Supporting Information

Discovery of GNF-5837, a selective TRK Inhibitor with efficacy in rodent cancer tumor models

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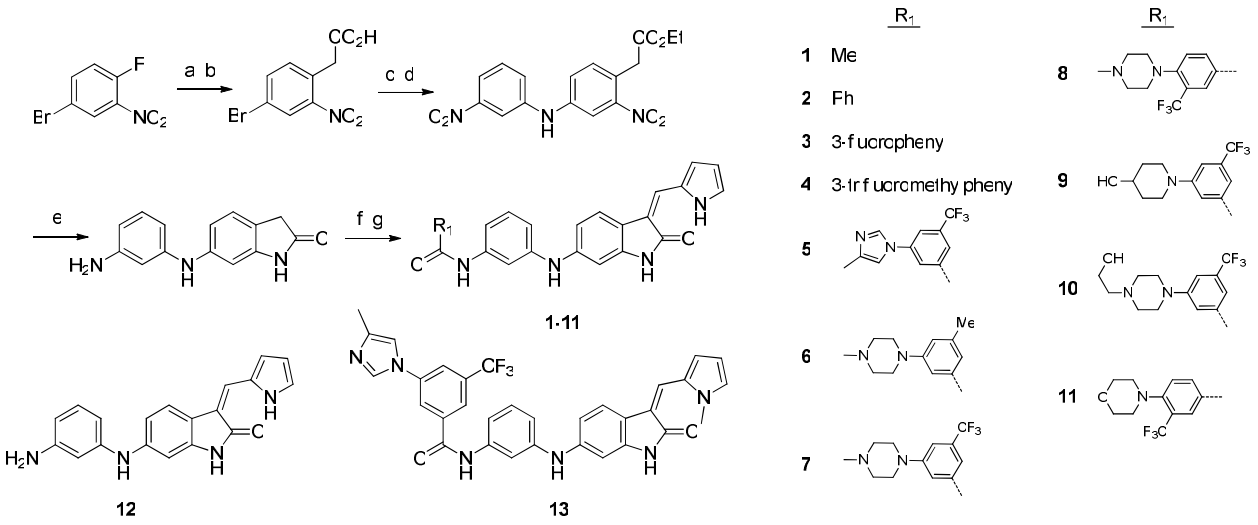
Experimental procedures

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

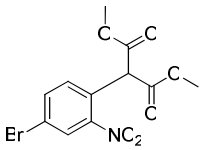
x 2.1 mm ID, 1.8 µm, part number 186003538 (Waters Corp). Eluent A was 0.1% (v/v) TFA in water. Eluent B was 0.1% (v/v) TFA in acetonitrile. All NMR spectra were recorded on a Bruker AVANCE-400 spectrometer operating at a frequency of 400.13 MHz for ^{1}H and 100.61 MHz for ^{13}C equipped with a 5mm 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. Elemental combustion analysis was performed by Midwest Microlab, LLC, Indianapolis, IN.

Scheme 1. Preparation of Oxindole Amides 1-11[a]



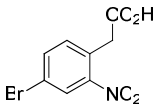
[a] Reagents and conditions: (a) $CH_2(CO_2Me)_2$, NaH, DMSO, 60 °C, 99%. (b) AcOH, HCl, 110 °C, 99%. (c) EtOH, Conc. H_2SO_4, reflux, 96%. (d) 3-Nitroaniline, $Pd(OAc)_2$, Xant phos, Cs_2CO_3, 80 °C, 85%. (e) H_2 (balloon), 10% Pd/C, AcOH, rt, 72%. (f) R-COOH, HATU, DIEA, DMF, rt, 47-99%. (g) 1*H*-pyrrole-2-carbaldehyde, EtOH, 80 °C, 35-91%.

Dimethyl 2-(4-bromo-2-nitrophenyl)malonate



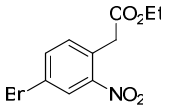
To a solution of dimethyl malonate (37 g, 0.284 mol) in DMSO (100 mL) was added 60% NaH in mineral oil (11.3 g, 0.284 mol) in portions at room temperature. The mixture was warmed to 60 ^{o}C for 15 minutes and then cooled to room temperature. 4-Bromo-1-fluoro-2-nitrobenzene (20.8 g 0.0945mol) was added drop wise to the above solution. The resulting mixture was heated to 60 ^{o}C overnight (approximately 14 hours). The reaction was cooled to room temperature and quenched with aqueous saturated NH_4Cl (100 mL). The mixture was extracted with EtOAc (3x150 mL). The combined organic layers were dried over Na_2SO_4, filtered and concentrated to afford dimethyl 2-(4-bromo-2-nitrophenyl)malonate (57.2 g). The crude product was used in the next step without further purification.

2-(4-Bromo-2-nitrophenyl)acetic acid



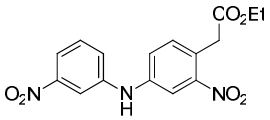
Crude dimethyl 2-(4-bromo-2-nitrophenyl)malonate (57.2 g, 0.0945 mol) was dissolved in 6N HCl (118 mL, 0.709 mol) and acetic acid (120 mL). The solution was heated to 110 ^{o}C overnight. The mixture was cooled to room temperature and all solvents were removed to afford 2-(4-Bromo-2-nitrophenyl)acetic acid. The resulting crude material was used in the next step without further purification.

Ethyl 2-(4-bromo-2-nitrophenyl)acetate



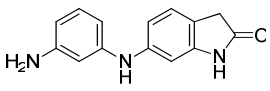
To a solution of crude 2-(4-bromo-2-nitrophenyl)acetic acid (37 g) in EtOH (150 mL) was added concentrated H_2SO_4 (0.5 mL). The reaction was heated to 80 ^oC overnight then cooled to room temperature and all the solvents were removed via rotavap. The mixture was purified by column chromatography with EtOAc/hexane as eluent to yield ethyl 2-(4-bromo-2-nitrophenyl)acetate (26.2 g, 96% yield).

Ethyl 2-(2-nitro-4-(3-nitrophenylamino)phenyl)acetate



A flask was charged with 3-nitroaniline (6.9 g, 0.05 mol), ethyl 2-(4-bromo-2-nitrophenyl)acetate (14.4 g, 0.05 mol), xantphos (868 mg, 1.5 mmol), $Pd(OAc)_2$ (225 mg, 1 mmol), Cs_2CO_3 (23 g, 0.07 mol) and 1,4-dioxane (100 mL). The reaction was heated to 110 ^oC overnight. The mixture was filtered through celite, concentrated and purified by column chromatography with EtOAc/hexane gradient as eluent to yield ethyl 2-(2-nitro-4-(3-nitrophenylamino)phenyl)acetate (14.6 g, 85% yield).

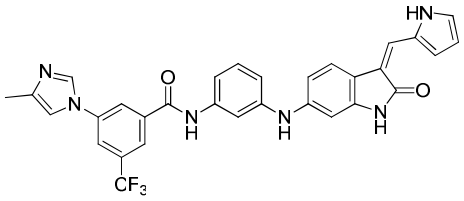
6-(3-Aminophenylamino)indolin-2-one



To a solution of ethyl 2-(2-nitro-4-(3-nitrophenylamino)phenyl)acetate (14.6 g, 0.042 mol) in AcOH (250 mL) was added 10% Pd/C (1.46 g). The mixture was stirred under a hydrgen balloon at room temperature overnight. The reaction was filtered through celite, concentrated and purified by column chromatography with EtOAc/hexane gradient as eluent to yield 6-(3-aminophenylamino)indolin-2-one (5.1 g, 51% yield). ^{1}H NMR (400 MHz, CD_3OD) δ 7.04 (d, *J* = 7.6 Hz, 1 H), 6.96 (t, *J* = 7.6 Hz, 1 H), 6.70 - 6.65 (m, 2 H), 6.52 (t, *J* = 2.0 Hz, 1 H), 6.45 (dd, *J* = 1.2, 7.6 Hz, 1 H), 6.28 (dd, *J* = 1.6, 8.0 Hz, 1 H), 3.41 (s, 2 H). MS *m/z* 240.4 $(M+1)^+$.

General experimental procedure for the synthesis of (1-11) is exemplified by compound 5.

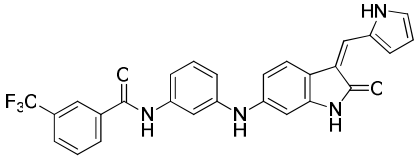
3-(4-Methyl-imidazol-1-yl)-N-{3-[2-oxo-3-(1H-pyrrol-2-ylmethylene)-2,3-dihydro-1H-indol-6-ylamino]-phenyl}-5-trifluoromethyl-benzamide (5)



To a solution of 6-(3-aminophenylamino)indolin-2-one (36 mg, 0.15 mmol) and 3-(4-methyl-imidazol-1-yl)-5-trifluoromethyl-benzoic acid (53 mg, 0.195 mmol) in DMF (1.5 mL) was added DIEA (78 μL, 0.45 mmol) and HATU (63 mg, 0.165 mmol). The reaction was stirred at ambient temperature overnight. The mixture was diluted with EtOAc and washed with 10% $Na_2S_2O_3$ aqueous solution and brine. The organic layer was separated, dried over $MgSO_4$, concentrated and used in the next step without further purification. MS *m/z* 492.1 $(M+1)^+$.

To a suspension of 3-(4-methyl-imidazol-1-yl)-N-[3-(2-oxo-2,3-dihydro-1H-indol-6-ylamino)-phenyl]-5-trifluoromethyl-benzamide (100 mg, 0.2 mmol) in EtOH (5 mL) was added pyrrole-2-carboxaldehyde (23 mg, 0.24 mmol) and piperidine (40 μL, 0.4 mmol). The reaction was heated to 80°C for 2 hours. All solvents were evaporated and the crude product was purified by prep-LC/MS and free-based to afford 3-(4-methyl-imidazol-1-yl)-N-{3-[2-oxo-3-(1H-pyrrol-2-ylmethylene)-2,3-dihydro-1H-indol-6-ylamino]-phenyl}-5-trifluoromethyl-benzamide (**5**) (83 mg, 73.1% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.78 (s, 1 H), 10.43 (s, 1 H), 8.45 – 8.42 (m, 2 H), 8.41 (s, 1 H), 8.24 (s, 1 H), 8.15 (s, 1 H), 7.72 (s, 1 H), 7.69 (s, 1 H), 7.51-7.47 (m, 2 H), 7.30-7.22 (m, 3 H), 6.89-6.85 (m, 1 H), 6.77 (dd, J = 2.0, 8.4 Hz, 1 H), 6.73-6.70 (m, 1 H), 6.67 (d, 1 H), 6.32-6.29 (m, 1 H), 2.19 (s, 3 H). ^{13}C NMR (100 Hz, DMSO-d_6) δ 169.8, 163.1, 143.5, 142.9, 140.2, 139.4, 138.9, 135.2 (d, J = 6.2 Hz), 130.7, (q, J = 322.2 Hz), 129.7, 129.3, 124.8, 124.3, 123.0, 122.6 (d, J = 4.6 Hz), 122.0, 121.8, 119.5, 119.0, 118.6, 117.6, 117.1, 114.2 (d, J = 3.3 Hz), 113.4, 112.2, 110.9, 109.9, 109.0, 98.5, 13.5. HRMS-ESI: Calculated monoisotopic mass (Da) of singly protonated ion with molecular formula $C_{31}H_{23}F_3N_6O_2$; calc, 569.1907; found, 569.1905.

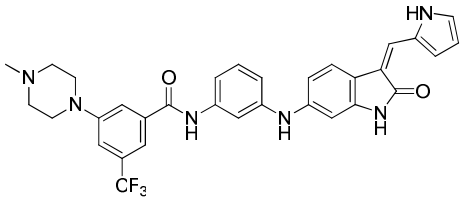
N-(3-((3-((1H-pyrrol-2-yl)methylene)-2-oxoindolin-6-yl)amino)phenyl)-3-(trifluoromethyl)benzamide (4)



^{1}H NMR (DMSO-d_6) δ 10.77 (s, 1 H), 10.40 (s, 1 H), 8.41 (s, 1 H), 8.29 – 8.23 (m, 2 H), 7.97 (d, J = 7.6 Hz 1 H), 7.78 (t, J = 8.0 Hz, 1 H), 7.69 (t, J = 2.0 Hz, 1 H), 7.49 (s, 1 H), 7.48 (d, J = 8.0

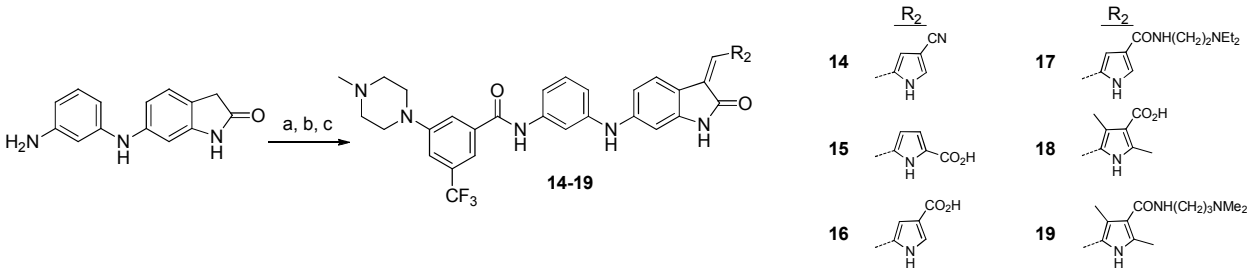
Hz, 1 H), 7.39 - 7.20 (m, 3 H), 6.85 (dt, *J* =1.6, 7.6 Hz, 1 H), 6.75 (dd, *J* = 2.0, 8.4 Hz, 1 H), 6.73 - 6.70 (m, 1 H), 6.67 (d, *J* = 2.0 Hz, 1 H), 6.32 - 6.29 (m, 1 H). ^{13}C NMR (100 Hz, DMSO-d_6) δ 169.8, 163.9, 143.4, 143.0, 140.2, 139.6, 135.8, 131.8, 129.7, 129.6, 129.3, 129.1 (q, *J* = 32.2 Hz), 128.0, 125.3, 124.3, 124.2, 123.9 (q, *J* = 234.8 Hz), 122.9, 119.5, 118.6, 117.6, 117.0, 113.3, 112.2, 110.9, 109.9, 109.1, 98.3. HRMS-ESI: Calculated monoisotopic mass (Da) of singly protonated ion with molecular formula $C_{27}H_{19}F_3N_4O_2$; calc, 489.1533; found, isotopic pattern suggests the presence of two species, representing the expected species (489.1520) as well as a lower mass species of 488.1451, tentatively assigned as the radical cation of $C_{27}H_{18}F_3N_4O_2$ (Calcd 488.1455).

N-(3-((3-((1H-pyrrol-2-yl)methylene)-2-oxoindolin-6-yl)amino)phenyl)-3-(4-methylpiperazin-1-yl)-5-(trifluoromethyl)benzamide (7)



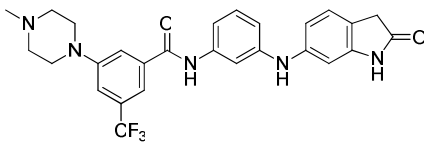
^{1}H NMR (DMSO-d_6) δ 10.77(s, 1 H), 10.27 (s, 1 H), 8.40 (s, 1 H), 7.69 (s, 1 H), 7.67 (s, 1 H), 7.60 (s, 1 H), 7.50 - 7.46 (m, 2 H), 7.37 (s, 1 H), 7.28 - 7.20 (m, 3 H), 6.86-6.82 (m, 1 H), 6.75 (dd, *J* = 2.0, 8.4 Hz, 1 H), 6.73-6.70 (m, 1 H), 6.67 (d, *J* = 1.6 Hz, 1 H), 6.32 - 6.29 (m, 1 H), 3.37 - 3.29 (m, 4 H), 2.50 - 2.45 (m, 4 H), 2.24 (d, 3 H). ^{13}C NMR (100 Hz, DMSO-d_6) δ 169.8, 164.4, 151.2, 143.4, 143.0, 140.2, 139.7, 136.7, 130.0 (q, *J* = 33.8 Hz), 129.7, 129.2, 124.3, 124.1 (q, *J* = 273.4 Hz), 122.9, 119.6, 118.6, 117.7, 117.3, 117.0, 113.4, 113.3, 112.3, 110.9, 109.9, 109.2, 98.3, 54.3, 47.3. HRMS-ESI: Calculated monoisotopic mass (Da) of singly protonated ion with molecular formula $C_{32}H_{29}F_3N_6O_2$; calc, 587.2377; found, 587.2374.

Scheme 2. Preparation of Oxindole Amides with Substituted Pyrroles 14-19[a]



[a] Reagents and conditions: (a) 3-(4-Methylpiperazin-1-yl)-5-(trifluoromethyl)benzoic acid, HATU, DIEA, DMF, rt, 75%. (b) Corresponding aldehyde, EtOH, 80 °C, 49-80%. (c) **16** to **17** and **18** to **19**, corresponding amine, HATU, DIEA, DMF, rt, 45-70%.

3-(4-Methylpiperazin-1-yl)-N-(3-((2-oxoindolin-6-yl)amino)phenyl)-5-(trifluoromethyl)benzamide

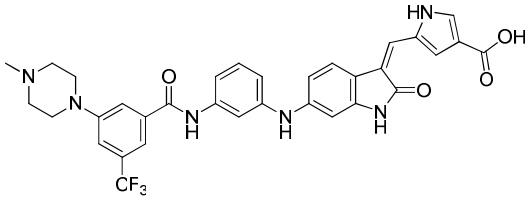


To a solution of 6-(3-amino-phenylamino)-1,3-dihydro-indol-2-one (120 mg, 0.5 mmol) and 3-(4-methylpiperazin-1-yl)-5-(trifluoromethyl)benzoic acid hydrochloride (162 mg, 0.5 mmol) in DMF (2.5 mL) was added DIEA (435 μL, 2.5 mmol) followed by addition of HATU (209 mg, 0.55 mmol). The reaction was stirred at ambient temperature overnight. The mixture was diluted with EtOAc and washed with 10% $Na_2S_2O_3$ aqueous solution and brine. The organic layer was separated, dried over $MgSO_4$ and concentrated. The crude product was purified by column chramatography with DCM/MeOH gradient as eluent to afford 3-(4-methylpiperazin-1-

yl)-N-(3-((2-oxoindolin-6-yl)amino)phenyl)-5-(trifluoromethyl)benzamide (190 mg, 75% yield). MS *m/z* 510.2 $(M+1)^+$.

General experimental procedure for the synthesis of (14-16 and 18) is exemplified by compound 16.

5-((6-((3-(3-(4-Methylpiperazin-1-yl)-5-(trifluoromethyl)benzamido)phenyl)amino)-2-oxoindolin-3-ylidene)methyl)-1H-pyrrole-3-carboxylic acid (16)

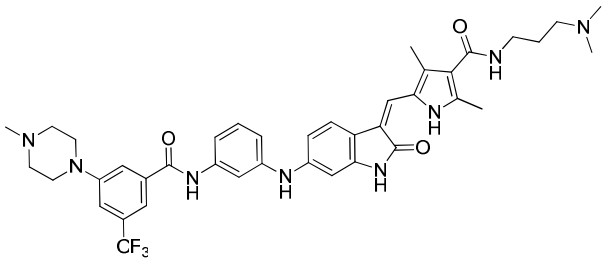


To a suspension of 3-(4-methylpiperazin-1-yl)-N-(3-((2-oxoindolin-6-yl)amino)phenyl)-5-(trifluoromethyl)benzamide (166 mg, 0.327 mmol) in EtOH (20 mL) was added pyrrole-2-carboxaldehyde (50 mg, 0.36 mmol) and piperidine (129 μL, 1.31 mmol). The mixture was heated to 80 ^oC for 2 hours. All solvents were evaporated and the crude product was purified by column chromatography with EtOAc/Hexane gradient as eluent to afford 5-((6-((3-(3-(4-methylpiperazin-1-yl)-5-(trifluoromethyl)benzamido)phenyl)amino)-2-oxoindolin-3-ylidene)methyl)-1H-pyrrole-3-carboxylic acid (**16**) (188 mg, 91.1% yield) and was converted to HCl salt. ^1H NMR (400 MHz, DMSO-d_6) δ 10.89 (s, 1 H), 10.30 (s, 1 H), 8.47 (s, 1 H), 7.73 (s, 1 H), 7.69 (s, 2 H), 7.60 (s, 1 H), 7.50 (s, 1 H), 7.49 (d, *J* = 8.0 Hz, 1 H), 7.37 (s, 1H), 7.25 (s, 1 H), 7.25 (d, *J* = 6.0 Hz, 1 H), 6.99 (s, 1 H), 6.85 (d, *J* = 6.4 Hz, 1 H), 6.76 (dd, *J* = 1.6, 8.4 Hz, 1 H), 6.67 (d, *J* = 1.6 Hz, 1 H), 3.32 (t, *J* = 4.8 Hz, 4 H), 2.47 (t, *J* = 4.8 Hz, 4 H), 2.23 (s, 3 H). ^{13}C NMR (100 Hz, DMSO-d_6) δ 169.8, 165.1, 164.4, 151.2, 143.8, 143.1, 140.7, 139.7, 136.7,

130.3, 130.0 (q, J = 31.2 Hz), 129.2, 128.0, 124.1 (q, J = 271.2 Hz), 122.1, 120.14, 120.12, 118.7, 118.2, 117.3, 116.2, 113.6, 113.4, 112.6, 109.7, 109.6, 98.1, 54.3, 47.3, 45.7. HRMS-ESI: Calculated monoisotopic mass (Da) of singly protonated ion with molecular formula $C_{33}H_{29}F_3N_6O_4$; calc, 631.2275; found, 631.2267. Anal. Calcd for $C_{33}H_{30}ClF_3N_6O_4$: C, 59.42; H, 4.53; N, 12.60; F, 8.54. Found: C, 60.51; H, 4.67; N, 12.67; F, 8.72.

General experimental procedure for the synthesis of (17 and 19) is exemplified by compound 19.

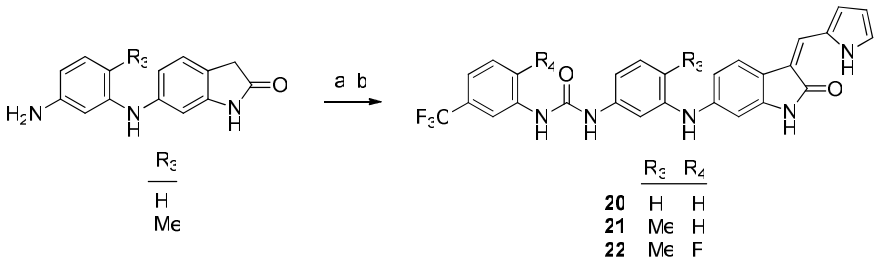
N-(3-(dimethylamino)propyl)-2,4-dimethyl-5-((6-((3-(3-(4-methylpiperazin-1-yl)-5-(trifluoromethyl)benzamido)phenyl)amino)-2-oxoindolin-3-ylidene)methyl)-1H-pyrrole-3-carboxamide (19)



To a solution of 2,4-dimethyl-5-((6-((3-(3-(4-methylpiperazin-1-yl)-5-(trifluoromethyl)benzamido)phenyl)amino)-2-oxoindolin-3-ylidene)methyl)-1H-pyrrole-3-carboxylic acid (**18**) (20 mg, 0.03 mmol) and N,N-dimethylpropane-1,3-diamine (5 µL, 0.033 mmol) in DMF (2.0 mL) was added DIEA (26 µL, 0.15 mmol) and HATU (12 mg, 0.03 mmol). The reaction was stirred at room temperature overnight. The mixture was diluted with EtOAc and washed with 10% $Na_2S_2O_3$ aqueous solution and brine. The organic layer was separated, dried over $MgSO_4$, concentrated and the crude product was purified by prep-LC-MS to afford N-

(3-(dimethylamino)propyl)-2,4-dimethyl-5-((6-((3-(3-(4-methylpiperazin-1-yl)-5-(trifluoromethyl)benzamido)phenyl)amino)-2-oxoindolin-3-ylidene)methyl)-1H-pyrrole-3-carboxamide (**19**) (10.0 mg, 45% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.20-11.08 (bs, 1 H), 10.80 (s, 1 H), 10.42 (s, 1 H), 10.36-10.29 (bs, 1 H), 7.81 (s, 1 H), 7.76-7.68 (m, 3 H), 7.63 (d, *J* = 8.4 Hz, 1 H), 7.49(s, 1 H), 7.41 (s, 1 H), 7.23 (d, *J* = 4.8 Hz, 1 H), 6.86-6.81 (m, 1 H), 6.77 (dd, *J* = 1.6, 8.4 Hz, 1 H), 6.70 (d, *J* = 2.0 Hz, 1 H), 4.10 (d, *J* = 13.2 Hz, 2 H), 3.50 (d, *J* = 11.6 Hz, 2 H), 3.34-3.24 (m, 4 H), 3.20-3.04 (m, 4 H), 2.81 (d, *J* = 4.4 Hz, 3 H), 2.75 (d, *J* = 4.8 Hz, 6 H), 2.44 (s, 3 H), 2.39 (s, 3 H), 1.94-1.86 (m, 2 H). MS *m/z* 743.2 $(M+1)^+$.

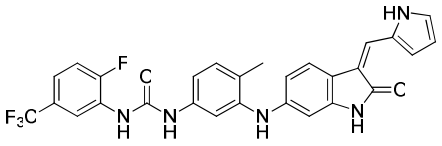
Scheme 3. Preparation of Oxindole Ureas 20-22[a]



[a] Reagents and conditions: (a) Corresponding isocyanate, TEA, THF, rt, 83-94%. (b) 1*H*-pyrrole-2-carbaldehyde, EtOH, 80 °C, 63-87%.

General experimental procedure for the synthesis of (20-22) is exemplified by compound 22.

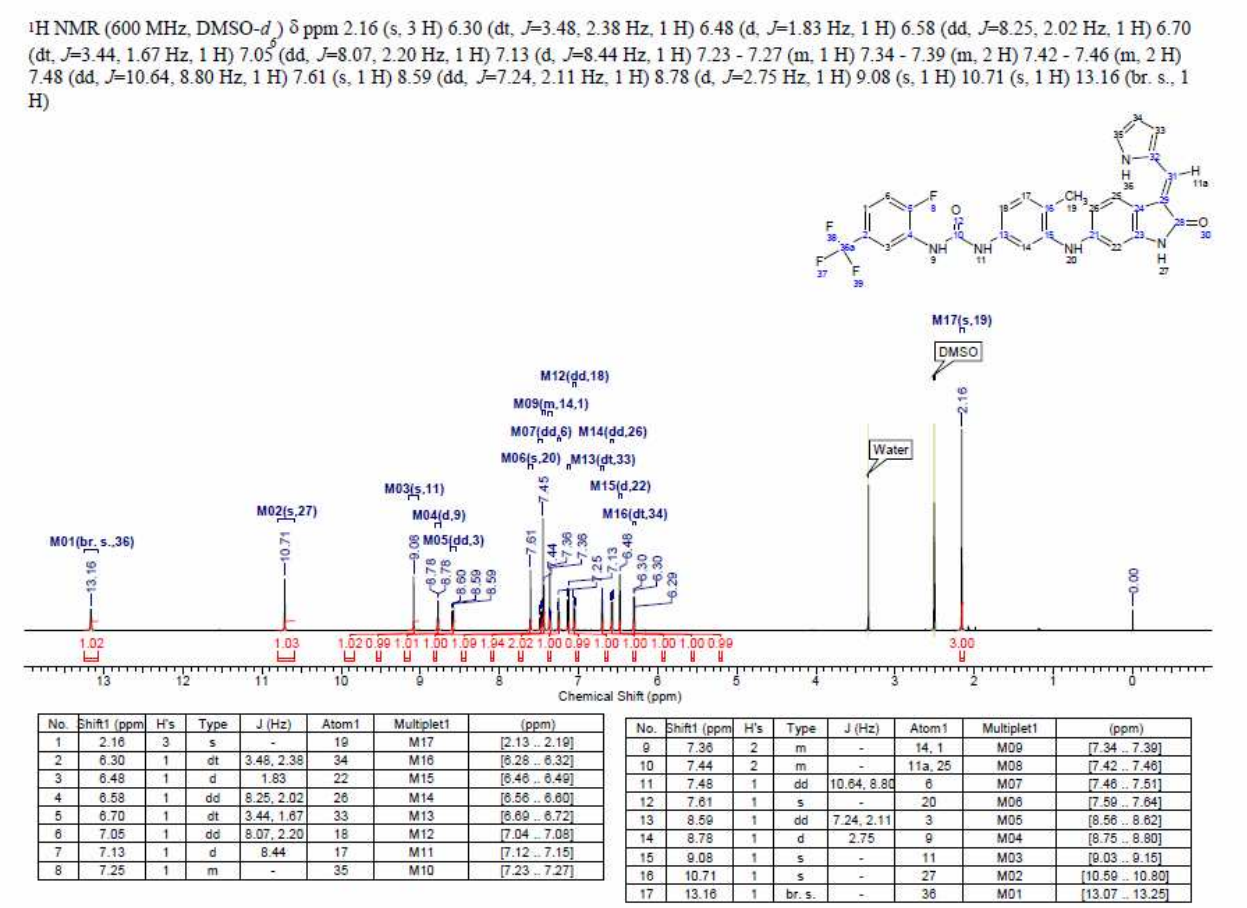
1-(3-((3-((1H-pyrrol-2-yl)methylene)-2-oxoindolin-6-yl)amino)-4-methylphenyl)-3-(2-fluoro-5-(trifluoromethyl)phenyl)urea (22)



To a solution of 6-((5-amino-2-methylphenyl)amino)indolin-2-one (prepared in an identical manner as 6-(3-aminophenylamino)indolin-2-one described above) (2.2 g, 8.69 mmol) in THF (100 mL) was added 1-fluoro-2-isocyanato-4-(trifluoromethyl)benzene (1.55 mL, 10.4 mmol) and TEA (3.6 mL, 26.1 mmol). The reaction was stirred at room temperature overnight then concentrated and purified by column chramatography with EtOAc/Hexance gradient as eluent to afford 1-(2-fluoro-5-(trifluoromethyl)phenyl)-3-(4-methyl-3-((2-oxoindolin-6-yl)amino)phenyl)urea (3.35 g, 84% yield).

To a suspension of 1-(2-fluoro-5-(trifluoromethyl)phenyl)-3-(4-methyl-3-((2-oxoindolin-6-yl)amino)phenyl)urea (3.35 g, 7.3 mmol) in ethanol (75 mL) was added 1H-pyrrole-2-carbaldehyde (764 mg, 8.0 mmol) and piperidine (1.1 mL, 11.0 mmol). The reaction was refluxed for 2 hours, concentrated and purified by column chramatography with EtOAc/Hexane gradient as eluent to afford 1-(3-((3-((1H-pyrrol-2-yl)methylene)-2-oxoindolin-6-yl)amino)-4-methylphenyl)-3-(2-fluoro-5-(trifluoromethyl)phenyl)urea (**22**) (3.1 g, 79% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.16 (s, 1 H), 10.72 (s, 1 H), 9.08 (s, 1 H), 8.78 (d, J = 2.8 Hz, 1 H), 8.58 (dd, J = 2.0, 7.2 Hz, 1 H), 7.62 (s, 1 H), 7.52 - 7.40 (m, 3 H), 7.38 - 7.32 (m, 2 H), 7.27 - 7.23 (m, 1 H), 7.13 (d, J = 8.4 Hz, 1 H), 7.04 (dd, J = 2.4, 8.4 Hz, 1 H), 6.71 - 6.68 (m, 1 H), 6.57 (dd, J = 2.0, 8.4 Hz, 1 H), 6.47 (d, J = 1.6 Hz, 1 H), 6.31 - 6.27 (m, 1 H), 2.15 (s, 3 H). ^{13}C NMR (100 Hz, DMSO-d_6) δ 169.8, 153.3 (d, J = 246.4 Hz), 151.9, 144.8, 141.3, 140.2, 137.4, 131.1, 129.7, 128.9 (d, J = 10.8 Hz), 123.8 (q, J = 275.3 Hz), 125.3 (dd, J = 31.7, 6.7 Hz), 124.1, 123.7, 122.5, 119.4, 119.0, 118.3, 117.8, 116.3, 116.1, 115.9 (d, J = 22.9 Hz), 112.2, 110.8, 110.2, 109.3, 97.6, 17.3. HRMS-ESI: Calculated monoisotopic mass (Da) of singly protonated ion with molecular formula $C_{28}H_{21}F_4N_5O_2$; calc, 536.1704; found, isotopic pattern suggests the presence of two species, representing the expected species (536.1692) as well as a

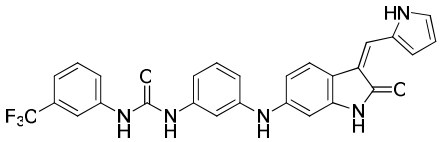
lower mass species of 535.1621, tentatively assigned as the radical cation of $C_{28}H_{20}F_4N_5O_2$ (Calcd 535.1626). Anal. Calcd for $C_{28}H_{21}F_4N_5O_2$: C, 62.80; H, 3.95; N, 13.08; F, 14.19. Found: C, 62.59; H, 4.02; N, 12.93; F, 14.05.



No.	Shift1 (ppm	H's	Type	J (Hz)	Atom1	Multiplet1	(ppm)
1	2.16	3	s	-	19	M17	[2.13 .. 2.19]
2	6.30	1	dt	3.48, 2.38	34	M16	[6.28 .. 6.32]
3	6.48	1	d	1.83	22	M15	[6.46 .. 6.49]
4	6.58	1	dd	8.25, 2.02	26	M14	[6.56 .. 6.60]
5	6.70	1	dt	3.44, 1.67	33	M13	[6.69 .. 6.72]
6	7.05	1	dd	8.07, 2.20	18	M12	[7.04 .. 7.08]
7	7.13	1	d	8.44	17	M11	[7.12 .. 7.15]
8	7.25	1	m	-	35	M10	[7.23 .. 7.27]

No.	Shift1 (ppm	H's	Type	J (Hz)	Atom1	Multiplet1	(ppm)
9	7.36	2	m	-	14, 1	M09	[7.34 .. 7.39]
10	7.44	2	m	-	11a, 25	M08	[7.42 .. 7.46]
11	7.48	1	dd	10.64, 8.80	6	M07	[7.46 .. 7.51]
12	7.61	1	s	-	20	M06	[7.59 .. 7.64]
13	8.59	1	dd	7.24, 2.11	3	M05	[8.56 .. 8.62]
14	8.78	1	d	2.75	9	M04	[8.75 .. 8.80]
15	9.08	1	s	-	11	M03	[9.03 .. 9.15]
16	10.71	1	s	-	27	M02	[10.59 .. 10.80]
17	13.16	1	br. s.	-	36	M01	[13.07 .. 13.25]

1-(3-((3-((1H-pyrrol-2-yl)methylene)-2-oxoindolin-6-yl)amino)phenyl)-3-(3-(trifluoromethyl)phenyl)urea (20)



^{1}H NMR (400MHz, DMSO-d_6) δ 10.76 (s, 1 H), 8.99 (s, 1 H), 8.76 (s, 1 H), 8.36 (s, 1 H), 8.02 (s, 1H), 7.56 (d, *J*= 8.4 Hz, 1 H), 7.52 (d, *J* = 8.0 Hz, 1 H), 7.48 (s, 1 H), 7.47 (d, *J* = 8.4 Hz, 1

H), 7.37 (t, *J* = 2.0 Hz, 1 H), 7.30 (d, *J* = 7.6 Hz, 1 H), 7.26 (dd, *J* = 2.4, 3.6 Hz, 1 H), 7.16 (t, *J* = 8.0 hz, 1 H), 6.90 (dd, *J* = 1.2, 8.0 Hz, 1 H), 6.77 - 6.70 (m, 3 H), 6.66 (d, *J* = 2.0 Hz, 1 H), 6.32 - 6.29 (m, 1 H). ^{13}C NMR (100 Hz, DMSO-d_6) δ 169.8, 153.2, 143.5 143.1, 140.5, 140.2, 129.9, 129.7, 129.4, 129.4 (q, *J* = 31.5 Hz), 124.2, 124.1 (q, *J* = 278.3 Hz), 122.8, 121.6, 119.6, 118.5, 118.0, 117.6, 116.8, 114.0, 111.1, 110.8, 110.2, 109.7, 107.3, 98.1. HRMS-ESI: Calculated monoisotopic mass (Da) of singly protonated ion with molecular formula $C_{27}H_{20}F_3N_5O_2$; calc, 504.1642; found, isotopic pattern suggests the presence of two species, representing the expected species (504.1630) as well as a lower mass species of 503.1553, tentatively assigned as the radical cation of $C_{27}H_{19}F_3N_5O_2$ (Calcd 503.1564). Anal. Calcd for $C_{27}H_{20}F_3N_5O_2 \cdot 0.5H_2O$: C, 63.28; H, 4.13; N, 13.67; F, 11.12. Found: C, 63.21; H, 4.19; N, 13.57; F, 11.02.

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 and RIE cells co-expressing full length TrkA and mNGF compared with parental Ba/F3 or RIE cells**.**

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.

Inhibition of c-kit dependent proliferation - Mo7e assay

Compounds were tested for inhibition of SCF dependent proliferation using human Mo7e cells in a 384 well format. Three-fold serially diluted test compounds (Cmax=10 mM) were evaluated for their antiproliferative activity of Mo7e cells stimulated with human recombinant SCF. After 48 hours of incubation at 37 ^{o}C, cell viability was measured by adding 25 uL of CellTiter Glo (Promega) to the cells and the luminescence was measured by a CLIPR CCD camera (Molecular Devices).

Inhibition of PDGFR dependent proliferation - Rat A10 assay

Rat A10 cells (ATCC) were resuspended in DMEM supplemented with 1% FBS and 10 ng/mL recombinant rat PDGF-BB at 20,000 cells/mL. The cells were aliquoted into 384 well plates at

50 μL/well and incubated for 4 hours at 37^{0}C. 0.5 μL of test compound 3-fold serially diluted in

DMSO was added to each well. The plates were returned to the incubator for a further 68 hours. 25 µL of CellTiter-Glo (Promega) was added to each well and the plates were incubated on the bench for 15 minutes. Luminescence was then read using a CLIPR CCD camera (Molecular Devices).

Rie-TRKA/NGF xenograft tumor model

3 x 10^6 of Rie cells expressing TrkA and NGF were implanted with a subcutaneous injection into the right hind flank of a Balb/c nude mouse. After implant, once tumors became palpable, animals were dosed with the vehicle, 25 mg/Kg, 50 mg/Kg, or 100 mg/Kg of GNF-5837 using oral gavages once a day for 10 days. Tumor volumes were measured by a caliper 3 times per week and were calculated using (L x W x H)/2.

Pharmacokinetic studies

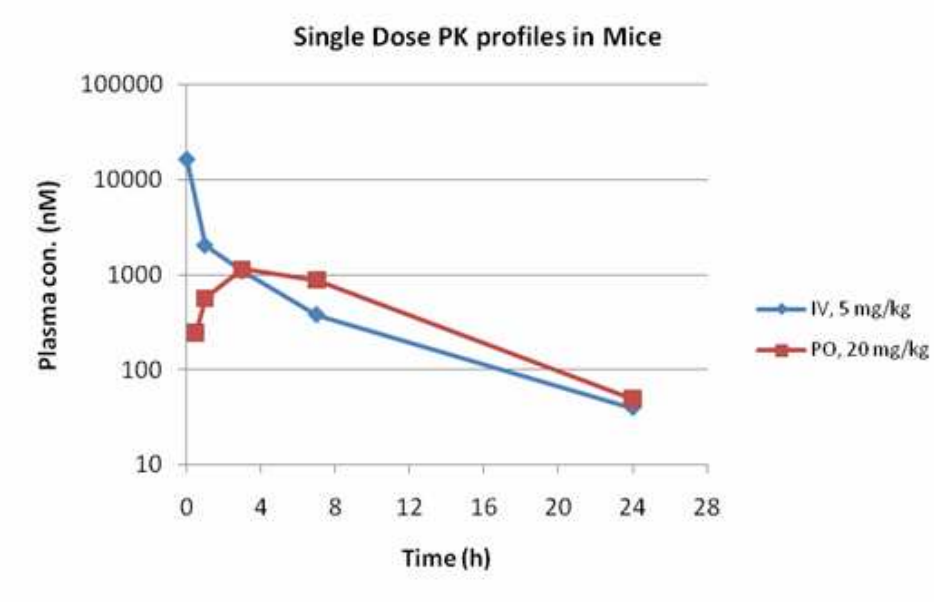
All animal related procedures at GNF were conducted under an IACUC approved protocol in compliance with Animal Welfare Act regulations and the Guide for the Care and Use of Laboratory Animals.

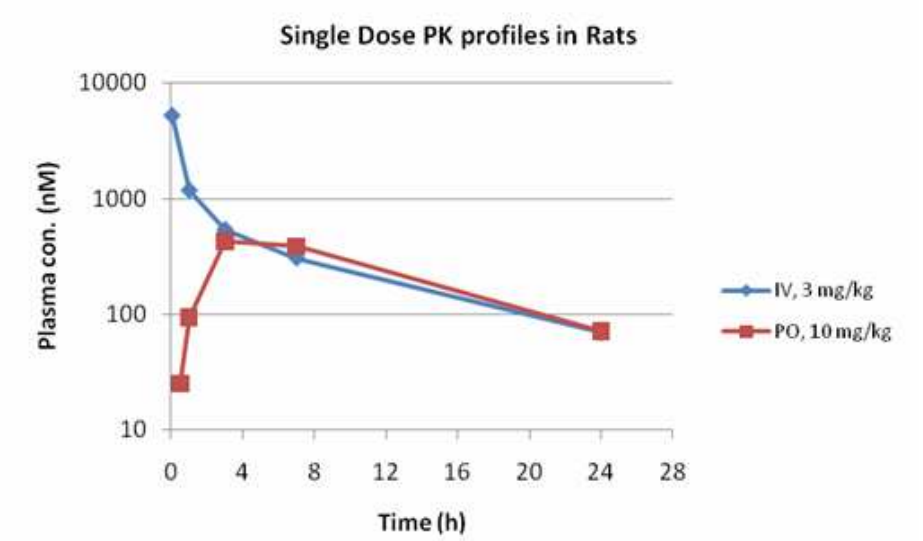
To determine the general PK properties, naïve male Balb/C mice (n=3, body weights 23-25 g, Taconic, Hudson, NY, USA) were administered 5 mg/kg GNF-5837 (free base) intravenously via the lateral tail vein or 20 mg/kg via oral gavage. Likewise, male Sprague-Dawley rats (n=3, body weights 230-280 g) were dosed GNF-5837 (free base) at 3 mg/kg i.v. or 10 mg/kg p.o. The dosing volumes were 3 and 10 ml/kg, respectively. The dosing solutions consisted of 2.0 to 2.5 mg/ml GNF-5837 in 75% polyethylene glycol 300 (PEG300) and 25% of dextrose (5 mg/ml) in distilled water. Five blood samples of 50 µl (mice) or 100 µl (rats) were collected serially at 2

min (i.v. only), 0.5 h (p.o. only), and 1, 3, 7 and 24 h after dosing. The blood samples were centrifuged and plasma separated and frozen until analysis by LC/MS/MS.

Distribution of GNF-5837 to brain was studied in mice (n=8) after single oral dose administration at 20 mg/kg, dosed in a vehicle of 75% polyethylene glycol 300 (PEG300) and 25% of dextrose (5 mg/ml). At allotted times after dosing (0.5 1, 3 and 7 h), two mice per dosing group were sacrificed, and blood and whole brain were collected. Blood was centrifuged and the plasma separated out for analysis. Brain samples were mixed with 4-fold weights of 5% acetonitrile in water and then homogenized. Plasma and brain concentrations were quantified using a Liquid Chromatography/Mass Spectrometry (LC/MS/MS) assay with a MDS SCIEX API-4000 triple quadruple mass spectrometer (MDS Inc., Toronto, Canada) coupled to a HPLC chromatograph (Agilent HP1100 Series, Agilent Technologies, Spain). The lower limit of quantitation (LLOQ) was 2 nM in plasma and 10 nM in brain tissue. Mean pharmacokinetic parameters were calculated by non-compartmental regression analysis using Winnonlin 4.3 software (Pharsight, Mountain View, CA, USA).

The mouse PK studies demonstrated that GNF-5837 exhibited low clearance (CL=8.2 ml/min/kg, ~10% of hepatic blood flow in mice), moderate volume of distribution (V_{ss}=1.4 l/kg), and moderate terminal half-life ($T_{1/2}$=4.4 h). The oral bioavailability in mice was 18%. The brain concentration after a 20 mg/kg oral dose was below limit of quantitation (< 10 nM) with corresponding maximum plasma levels of 2 μM, indicating negligible brain distribution of GNF-5837. In rats, GNF-5837 exhibited low clearance (CL= 9.0 ml/min/kg, ~20% of hepatic blood flow), moderate volume of distribution (V_{ss}=3.6 l/kg), and moderate terminal half-life ($T_{1/2}$=7.5 h). The oral bioavailability was 19%.





TrkC Crystallography

The recombinant kinase domain consisting of residues 530-839 of TrkC (Genbank: NM_002530) 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 **20**. 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 2.3 Å were collected on Beamline 5.0.3 at the ALS, Berkeley, and the structure was solved by molecular replacement using an in-house TrkC crystal structure as starting model.

Additional Data for GNF-5837 (22)

Table 1. Summary of cellular data for compound **22** (IC_{50}, μM)

Assay	**22**	Ba/F3 [a]	**22**
Ba/F3 [a]-ABL [b]	8.2	Mo7e (c-kit)	1.0
Ba/F3 ¯ALK[c]	6.8	Ba/F3 ¯MET	5.8
Ba/F3 ¯ALK	3.4	Ba/F3 ¯LYN	7.3
Ba/F3 ¯BMX	6.7	Ba/F3 ¯PDGFRβ	0.87
Ba/F3 ¯EPHA3	5.9	RatA10 (PDGFR)	0.5
Ba/F3 ¯EPHB2	5.8	Ba/F3 ¯RET	5.3
Ba/F3 ¯FGFR3	9.3	Ba/F3 ¯RON	4.7
Ba/F3 ¯FGFR4	7.0	Ba/F3 ¯ROS	6.0
Ba/F3 ¯FGR	3.6	Ba/F3 ¯SRC	7.3
Ba/F3 ¯FLT1	4.4	Ba/F3 ¯TIE1	5.7
Ba/F3 ¯FLT3	5.1	Ba/F3 ¯TRKA	0.011
Ba/F3 ¯FMS	4.0	Ba/F3 ¯TRKB	0.009
Ba/F3 ¯IGF1R	7.6	Ba/F3 ¯TRKC	0.007
Ba/F3 ¯INSR	8.0	Ba/F3 ¯TRKA-NGF	0.042
Ba/F3 ¯JAK2	2.9	RIE[d]-TRKA-NGF	0.017
Ba/F3 ¯KDR	3.0	Ba/F3 ¯ZAP70	4.7

Ba/F3 -KIT	0.91

[a] Ba/F3 cells rendered IL-3 independent by stable transduction with the indicated kinase fused with a Tel dimerization partner unless otherwise specified, [b] Bcr-abl , [c] NMP-ALK., [d]Rat intestinal epithelial

Table 2. Compound **22** Kinase Selectivity in a Biochemical Kinase Panel (Invitrogen)

Kinase	% Inhib.[a]	Kinase	% Inhib.[a]	Kinase	% Inhib.[a]	Kinase	% Inhib.[a]
ABL1	26	DYRK1A	0	KDR	9	PDK1	1
ABL1 T315I	2	EGFR	-6	KIT	1	PIM2	-2
ACVR1B	-7	EPHB4	4	LCK	21	PLK1	-8
AKT1	2	FES	-6	LYNA	24	PRKACA	-6
BMX	-5	FGFR3	-5	MAP2K1	14	PRKCA	10
BRAF	15	FLT3	37	MAP2K6	4	PTK2 (FAK)	-4
BRAF V599E	10	GRK4	-7	MAP3K8	13	ROCK2	1
BTK	0	GSK3B	5	MAPK1	-1	RPS6KA1	5
CAMK2A	5	IGF1R	-4	MAPK13	5	RPS6KB1	1
CDK1/cyclin A	-1	IKBKB	2	MAPK14	5	STK22D	9
CDK1/cyclin B	5	INSR	0	MAPK3	4	STK6	4
CHEK2	4	IRAK4	3	MET	4	SYK	-2
CSF1R	22	ITK	2	NEK2	-2	TEK	-4
CSNK1A1	-5	JAK2	-3	PAK3	9	ZAP70	-4
CSNK2A1	0	JAK3	-5	PDGFRA	15		

[a] Values indicate percentage of kinase activity inhibition using compound **22** at 10 μM 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: phenacetin (CYP1A2), 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-177 was found to inhibit CYP3A4_II (3.2 μM) when testosterone was used as probe substrate, suggesting a potential for a drug-drug interaction with compounds cleared exclusively or predominantly through CYP3A4-mediated metabolism. GNF-177 did not significantly inhibit CYP1A2, CYP2C9, CYP2C19, CYP2D6 or CYP3A4_I metabolism, indicating minimal drug-drug potential with compounds cleared predominantly via these metabolic pathways.

Table 3. Inhibition of CYP Isoenzymes

Compound	**CYP1A2**	**CYP2C9**	**CYP2C19**	**CYP2D6**	**CYP3A4_I**	**CYP3A4_II**
22	12.96	>10	>10	>10	>10	3.2

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-5837 (**22**) 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. **22** showed a low intrinsic clearance (CLint < 50 µl/min/mg) in mouse, rat and human liver microsomes. The results suggest that **22** is quite stable in hepatic microsomes.

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

Compound	**CL(int) Human**	**CL(int) Mouse**	**CL(int) Rat**
22	<6.926±0	<12.465±7.818	8.306±1.131

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

Genetic toxicity: MiniAmes

Compound **22** was tested for bacterial reverse mutation in a miniscreen Ames test, at concentrations of 1, 3, 10, 30 and 100 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 **22** showed weak binding activity with IC_{50} values of 3.29 µM. In the hERG patch clamp test using stably transfected HEK293 cells, compound **22** did not show inhibition of hERG channel activity (IC_{50} > 30 µM).

High Throughput Solubility Data for Compound 1-22

The protocol for this assay is described: Zhou, L; Yang, L.; Tilton, S.; Wang, J. Development of a High Throughput Equilibrium SolubilityAssay Using Miniaturized Shake-Flask Method in Early Drug Discovery.J. Pharm. Sci, 2007, *96*, 3052-3071.

Table 5. High throughput solubility data

Compound	HT Solubility at pH 6.8 (μM)
1	4.5
2	<2
3	ND
4	<2
5	<3.75
6	<2
7	3.6
8	<2
9	<2
10	5.7
11	ND
12	<3.1
13	<2
14	<2
15	3
16	ND
17	>175
18	<2
19	>100
20	<2.5
21	<19.5
22	<2