepared with assay buffer (20 mM HEPES, 0.01% Triton X-100, 2 mM DTT, pH 7.5) and mixed and incubated in a polypropylene 384 well microplate for 1 or 5 h at room temperature, depending on kinase)

2) 60 uL of Termination Buffer (QuickScout Screening Assist MSA; Cama Biosciences) was added to the well.

3) The reaction mixture was applied to a LabChip3000 system (Caliper Life Science), and the product and substrate peptide peaks were separated and quantitated.

4) The kinase reaction was evaluated by the product ratio calculated from peak heights of product (P) and substrate (S) peptides (P/(P+S)).

**Data Analysis**

The readout value of reaction control (complete reaction mixture) was set as 0% inhibition, and the readout value of background (Enzyme(-)) was set as 100% inhibition, then the percent inhibition of each test solution was calculated.

## ATP ActivX probe assay

### Liquid Chromatography (LC) and Mass Spectrometry (MS) Analysis

LC-MS analysis was performed on a Thermo Fisher LTQ Orbitrap XL with ETD linked to an Easy nLCII pump and autosampler (Proxeon/Thermo Fisher). Each sample (3uL) was injected onto a 50mm Biobasic C18 picofrit column (PF7515-50H052, New Objective Inc.) with a fixed flow rate of 300nL/minute. The total chromatography gradient was 140 minutes: 2%-30% acetonitrile in 0.1% formic acid from 0-80 minutes. MS1 scans were performed in the Orbitrap (300-2000 m/z, 60k resolution, AGC $1\times10^6$). The top five precursors were selected for MS2 analysis. The MS2 scan was performed in the ion trap (CID, AGC 2×104, normalized collision energy 35, max injection time of 100ms, with neutral loss)

### Data Analysis

MS2 Identifications

Mass spectrometry data analysis was performed using Proteome Discoverer 1.4 (PD) and Pinpoint 1.4 by Thermo Fisher Scientific. For MS2 identifications, raw files were search in PD using the SEQUEST algorithm against a freely available database containing the kinase domain of all human kinases (http://kinase.com/kinbase/FastaFiles/). Database searching matched MS/MS spectra with fully tryptic peptides assuming a 10 ppm precursor ion and a product ion tolerance of 0.6 Da. Carbamidomethylation of cysteine residues (+57.021 Da), desthiobiotin of

lysine residues (+196.121), phosphorylation of serine, threonine, or tyrosine residues (+79.966), oxidation of methionine residues (+15.995), and N-terminal acetylation (+42.011) were set as variable modifications. The maximum number of missed cleavages by trypsin was set to two. The data was filtered using Percolator (University of Washington) to a false discovery rate of less than 1%.

Label Free Quantitation

Peptide spectra libraries generated from PD SEQUEST searches were imported into Pinpoint 1.4 if the false discovery rate (FDR) was less than 1% and they contained the desthiobiotin variable modification. Instrument .raw files were imported in quintuplet (3 technical replicates) for each dose [Control, 0.01, 0.1, 1.0, 10, 100, 1000 nM] into Pinpoint for analysis. The four most abundant isotopes of each precursor were selected for targeted statistical analysis (retention time window: +/- 1 minute, peak width: 1 min, minimum signal threshold: 3000). Area was computed on smoothed data (7 smoothing points). The integrated peak area for each peptide was manually confirmed or adjusted. Average peak area for each peptide and dose was determined. From this, the % of control was determined and graphed vs. dose on a semi-log scale using Graphpad Prism. The inhibitor concentration that resulted in the detection of 50% of the control ($IC_{50}$) was then determined for each peptide/protein.

## Calculation of free concentration of inhibitors to reach 90% inhibition

The amount of kinase inhibitor required for 90% inhibition *in vivo* was estimated using Michaelis-Menton kinetic equations. First, the Ki was estimated from the $IC_{50}$ as determined by Carna Biosciences, using a Cheng-Prusoff equation (eq. 1). Next, as a way of approximating the in vivo effectiveness of our compound, the amount of substrate ATP consistent with inhibiting p-

Mer by 50% at 2.7 nM for an inhibitor with a Ki of 193 pM, was determined to be 468.8 uM ATP (eq. 2). Applying this concentration of ATP substrate, along with the corresponding Km for ATP and Ki for inhibition of each enzyme, we calculated the amount of free inhibitor required for 90% inhibition (eq. 3). For our compound **11** (Figure 8) and for reference kinase inhibitors (Figure 9) reported by Carna, the free inhibitor was calculated from knowing the percent protein bound and the Cmax and displayed as a guide across the plots (in red). (It has been noted that Imatinib preferentially inhibits inactive ABL rather than active ABL as presented here)

(eq. 1) $K_i = IC50/(1+[S]/Km)$

Uninhibited velocity;

$V = V_{max}*[S]/(K_m + [S])$

Inhibited velocity;

$V_i = V_{max}*[S]/(Km_{app} + [S])$

Calculate,

$\%\ inhibition = 100 * (1-V_i/V) = 100 * (1-(Km + [S])/(Km_{app} + [S])$

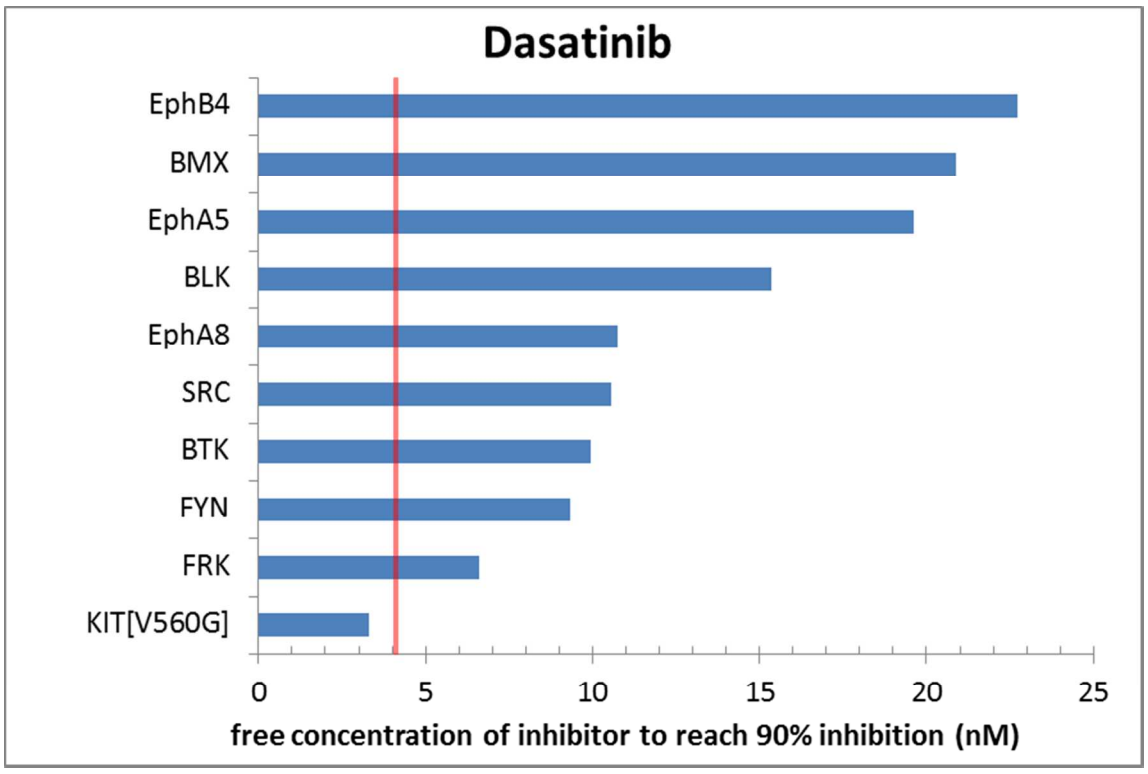
By substituting $Km_{app} = Km(1+[i]/K_i)$

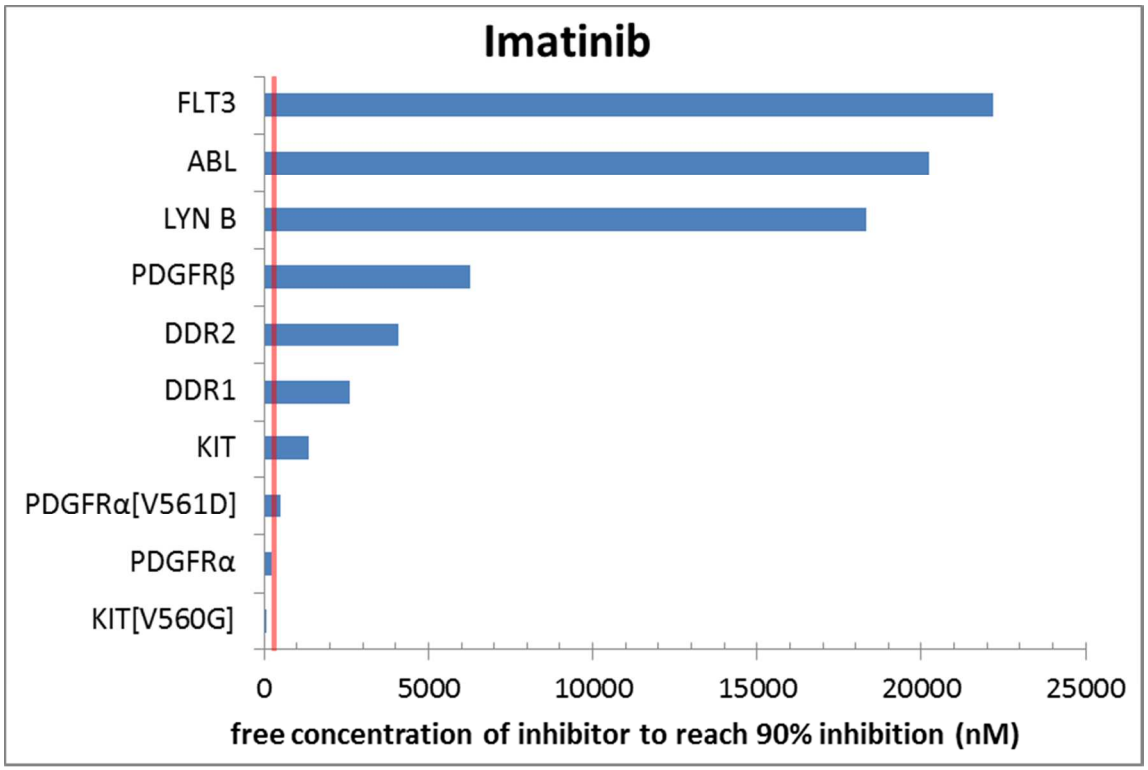
And rearranging this equation to solve for [S], we get;

(eq. 2) $[S]=100* (Km_{app} – Km- Km_{app}*\%inhibition/100)/\%inhibition$

Or, rearranging this equation to solve for [i], we get;

(eq. 3) $[i]= ((((km+[S])/(-\%inhibition/100 + 1)-[S])/Km)-1)*Ki$


Dasatinib
EphB4
BMX
EphA5
BLK
EphA8
SRC
BTK
FYN
FRK
KIT[V560G]
0
5
10
15
20
25
free concentration of inhibitor to reach 90% inhibition (nM)


Imatinib
FLT3
ABL
LYN B
PDGFRβ
DDR2
DDR1
KIT
PDGFRα[V561D]
PDGFRα
KIT[V560G]
0
5000
10000
15000
20000
25000
free concentration of inhibitor to reach 90% inhibition (nM)

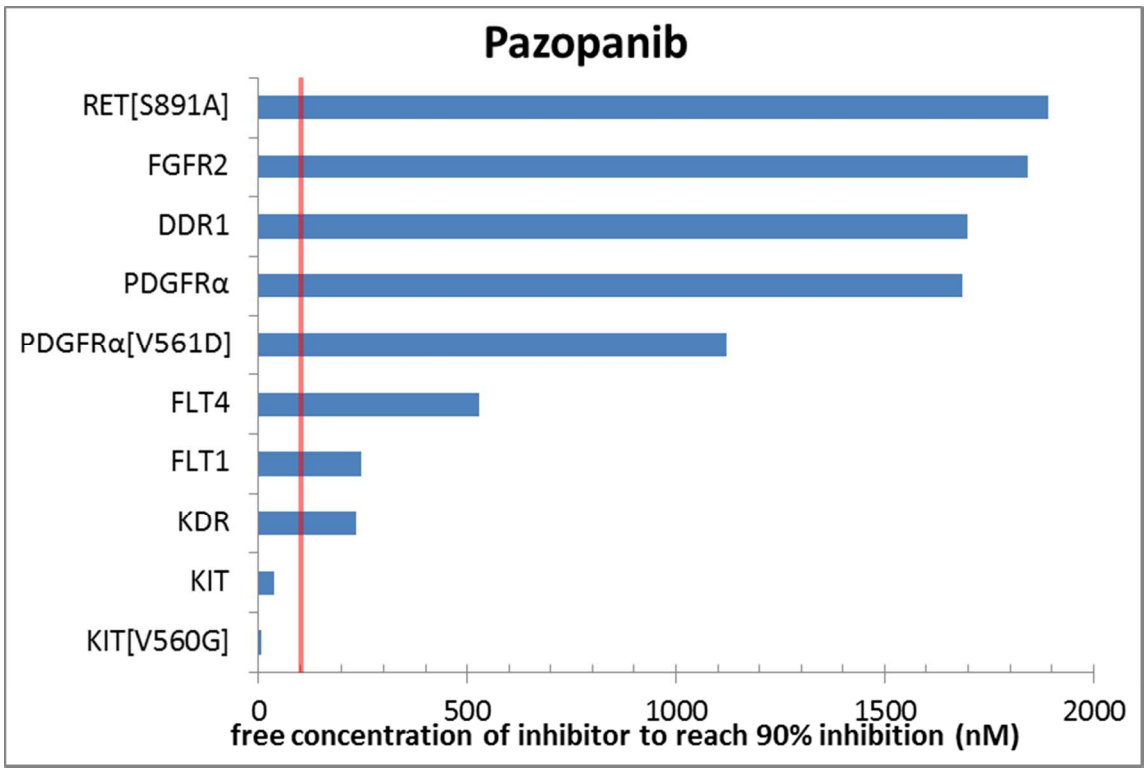



**Figure 9**. The predicted free concentration of dasatinib, imatinib and pazopanib required *in vivo* for 90% inhibition of the top 10 kinases.

## PK Study

A group of 9 male Swiss albino mice (group I) were dosed intravenously (IV) with solution formulation of the desired compound. Another group of 9 male Swiss albino mice (group II) were dosed orally (PO) with suspension formulation of the desired compound in 0.5% (w/v) NaCMC with 0.1% (v/v) Tween-80 in water or with solution in saline. From each mouse, three blood samples (60 µL) were collected from retro orbital plexus such that samples were obtained at 0, 0.08, 0.25, 0.5, 1, 2, 4, 8 & 24 h (iv) & 0, 0.25, 0.5, 1, 2, 4, 6, 8 & 24 h (po) post dose. At each time point blood samples were collected from three mice. Immediately after collection, plasma was harvested by centrifugation of blood and was stored below -70ºC until analysis. All samples were processed for analysis by precipitation using albendazole as internal standard and

analyzed with partially validated MS -MS method (LLOQ was 1.038 ng/mL). Pharmacokinetic parameters were calculated using the Non-compartmental analysis tool of WinNonlin® Enterprise software (version 5.2).

## Plasma Protein Binding

A 1 mM stock solution of the test compound was prepared in DMSO and diluted 200-fold in human/mice/rat plasma to prepare a concentration of 5 μM. The final DMSO concentration in plasma was 0.5%. Rapid equilibrium dialysis was performed with a rapid equilibrium dialysis (RED) device containing dialysis membrane with a molecular weight cut-off of 8,000 Daltons. Each dialysis insert contains two chambers. The red chamber is for plasma while the white chamber is for buffer. A 200 μL aliquot of warfarin or test compound at 5 μM (triplicates) was separately added to the plasma chamber and 350 μL of phosphate buffer saline (pH 7.4) was added to the buffer chamber of the inserts. After sealing the RED device with an adhesive film, dialysis was performed at 37 ºC with shaking at 100 rpm for 4 h. Following dialysis, an aliquot of 50 μL was removed from each well (both plasma and buffer side) and diluted with equal volume of opposite matrix (dialyzed with the other matrix) to nullify the matrix effect. An aliquot of 100 μL was submitted for LC-MS/MS analysis. The peak area ratios of analyte versus internal standard obtained were used to determine the fraction of compound bound to plasma proteins and the corresponding free fraction.