Supporting Information

Design and synthesis of $mGlu_2$ NAMs with improved potency and CNS penetration based on a truncated picolinamide core

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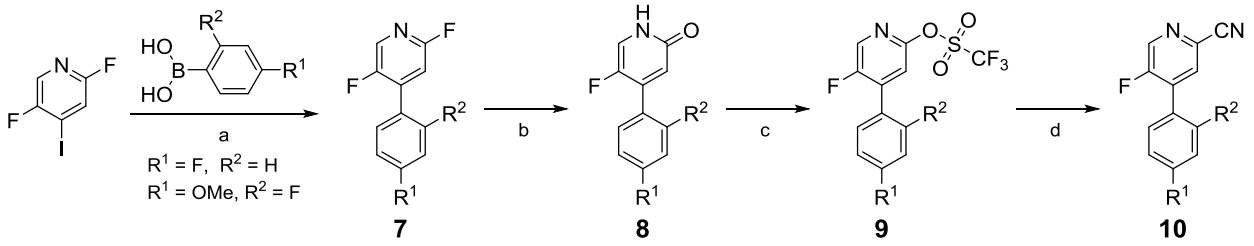
Experimental Procedures and Spectroscopic Data

General. All NMR spectra were recorded on a 400 MHz AMX Bruker NMR spectrometer. ^{1}H and ^{13}C chemical shifts are reported in δ values in ppm downfield with the deuterated solvent as the internal standard. Data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, b = broad, m = multiplet), integration, coupling constant (Hz). Low resolution mass spectra were obtained on an Agilent 6120 or 6150 with ESI source. Method A: MS parameters were as follows: fragmentor: 70, capillary voltage: 3000 V, nebulizer pressure: 30 psig, drying gas flow: 13 L/min, drying gas temperature: 350 ºC. Samples were introduced via an Agilent 1290 UHPLC comprised of a G4220A binary pump, G4226A ALS, G1316C TCC, and G4212A DAD with ULD flow cell. UV absorption was generally observed at 215 nm and 254 nm with a 4 nm bandwidth. Column: Waters Acquity BEH C18, 1.0 x 50 mm, 1.7 um. Gradient conditions: 5% to 95% CH_3CN in H_2O (0.1% TFA) over 1.4 min, hold at 95% CH_3CN for 0.1 min, 0.5 mL/min, 55 ºC. Method B: MS parameters were as follows: fragmentor: 100, capillary voltage: 3000 V, nebulizer pressure: 40 psig, drying gas flow: 11 L/min, drying gas temperature: 350 ºC. Samples were introduced via an Agilent 1200 HPLC comprised of a degasser, G1312A binary pump, G1367B HP-ALS, G1316A TCC, G1315D DAD, and a Varian 380 ELSD (if applicable). UV absorption was generally observed at 215 nm and 254 nm with a 4 nm bandwidth. Column: Thermo Accucore C18, 2.1 x 30 mm, 2.6 um. Gradient conditions: 7% to 95% CH_3CN in H_2O (0.1% TFA) over 1.6 min, hold at 95% CH_3CN for 0.35 min, 1.5 mL/min, 45 ºC. High resolution mass spectra were obtained on an Agilent 6540 UHD Q-TOF with ESI source. MS parameters were as follows: fragmentor: 150, capillary voltage: 3500 V, nebulizer pressure: 60 psig, drying gas flow: 13 L/min, drying gas temperature: 275 ºC. Samples were introduced via an Agilent 1200 UHPLC comprised of a G4220A binary pump, G4226A

ALS, G1316C TCC, and G4212A DAD with ULD flow cell. UV absorption was observed at 215 nm and 254 nm with a 4 nm bandwidth. Column: Agilent Zorbax Extend C18, 1.8 µm, 2.1 x 50 mm. Gradient conditions: 5% to 95% CH_3CN in H_2O (0.1% formic acid) over 1 min, hold at 95% CH_3CN for 0.1 min, 0.5 mL/min, 40 ºC. Optical specific rotations were obtained using JASCO P-2000 Digital Polarimeter equipped with Tungsten-Halogen lamp (WI), 589 nm wavelength, photomultiplier tube (1P28-01) detector and CG2-100 Cylindrical glass cell, 2.5ø x 100 mm. For compounds that were purified on a Gilson preparative reversed-phase HPLC, the system comprised of a 333 aqueous pump with solvent-selection valve, 334 organic pump, GX-271 or GX-281 liquid hander, two column switching valves, and a 155 UV detector. UV wavelength for fraction collection was user-defined, with absorbance at 254 nm always monitored. Method 1: Phenomenex Axia-packed Luna C18, 30 x 50 mm, 5 µm column. Mobile phase: CH_3CN in H_2O (0.1% TFA). Gradient conditions: 0.75 min equilibration, followed by user defined gradient (starting organic percentage, ending organic percentage, duration), hold at 95% CH_3CN in H_2O (0.1% TFA) for 1 min, 50 mL/min, 23 ºC. Method 2: Phenomenex Axia-packed Gemini C18, 50 x 250 mm, 10 um column. Mobile phase: CH_3CN in H_2O (0.1% TFA). Gradient conditions: 7 min equilibration, followed by user defined gradient (starting organic percentage, ending organic percentage, duration), hold at 95% CH_3CN in H_2O (0.1% TFA) for 7 min, 120 mL/min, 23 ºC. Chiral separation was performed on a Thar (Waters) Investigator SFC Column: Chiral Technologies CHIRALPAK IF, 4.6 x 250 mm, 5 µm column. Gradient conditions: 20% to 50% IPA in CO_2 over 7 min, hold at 50% CO_2 for 1 min. Flow rate: 3.5 mL/min. Column temperature: 40 ºC. System backpressure: 100 bar. Solvents for extraction, washing and chromatography were HPLC grade. All reagents were purchased from Aldrich Chemical Co. and were used without purification.

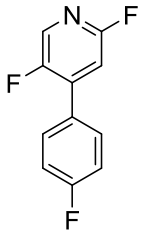
Experimental Procedures and Spectroscopic Data

Preparation of Intermediate 10. The referenced intermediate was prepared via the route pictured immediately below.



Reagents and Conditions: (a) $PdCl_2$(dppf), 1M aq. Na_2CO_3, DME, 100°C; (b) AcOH, H_2O, 130°C; (c) *N*-Phenyl triflimide, TEA, DCM, DMF, 0°C; (d) $Zn(CN)_2$, Tetrakis, DMF, microwave 140°C, 15 min.

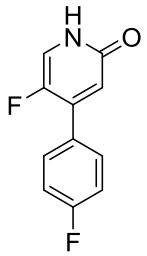
2,5-Difluoro-4-(4-fluorophenyl)pyridine (7)



2,5-difluoro-4-iodo-pyridine **6** (5.00 g, 20.7 mmol, 1.0 eq), 4-fluorophenylboronic acid (6.10 g, 43.6 mmol, 2.1 eq), [1,1'-bis(diphenylphosphino)-ferrocene]dichloropalladium(II) (1.52 g, 2.07 mmol, 0.10 eq) and 1M aqueous sodium carbonate (50.0 mL, 50.0 mmol, 2.4 eq) were dissolved in dimethoxyethane (100 mL) in a round-bottom flask. The reaction was heated at 100 ºC overnight and then cooled and diluted with ethyl acetate. The mixture was washed with water and brine, and the aqueous phase was back-extracted with ethyl acetate. The combined organic layers were dried (MgSO4), filtered, and concentrated *in vacuo*. Purification by flash liquid chromatography on silica gel afforded 4.00 g (92%) of the title compound **7** as a white solid. ^{1}H

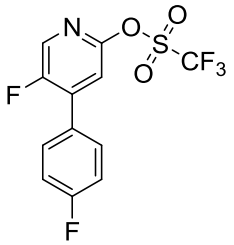
NMR (400 MHz, $CDCl_3$) δ= 8.12 (t, *J* = 1.64 Hz, 1H), 7.63-7.57 (m, 2H), 7.23-7.17 (m, 2H), 7.00 ppm (q, *J* = 2.56 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): *δ* = 163.77 (d, *J*(C,F) = 250.81 Hz), 159.85 (dd, *J*(C,F) = 236.53, 1.67 Hz), 154.72 (dd, *J*(C,F) = 251.52, 4.76 Hz), 140.68 (q, *J*(C,F) = 8.57 Hz), 135.62 (q, *J*(C,F) = 16.89 Hz), 130.82 (dd, *J*(C,F) = 8.53, 3.61 Hz), 128.42-128.34 (m), 116.30 (d, *J*(C,F) = 21.89 Hz), 109.45 ppm (d, *J*(C,F) = 41.64 Hz). HRMS (ESI): calculated for $C_{11}H_6F_3N$ [M]: 209.0452; found: 209.0451. LCMS R_T = 0.813, ES-MS $[M+1]^+$: 210.2.

5-Fluoro-4-(4-fluorophenyl)pyridin-2(1*H*)-one (8)



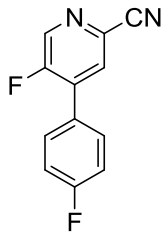
Intermediate **7** (4.00 g, 19.1 mmol, 1.0 eq) was dissolved in acetic acid (200 mL) and water (96 mL) in a sealed vessel. The reaction was heated at 130 ºC for 5 days, cooled, and concentrated to give 4.06 g (~100%) of the title compound as yellow solid **8** that was used without further purification. ^1H NMR (400 MHz, DMSO-*d6*) δ = 7.82 (d, *J* = 4.48 Hz, 1H), 7.67-7.62 (m, 2H), 7.37-7.31 (m, 2H), 6.55 ppm (d, *J* = 6.45 Hz, 1H), missing exchangeable proton (N-H); ^{13}C NMR (100 MHz, DMSO-*d6*): *δ* = 162.76 (d, *J*(C,F) = 247.27 Hz), 160.64, 147.48 (d, *J*(C,F) = 233.65 Hz), 141.25 (d, *J*(C,F) = 15.36 Hz), 130.87 (dd, *J*(C,F) = 8.53, 3.20 Hz), 129.24-129.19 (m), 126.73 (d, *J*(C,F) = 33.15 Hz), 115.82 (d, *J*(C,F) = 21.59 Hz), 114.99 ppm. HRMS (ESI): calculated for $C_{11}H_7F_2NO$ [M]: 207.0496; found: 207.0495. LCMS R_T = 0.572, ES-MS $[M+1]^+$: 208.2.

5-Fluoro-4-(4-fluorophenyl)pyridin-2-yl trifluoromethanesulfonate (9)



Intermediate **8** (2.34 g, 11.3 mmol, 1.0 eq) and triethylamine (3.31 mL, 23.7 mmol, 2.1 eq) were dissolved in dichloromethane (28 mL) and DMF (28 mL). The reaction was cooled to 0 ºC and *N*-phenyl-bis(trifluoromethanesulfonimide) (4.84 g, 13.6 mmol, 1.2 eq) was added. The reaction was stirred until judged complete by LCMS at which point it was neutralized with saturated sodium bicarbonate and extracted with dichloromethane (2x). The combined organics were dried (MgSO4), filtered, and concentrated *in vacuo*. Purification by flash chromatography on silica gel afforded 3.57 g (93%) of the title compound **9** as a white solid. ^{1}H NMR (400 MHz, DMSO-*d6*) δ = 8.60 (d, *J* = 2.2 Hz, 1H), 7.97 (d, *J* = 5.3 Hz, 1H), 7.83-7.79 (m, 2H), 7.45-7.40 ppm (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 164.05 (d, J(C,F) = 251.47 Hz), 156.41 (d, J(C,F) = 259.09 Hz), 151.80 (d, J(C,F) = 2.43 Hz), 140.53 (d, J(C,F) = 13.39 Hz), 137.29 (d, J(C,F) = 30.12 Hz), 130.97 (dd, J(C,F) = 8.64, 3.71 Hz), 127.57-127.52 (m), 118.76 (d, J(C,F) = 320.90 Hz), 116.57 (d, J(C,F) = 22.28 Hz), 115.89 ppm. HRMS (ESI): calculated for $C_{12}H_6F_5NO_3S$ [M]: 338.9989; found: 338.9990. LCMS R_T = 1.073, ES-MS $[M+1]^+$: 340.2.

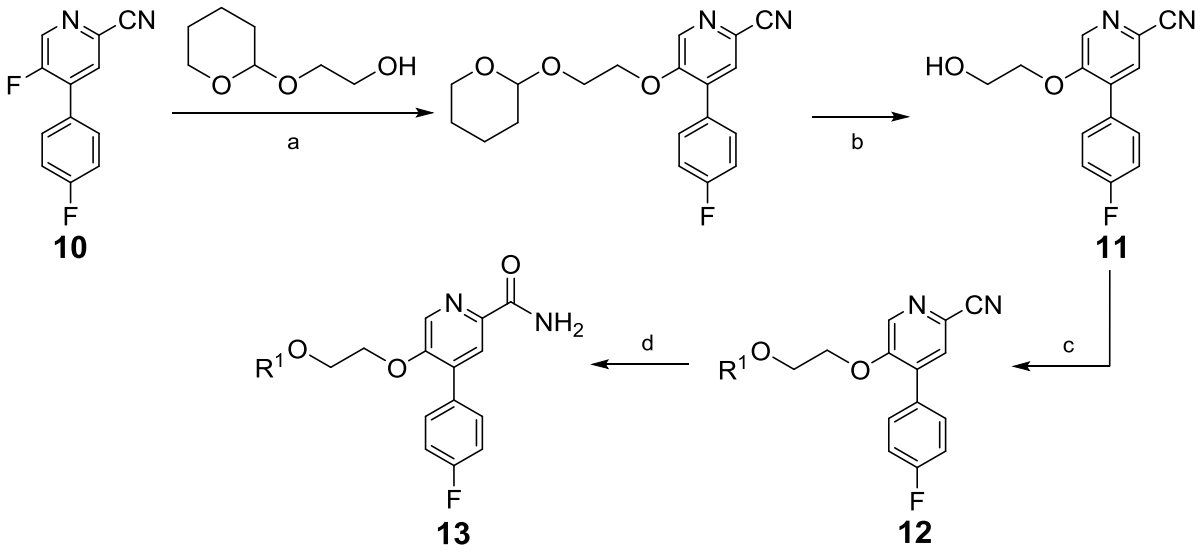
5-Fluoro-4-(4-fluorophenyl)picolinonitrile (10)



Intermediate **9** (6.32 g, 18.6 mmol, 1.0 eq), zinc cyanide (2.63 g, 22.4 mmol, 1.2 eq) and tetrakis(triphenylphosphine)palladium(0) (2.15 g, 1.86 mmol, 0.10 eq) were dissolved in DMF (56 mL) in a microwave vial and heated in a microwave reactor at 140 ºC for 15 min. The reaction was concentrated and purified by flash liquid chromatography on silica gel to afford

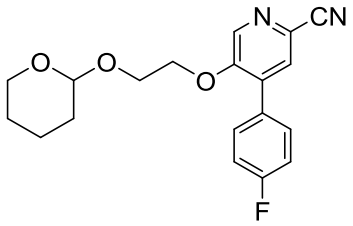
2.96 g (73%) of the title compound **10** as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ = 8.62 (d, *J* = 2.21 Hz, 1H), 7.81 (d, *J* = 6.07 Hz, 1H), 7.63-7.59 (m, 2H), 7.24-7.21 ppm (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): *δ* = 164.04 (d, *J*(C,F) = 252.17 Hz), 157.79 (d, *J*(C,F) = 266.82 Hz), 141.26 (d, *J*(C,F) = 28.36 Hz), 136.49 (d, *J*(C,F) = 11.86 Hz), 130.97 (dd, *J*(C,F) = 8.61, 3.62 Hz), 130.45 (d, *J*(C,F) = 5.48 Hz), 129.63 (d, *J*(C,F) = 2.56 Hz), 126.98-126.93 (m), 116.66 (d, *J*(C,F) = 21.91 Hz), 116.63 ppm. HRMS (ESI): calculated for $C_{12}H_6F_2N_2$ [M]: 216.0499; found: 216.0497. LCMS R_T = 0.817, ES-MS $[M+1]^+$: 217.4.

Preparation of Analogs 12 and 13. The referenced compounds were prepared via the route pictured immediately below.



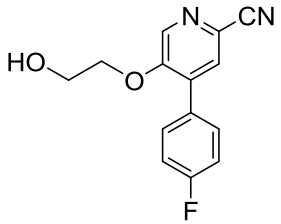
Reagents and Conditions: (a) NaH, DMF; (b) $pTSA{\cdot}H_2O$, DCM, EtOH; (c) R^1-OH, PPh_3, D^tBAD, THF, 0°C to r.t.; (d) $KOSiMe_3$, THF, reflux.

4-(4-fluorophenyl)-5-(2-((tetrahydro-2H-pyran-2-yl)oxy)ethoxy) picolinonitrile.



To a solution of 2-(tetrahydro-2*H*-pyran-2-yloxy)ethanol (301 µL, 2.22 mmol, 1.2 eq.) and sodium hydride (93.5 mg, 3.7 mmol, 2.0 eq.) in DMF (9.25 mL) was added intermediate **10** (400 mg, 1.85 mmol, 1.0 eq.). After three hours, the reaction was quenched with saturated aqueous ammonium chloride, diluted with ethyl acetate and washed with water and brine twice. The combined organics were dried (MgSO4), filtered, and concentrated *in vacuo* to give 633 mg of the title compound, which was used without further purification. ^{1}H NMR (400 MHz, $CDCl_3$) δ = 8.46 (s, 1H), 7.65 (s, 1H), 7.63-7.58 (m, 2H), 7.17-7.11 (m, 2H), 4.62 (t, *J* = 3.16 Hz, 1H), 4.37 (t, *J* = 4.63 Hz, 2H), 4.08-4.03 (m, 1H), 3.78-3.72 (m, 2H), 3.49-3.44 (m, 1H), 1.81-1.65 ppm (m, 2H), 1.63-1.51 (m, 4H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 163.34 (d, *J*(C,F) = 250.07 Hz), 154.25, 137.43, 137.09, 131.28 (d, *J*(C,F) = 8.36 Hz), 129.84 (d, *J*(C,F) = 3.55 Hz), 129.41, 126.72, 117.56, 115.75 (d, *J*(C,F) = 21.73 Hz), 99.12, 69.24, 65.51, 62.10, 30.54, 25.45, 19.26 ppm. HRMS (ESI): calculated for $C_{19}H_{19}FN_2O_3$ [M]: 342.1380; found: 342.1380. LCMS R_T = 1.299, ES-MS $[M+1]^+$: 343.3.

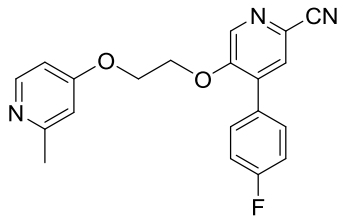
4-(4-fluorophenyl)-5-(2-hydroxyethoxy) picolinonitrile (11)



A mixture of 4-(4-fluorophenyl)-5-(2-((tetrahydro-2H-pyran-2-yl)oxy)ethoxy) picolinonitrile (633 mg, 1.85 mmol, 1.0 eq.) and *p*-toluenesulfonic acid monohydrate (35.2 mg, 0.19 mmol, 0.10 eq.) in dichloromethane (4.62 mL) and ethanol (4.62 mL) was stirred for 18 hours. The reaction was washed with 10% aqueous potassium carbonate solution. The organic layer was

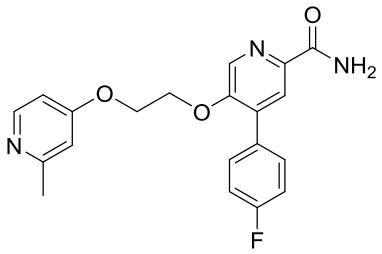
separated, dried ($MgSO_4$), filtered and concentrated *in vacuo*. Purification by flash chromatography on silica gel afforded 312 mg (65%, 2 steps) of the title compound **11**. ^{1}H NMR (400 MHz, $CDCl_3$) δ = 8.45 (s, 1H), 7.65 (s, 1H), 7.58-7.53 (m, 2H), 7.21-7.15 (m, 2H), 4.30 (t, *J* = 4.43 Hz, 2H), 3.99-3.95 (m, 2H), 1.70 ppm (t, *J* = 6.10 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): *δ* = 163.38 (d, *J*(C,F) = 248.32 Hz), 153.98, 137.71, 137.02, 131.08 (d, *J*(C,F) = 8.19 Hz), 129.72 (d, *J*(C,F) = 3.46 Hz), 129.56, 127.04, 117.43, 116.02 (d, *J*(C,F) = 21.42 Hz), 71.11, 61.12 ppm. HRMS (ESI): calculated for $C_{14}H_{11}FN_2O_2$ [M]: 258.0805; found: 258.0800. LCMS R_T = 0.849, ES-MS $[M+1]^+$: 259.2.

4-(4-fluorophenyl)-5-(2-((2-methylpyridin-4-yl)oxy)ethoxy) picolinonitrile (12b)



Intermediate **11** (15 mg, 0.058 mmol, 1.0 eq) and 4-hydroxy-2-methylpyridine (12.7 mg, 0.116 mmol, 2.0 eq) were dissolved in THF (0.5 mL) and cooled to 0 ºC. Triphenylphosphine (33.5 mg, 0.128 mmol, 2.2 eq) and di-*tert*-butylazodicarboxylate (21.4 mg, 0.093 mmol, 1.6 eq) were added, and the reaction was stirred at room temperature until judged complete by LCMS. The reaction was concentrated and purified using reverse-phase HPLC to afford 1.9 mg (9.4%) of the title compound. ^{1}H NMR (400 MHz, $CDCl_3$) δ = 8.49 (s, 1H), 8.33 (d, *J* = 5.75 Hz, 1H), 7.67 (s, 1H), 7.57-7.51 (m, 2H), 7.11-7.05 (m, 2H), 6.65 (d, *J* = 2.33 Hz, 1H), 6.62 (dd, *J* = 5.75, 2.45 Hz, 1H), 4.56-4.53 (m, 2H), 4.35-4.33 (m, 2H), 2.52 ppm (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): *δ* = 164.73, 163.33 (d, *J*(C,F) = 250.43 Hz), 160.49, 153.80, 150.68, 137.82, 137.12, 131.13 (d, *J*(C,F) = 8.51 Hz), 129.52, 129.43 (d, *J*(C,F) = 3.31 Hz), 127.26, 117.38, 115.85 (d, *J*(C,F) = 21.75 Hz), 109.35, 107.53, 68.02, 65.76, 24.72 ppm. HRMS (ESI): calculated for $C_{20}H_{16}FN_3O_2$ [M]: 349.1227; found: 349.1231. LCMS R_T = 0.658, ES-MS $[M+1]^+$: 350.4.

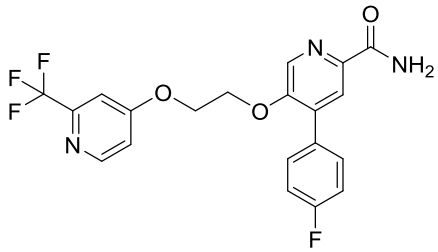
4-(4-fluorophenyl)-5-(2-((2-methylpyridin-4-yl)oxy)ethoxy) picolinamide (13b)



Intermediate **12b** (1.9 mg, 0.005 mmol, 1.0 eq.) and potassium trimethylsilanolate (1.46 mg, 0.011 mmol, 2.1 eq.) in THF (1 mL) were added to a small vial. The vial was capped and the reaction was heated to reflux. After two hours, the reaction was quenched with 2N hydrogen chloride solution and concentrated *in vacuo*. The compound was purified by reverse phase HPLC to afford 1.7 mg (85%) of the title compound **13b**. ^{1}H NMR (400 MHz, $CDCl_3$) δ = 8.36 (d, J = 3.98 Hz, 2H), 8.23 (s, 1H), 7.74 (s, 1H), 7.67-7.61 (m, 2H), 7.11-7.05 (m, 2H), 6.68 (d, J = 2.32 Hz, 1H), 6.66 (dd, J = 5.80, 2.48 Hz, 1H), 5.55 (s, 1H), 4.54-4.52 (m, 2H), 4.37-4.35 (m, 2H), 2.54 ppm (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 166.58, 164.87, 163.09 (d, *J*(C,F) = 248.80 Hz), 160.47, 153.98, 150.68, 143.89, 137.97, 134.36, 131.24 (d, *J*(C,F) = 8.22 Hz), 130.77 (d, *J*(C,F) = 3.44 Hz), 124.06, 115.54 (d, *J*(C,F) = 21.60 Hz), 109.42, 107.60, 68.00, 65.96, 24.75 ppm. HRMS (ESI): calculated for $C_{20}H_{18}FN_3O_3$ [M]: 367.1332; found: 367.1335. LCMS R_T = 0.634, ES-MS $[M+1]^+$: 368.2.

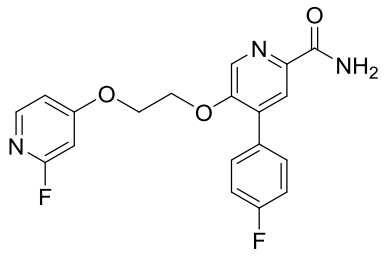
*The following compounds were prepared analogous to compound **13b**,, and >98% pure by LCMS.*

4-(4-fluorophenyl)-5-(2-((2-(trifluoromethyl)pyridin-4-yl)oxy)ethoxy) picolinamide (13a)



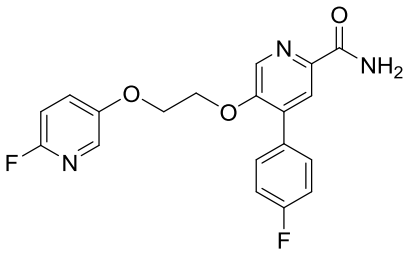
LCMS R_T = 0.954, ES-MS $[M+1]^+$: 422.2.

4-(4-fluorophenyl)-5-(2-((2-fluoropyridin-4-yl)oxy)ethoxy) picolinamide (13c)



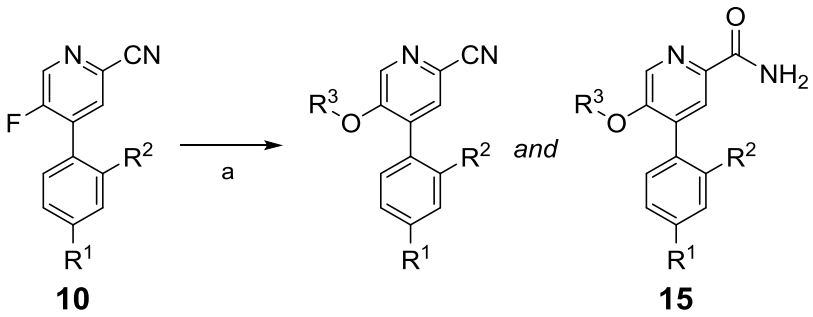
LCMS R_T = 0.883, ES-MS $[M+1]^+$: 372.2.

4-(4-fluorophenyl)-5-(2-((6-fluoropyridin-3-yl)oxy)ethoxy) picolinamide (13d)



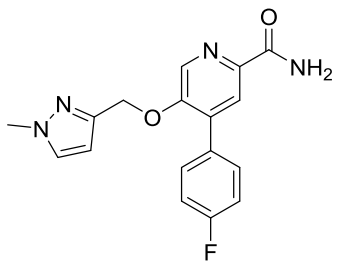
LCMS R_T = 0.908, ES-MS $[M+1]^+$: 372.2.

Preparation of Analogs 15/16. The referenced compounds were prepared via the route pictured immediately below.



Reagents and conditions: (a) NaH, R^3-OH, DMF.

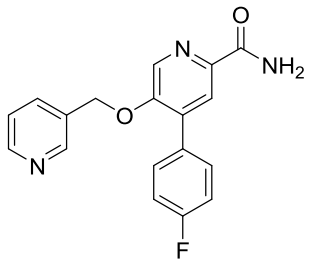
4-(4-fluorophenyl)-5-((1-methyl-1H-pyrazol-3-yl)methoxy) picolinamide (15m)



A solution of (1-methyl-1*H*-pyrazol-3-yl) methanol (16.3 mg, 0.15 mmol, 2.1 eq.) and sodium hydride (3.3 mg, 0.14 mmol, 2.0 eq.) in DMF (1 mL) was stirred for 20 minutes. Intermediate **10** (15 mg, 0.069 mmol, 1.0 eq.) was added, and the reaction was stirred for two hours. Purification by reverse phase HPLC afforded 10.3 mg (48%) of the cyano product and 6.5 mg (29%) of the amide product **15m**, which was presumably formed due to residual water present in the solvent or reactants. ^{1}H NMR (400 MHz, $CDCl_3$) δ = 8.43 (s, 1H), 8.18 (s, 1H), 7.70 (s, 1H), 7.65-7.60 (m, 2H), 7.31 (d, *J* = 2.21 Hz, 1H), 7.14-7.07 (m, 2H), 6.21 (d, *J* = 2.23 Hz, 1H), 5.46 (s, 1H), 5.24 (s, 2H), 3.89 ppm (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 166.78, 163.04 (d, *J*(C,F) = 248.37 Hz), 154.02, 147.45, 143.27, 137.76, 134.70, 131.44, 131.32 (d, *J*(C,F) = 8.27 Hz), 131.16 (d, *J*(C,F) = 3.34 Hz), 123.94, 115.43 (d, *J*(C,F) = 21.54 Hz), 105.41, 65.74, 39.09 ppm. HRMS (ESI): calculated for $C_{17}H_{15}FN_4O_2$ [M]: 326.1179; found: 326.1182. LCMS R_T = 0.842, ES-MS $[M+1]^+$: 327.4.

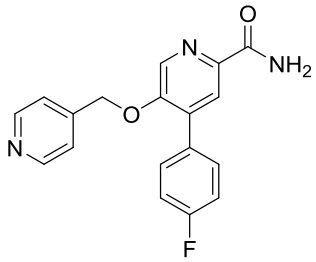
*The following compounds were prepared analogous to compound **15m,** and >98% pure by LCMS.*

4-(4-fluorophenyl)-5-(pyridin-3-ylmethoxy) picolinamide (15b)



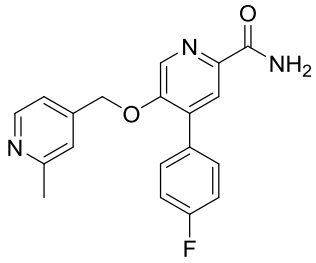
LCMS R_T = 0.624, ES-MS $[M+1]^+$: 324.4.

4-(4-fluorophenyl)-5-(pyridin-4-ylmethoxy) picolinamide (15a)



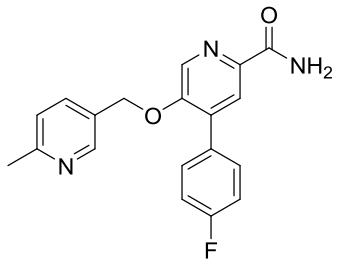
LCMS R_T = 0.623, ES-MS $[M+1]^+$: 324.4.

4-(4-fluorophenyl)-5-((2-methylpyridin-4-yl)methoxy) picolinamide (15c)



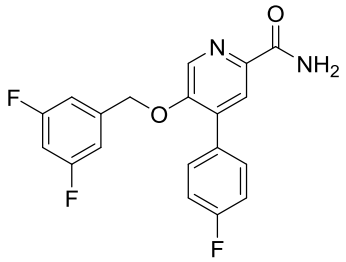
LCMS R_T = 0.649, ES-MS $[M+1]^+$: 338.4.

4-(4-fluorophenyl)-5-((6-methylpyridin-3-yl)methoxy) picolinamide (15d)



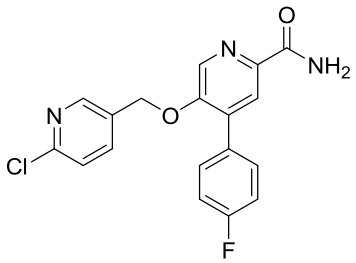
LCMS R_T = 0.644, ES-MS $[M+1]^+$: 338.3.

5-((3,5-difluorobenzyl)oxy)-4-(4-fluorophenyl) picolinamide (15g)



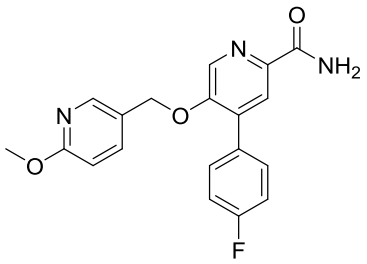
LCMS R_T = 1.105, ES-MS $[M+1]^+$: 359.3.

5-((6-chloropyridin-3-yl)methoxy)-4-(4-fluorophenyl) picolinamide (15e)



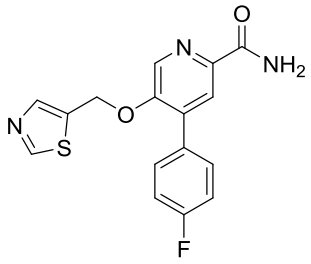
LCMS R_T = 0.980, ES-MS $[M+1]^+$: 358.4.

4-(4-fluorophenyl)-5-((6-methoxypyridin-3-yl)methoxy) picolinamide (15f)



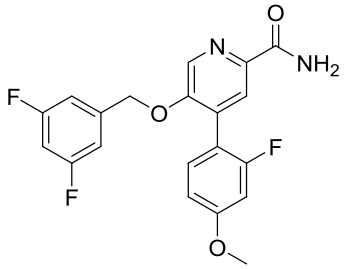
LCMS R_T = 0.935, ES-MS $[M+1]^+$: 354.3.

4-(4-fluorophenyl)-5-(thiazol-5-ylmethoxy) picolinamide (15l)



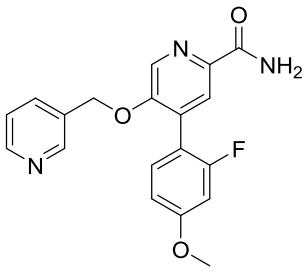
LCMS R_T = 0.820, ES-MS $[M+1]^+$: 330.4.

5-((3,5-difluorobenzyl)oxy)-4-(2-fluoro-4-methoxyphenyl) picolinamide (16a)



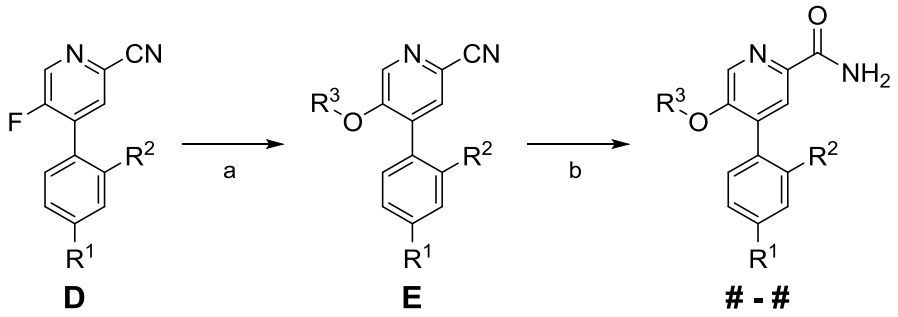
LCMS R_T = 1.083, ES-MS $[M+1]^+$: 389.2.

4-(2-fluoro-4-methoxyphenyl)-5-(pyridin-3-ylmethoxy) picolinamide (16b)



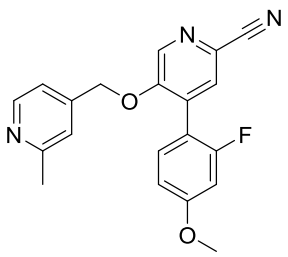
LCMS R_T = 0.602, ES-MS $[M+1]^+$: 354.2.

Preparation of Analogs 16c – 16f. The referenced compounds were prepared via the route pictured immediately below.



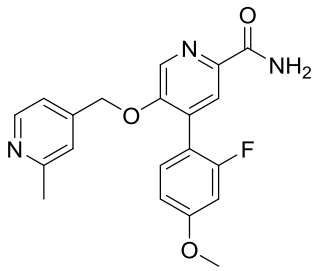
Reagents and Conditions: (a) NaH, R^3-OH, THF; (b) $KOSiMe_3$, THF, reflux.

4-(2-fluoro-4-methoxyphenyl)-5-((2-methylpyridin-4-yl)methoxy)picolinonitrile.



Sodium hydride (2.1 mg, 0.08 mmol, 2.0 eq.) was added to a solution of 2-methyl-4-hydroxymethylpyridine (4.7 µL, 0.05 mmol, 1.2 eq.) in THF (0.5 mL). After five minutes, intermediate **10** (10 mg, 0.04 mmol, 1.0 eq.) in THF (0.5 mL) was added to the reaction. Upon stirring for one hour, the reaction was quenched with a saturated ammonium chloride solution and concentrated in vacuo to give 14.2 mg (100%) of the title compound that was used without further purification. ^{1}H NMR (400 MHz, $CDCl_3$) δ = 8.48 (d, *J* = 5.11, 1H), 8.38 (s, 1H), 7.66 (s, 1H), 7.31 (t, *J* = 8.46 Hz, 1H), 7.09 (s, 1H), 7.03 (d, *J* = 5.05 Hz, 1H), 6.82 (dd, *J* = 8.43, 2.41 Hz, 1H), 6.76 (dd, *J* = 11.95, 2.41 Hz, 1H), 5.23 (s, 2H), 3.87 (s, 3H), 2.54 ppm (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): *δ* = 162.27 (d, *J*(C,F) = 11.11 Hz), 160.54 (d, *J*(C,F) = 249.79 Hz), 159.19, 153.93, 149.74, 144.55, 136.62, 134.15, 131.65 (d, *J*(C,F) = 4.78 Hz), 130.70 (d, *J*(C,F) = 1.77 Hz), 126.90, 120.65, 118.22, 117.36, 113.51 (d, *J*(C,F) = 15.57 Hz), 110.54 (d, *J*(C,F) = 2.83 Hz), 102.14 (d, *J*(C,F) = 25.66 Hz), 69.66, 55.90, 24.63 ppm. HRMS (ESI): calculated for $C_{20}H_{16}FN_3O_2$ [M]: 349.1227; found: 349.1231. LCMS R_T = 0.765, ES-MS $[M+1]^+$: 350.2.

4-(2-fluoro-4-methoxyphenyl)-5-((2-methylpyridin-4-yl)methoxy)picolinamide (16c)

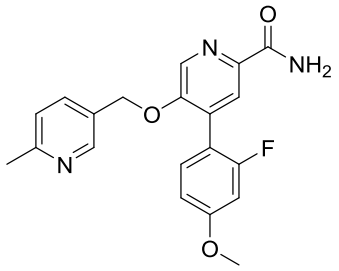


4-(2-Fluoro-4-methoxyphenyl)-5-((2-methylpyridin-4-yl)methoxy)picolinonitrile (14.2 mg, 0.04 mmol, 1.0 eq.) and potassium trimethylsilanolate (10.95 mg, 0.085 mmol, 2.1 eq.) in THF (1 mL) were added to a small vial. The vial was capped and the reaction was heated to reflux. After two hours, the reaction was quenched with 2N hydrogen chloride solution and concentrated in vacuo. The compound was purified by reverse phase HPLC to afford 4.8 mg (32%) of the title compound. ^{1}H NMR (400 MHz, $CDCl_3$) δ = 8.47 (d, *J* = 5.11, 1H), 8.22 (s, 1H), 8.18 (s, 1H), 7.67 (s, 1H), 7.33 (t, *J* = 8.45 Hz, 1H), 7.11 (s, 1H), 7.05 (d, *J* = 5.11 Hz, 1H), 6.81 (dd, *J* = 8.58, 2.52 Hz, 1H), 6.74 (dd, *J* = 11.85, 2.45 Hz, 1H), 5.49 (s, 1H), 5.22 (s, 2H), 3.87 (s, 3H), 2.54 ppm (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): *δ* = 166.52, 161.85 (d, *J*(C,F) = 4.55 Hz), 160.56 (d,

J(C,F) = 243.18 Hz), 159.07, 154.16, 149.65, 145.33, 143.49, 134.42, 133.51, 131.80 (d, *J*(C,F) = 5.0 Hz), 125.26, 120.65, 118.26, 115.08 (d, *J*(C,F) = 15.92 Hz), 110.29 (d, *J*(C,F) = 3.0 Hz), 101.96 (d, *J*(C,F) = 25.85 Hz), 69.58, 55.83, 24.65 ppm. HRMS (ESI): calculated for $C_{20}H_{18}FN_3O_3$ [M]: 367.1332; found: 367.1336. LCMS R_T = 0.637, ES-MS $[M+1]^+$: 368.4.

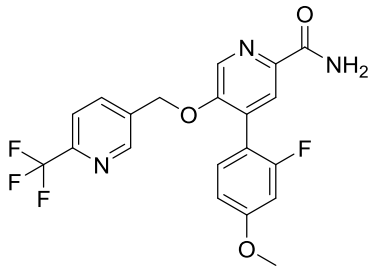
*The following compounds were prepared analogous to compound **16c**, and >98% pure by LCMS.*

4-(2-fluoro-4-methoxyphenyl)-5-((6-methylpyridin-3-yl)methoxy) picolinamide (16d)



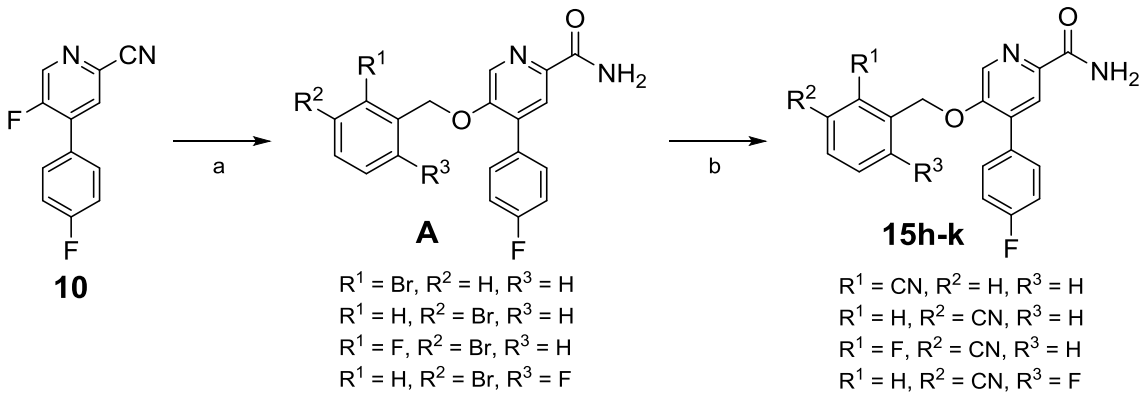
LCMS R_T = 0.665, ES-MS $[M+1]^+$: 368.4.

4-(2-fluoro-4-methoxyphenyl)-5-((6-(trifluoromethyl)pyridin-3-yl)methoxy)picolinamide (16f)



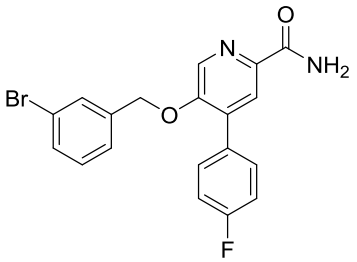
LCMS R_T = 1.035, ES-MS $[M+1]^+$: 422.3.

Preparation of Analogs 15h -k. The referenced compounds were prepared via the route pictured immediately below.



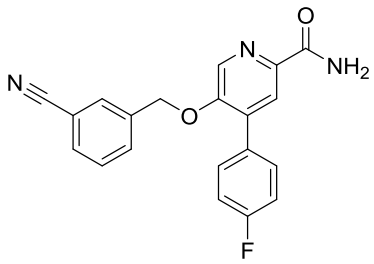
Reagents and Conditions: (a) NaH, Br-benzyl alcohol, DMF; (b) $Pd(PPh_3)_4$, $Zn(CN)_2$, DMF, microwave 140 °C, 45 min.

5-((3-bromobenzyl)oxy)-4-(4-fluorophenyl) picolinamide (A)



A solution of 3-bromobenzyl alcohol (36.3 mg, 0.19 mmol, 2.1 eq.) and sodium hydride (4.4 mg, 0.19 mmol, 2.0 eq.) in DMF (1 mL) was stirred for 20 minutes. Intermediate **D** (20 mg, 0.093 mmol, 1.0 eq.) was added, and the reaction was stirred for two hours. Purification by reverse phase HPLC afforded 24.5 mg (66%) of the amide product, which was presumably formed due to residual water present in the solvent or reactants. ^{1}H NMR (400 MHz, $CDCl_3$) δ = 8.29 (s, 1H), 8.22 (s, 1H), 7.70 (s, 1H), 7.65-7.61 (m, 2H), 7.52-7.45 (m, 3H), 7.18-7.13 (m, 3H), 5.55 (s, 1H), 5.21 ppm (s, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 166.69, 163.15 (d, J(C,F) = 249.64 Hz), 153.78, 143.63, 138.21, 137.99, 134.40, 131.54, 131.27 (d, J(C,F) = 8.5 Hz), 130.84 (d, J(C,F) = 3.35 Hz), 130.44, 130.10, 125.49, 124.16, 122.96, 115.61 (d, J(C,F) = 21.65 Hz), 70.71 ppm. HRMS (ESI): calculated for $C_{19}H_{14}BrFN_2O_2$ [M]: 400.0223; found: 400.0223. LCMS R_T = 1.001, ES-MS $[M+1]^+$: 401.2.

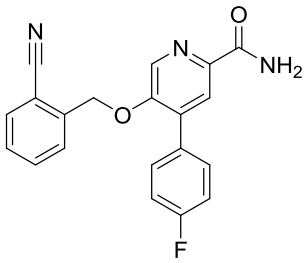
5-((3-cyanobenzyl)oxy)-4-(4-fluorophenyl) picolinamide (15h)



Intermediate **A** (24 mg, 0.06 mmol, 1.0 eq.), zinc cyanide (8.4 mg, 0.071 mmol, 1.2 eq.), tetrakis(triphenylphosphine)palladium(0) (6.9 mg, 0.006 mmol, 0.1 eq.), and DMF (600 µL) were added to a small microwave vial. The vial was capped and placed in the microwave reactor at 140 ºC for 45 minutes. Purification by reverse phase HPLC afforded 8.4 mg (40%) of the title compound. ^{1}H NMR (400 MHz, $CDCl_3$) δ = 8.29 (s, 1H), 8.23 (s, 1H), 7.69 (s, 1H), 7.64-7.58 (m, 4H), 7.57-7.54 (m, 1H), 7.52-7.47 (m, 1H), 7.18-7.13 (m, 2H), 5.50 (s, 1H), 5.26 ppm (s, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 166.42, 163.23 (d, J(C,F) = 249.48 Hz), 153.55, 144.06, 138.36, 137.42, 134.36, 132.15, 131.24 (d, J(C,F) = 7.90 Hz), 131.23, 130.80 (d, J(C,F) = 3.36 Hz), 130.52, 129.80, 124.27, 118.44, 115.72 (d, J(C,F) = 21.45 Hz), 113.19, 70.38 ppm. HRMS (ESI): calculated for $C_{20}H_{14}FN_3O_2$ [M]: 347.1070; found: 347.1074.LCMS R_T = 0.999, ES-MS [M+1]+: 348.3.

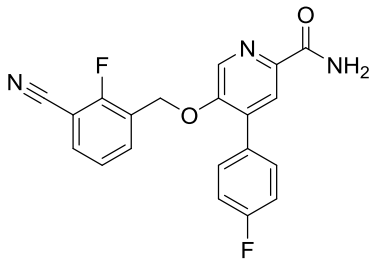
*The following compounds were prepared analogous to compound **15h**, and >98% pure by LCMS.*

5-((2-cyanobenzyl)oxy)-4-(4-fluorophenyl) picolinamide (15i)



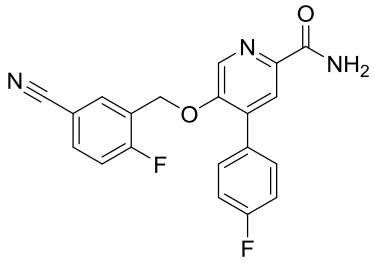
LCMS R_T = 0.974, ES-MS $[M+1]^+$: 348.3.

5-((3-cyano-2-fluorobenzyl)oxy)-4-(4-fluorophenyl) picolinamide (15j)



LCMS R_T = 0.980, ES-MS $[M+1]^+$: 366.2.

5-((5-cyano-2-fluorobenzyl)oxy)-4-(4-fluorophenyl) picolinamide (15k)



LCMS R_T = 0.990, ES-MS $[M+1]^+$: 366.3.

Molecular Pharmacology Methods

$mGlu_3$ and $mGlu_2$ Ca^{2+} flux assays (CRC format)

$G_{\alpha 15}$/TREx cells stably expressing rat $mGlu_3$ or $G_{\alpha 15}$ HEK293 cells stably expressing rat $mGlu_2$ were plated in black-walled, clear-bottomed, poly-D-lysine coated 384-well plates in 20 μL of assay medium (For $mGlu_3$ assay: DMEM containing 10% dialyzed FBS, 20 mM HEPES,

25 ng/mL tetracycline, and 1 mM sodium pyruvate; For $mGlu_2$ assay: DMEM containing 10% dialyzed FBS, 20 mM HEPES, and 1 mM sodium pyruvate) at a density of 20K cells/well ($mGlu_3$) or 12K cells/well ($mGlu_2$). The cells were grown overnight at 37 ºC in the presence of 5% CO_2. The next day, medium was removed and the cells incubated with 20 µL of 2.3 µM Fluo-4 AM prepared as a 2.3 mM stock in DMSO and mixed in a 1:1 ratio with 10% (w/v) pluronic acid F-127 and diluted in assay buffer (Hank's balanced salt solution, 20 mM HEPES, and 2.5 mM probenecid) for 60 minutes at room temperature ($mGlu_3$) or 45 minutes at 37 ºC ($mGlu_2$). Dye was removed, 20 µL of assay buffer was added, and the plate was incubated for 10 ($mGlu_3$) or 5 ($mGlu_2$) minutes at room temperature.

Ca^{2+} flux was measured using the Functional Drug Screening System (FDSS7000, Hamamatsu, Japan). After establishment of a fluorescence baseline for about 3 seconds, the test compounds were added to the cells, and the response in cells was measured. 2.3 minutes later an EC_{20} concentration of the $mGlu_{2/3}$ receptor agonist glutamate was added to the cells, and the response of the cells was measured for 1.9 minutes; an EC_{80} concentration of agonist was added and readings taken for an additional 1.7 minutes. All test compounds were dissolved and diluted to a concentration of 10 mM in 100% DMSO. Compounds were then serially diluted 1:3 in DMSO into 10 point concentration response curves, transferred to daughter plates, and further diluted into assay buffer to a 2x stock. Calcium fluorescence measures were recorded as fold over basal fluorescence; raw data was then normalized to the maximal response to glutamate. Antagonism of the agonist response of the $mGlu_3$ or $mGlu_2$ receptor was observed as a decrease in response to nearly maximal concentrations of glutamate in the presence of compound compared to the response to glutamate in the absence of compound.

The raw data file containing all time points was used as the data source in the analysis template. This was saved by the FDSS as a tab-delimited text file. Data were normalized using a static ratio function (F/F_0) for each measurement of the total 360 values per well divided by each well's initial value. Data were then reduced to peak amplitudes (Max – Initial Min) using a time range that starts approximately 3 seconds prior to the glutamate EC_{80} addition and continues for approximately 90 seconds. This is sufficient time to capture the peak amplitude of the cellular calcium response. Individual amplitudes were expressed as % EC_{Max} by multiplying each amplitude by 100 and then dividing the product by the mean of the amplitudes derived from the glutamate EC_{Max}-treated wells. IC_{50} values for test compounds were generated by fitting the normalized values versus the log of the test compound concentration (in mol/L) using a 4 parameter logistic equation where none of the parameters were fixed. Each of the three values collected at each concentration of test compound were weighted evenly.

A compound was designated as a negative allosteric modulator (NAM) if the compound showed a concentration-dependent decrease in the glutamate EC_{80} addition. For NAMs, potency (IC_{50}) and maximum response (% Glu Max), i.e. the amplitude of response in the presence of 30 µM test compound as a percentage of the maximal response to glutamate, are reported. For NAMs that show a decrease in the EC_{80} response, but do not hit a plateau, the average of the maximum response at a single concentration (30 µM) was determined (% Glu Max) and potencies were reported as ">10,000 nM". Compounds with no measurable activity are designated as ">30,000 nM" since the top concentration of compound tested in the assay is 30 µM.

$mGlu_3$ Ca^{2+} flux assay (fold-shift format)

$G_{\alpha 15}$/TREx cells stably expressing rat $mGlu_3$ were prepared as described above. Compounds were diluted by half-log in DMSO and further diluted into assay buffer to a 2x stock which was applied to cells at t = 3 s. Cells were incubated with the test compounds for 2.3 minutes and then stimulated with varying concentrations of glutamate, and readings taken for an additional 2.6 minutes. Data were collected at 1 Hz. Concentration response curves were generated using a four point logistical equation with XLfit curve fitting software for Excel (IDBS, Guildford, U.K.) or GraphPad Prism (GraphPad Software, Inc., La Jolla, CA).

The raw data file containing all time points was used as the data source in the analysis template. This was saved by the FDSS as a tab-delimited text file. Data were normalized using a static ratio function (F/F_0) for each measurement of the total 360 values per well divided by each well's initial value. Data were then reduced to peak amplitudes (Max – Initial Min) using a time range that starts approximately 3 seconds prior to the glutamate EC_{20}/EC_{80} addition and continues for approximately 90-120 seconds. This is sufficient time to capture the peak amplitude of the cellular calcium response. Individual amplitudes were expressed as % E_{Max} by multiplying each amplitude by 100 and then dividing the product by the mean of the amplitudes derived from the glutamate EC_{Max}-treated wells. EC_{50} values for test compounds were generated by fitting the normalized values versus the log of the test compound concentration (in mol/L) using a 4 parameter logistic equation where none of the parameters were fixed. Each of the three values collected at each concentration of test compound were weighted evenly.

A compound was designated as a positive allosteric modulator (PAM) if the compound showed a concentration-dependent increase in the glutamate EC_{20} addition. For PAMs, potency (EC_{50}) and maximum response (% Glu Max), i.e. the amplitude of response in the presence of 30 μM test compound as a percentage of the maximal response to glutamate, are reported. For

PAMs that show an increase in the EC_{20} response, but do not hit a plateau, the average of the maximum response at a single concentration (30 μM) was determined (% Glu Max) and potencies were reported as ">10,000 nM". A compound was designated as a negative allosteric modulator (NAM) if the compound showed a concentration-dependent decrease in the glutamate EC_{80} addition. For NAMs, potency (IC_{50}) and maximum response (% Glu Max), i.e. the amplitude of response in the presence of 30 μM test compound as a percentage of the maximal response to glutamate, are reported. For NAMs that show a decrease in the EC_{80} response, but do not hit a plateau, the average of the maximum response at a single concentration (30 μM) was determined (% Glu Max) and potencies were reported as ">10,000 nM". Compounds with no measurable activity are designated as ">30,000 nM" since the top concentration of compound tested in the assay is 30 μM.

$mGlu_{1,5}$ fold-shift selectivity assay

Human $mGlu_1$ TREx293 cells were plated in black-walled, clear-bottomed, poly-D-lysine coated 384-well plates (BD Biosciences, San Jose, CA) at a density of 20,000 cells/well in 20 μL of assay medium (DMEM supplemented with 10% dialyzed FBS, 20 mM HEPES, and 1 mM sodium pyruvate) containing tetracycline (TET) to induce the $mGlu_1$ expression; 50ng/mL TET was used. The cells were grown overnight at 37 ºC in the presence of 5% CO_2. The next day, cells were washed with assay buffer (Hank's balanced salt solution, 20 mM HEPES, and 2.5 mM probenecid (Sigma-Aldrich, St. Louis, MO)) using an ELX405 microplate washer (BioTek) leaving 20 μL/well. Immediately cells were incubated with 20 μl/well of Fluo-4 AM (Invitrogen) calcium indicator dye solution (1.15 μM final concentration) for 45 m at 37 ºC. The Fluo-4 dye prepared as a DMSO stock, was mixed in a 1:1 ratio with 10% pluronic acid F-127 and then

diluted in assay buffer. The dye was then removed and washed with assay buffer using an ELX405, leaving 20 μL/well. Ca^{2+} flux was measured using the Functional Drug Screening System (FDSS7000, Hamamatsu, Japan). Compounds were diluted by half-log in DMSO and further diluted into assay buffer to a 2x stock which was applied to cells at t = 3 s. Cells were incubated with the test compounds for 2.3 minutes and then stimulated with varying concentrations of glutamate, and readings taken for an additional 2.6 minutes. Data were collected at 1 Hz. Concentration response curves were generated using a four point logistical equation with XLfit curve fitting software for Excel (IDBS, Guildford, U.K.) or GraphPad Prism (GraphPad Software, Inc., La Jolla, CA).

$mGlu_{4/6/7/8}$ fold-shift selectivity assay

Compound **15m** activity at the group III mGlu receptors was assessed using thallium flux through G-protein-coupled inwardly rectifying potassium (GIRK) channels, a method that has been described in detail (Niswender et al. *Mol. Pharmacol.* 2008, reference 45). These cell lines were grown in growth media containing 45% DMEM, 45% F-12, 10% FBS, 20 mM HEPES, 2 mM L-glutamine, antibiotic/antimycotic, nonessential amino acids, 700 μg/mL G418, and 0.6 μg/mL puromycin at 37 ºC in the presence of 5% CO_2. Briefly, HEK/GIRK cells expressing rat $mGlu_4$, human $mGlu_6$, rat $mGlu_7$, or rat $mGlu_8$ were plated into 384 well, black-walled, clear-bottom poly-D-lysine coated plates at a density of 15,000 cells/20 μL/well in assay medium and incubated overnight at 37 ºC in the presence of 5% CO_2. The following day, the medium from the cells and 20 μL/well of 1.7 μM concentration of the indicator dye BTC-AM (Invitrogen, Carlsbad, CA) in assay buffer was added. Cells were incubated for 1 hour at room temperature and the dye was replaced with 20 μL/well of assay buffer. After establishment of a fluorescence

baseline for about 3 seconds, test compound was added to the cells at 2x final concentration, and the response in cells was measured. 2.3 min later the appropriate concentration of agonist (L-AP4 for $mGlu_7$, glutamate for all other mGlu receptors) was added and readings taken for an additional 2.6 minutes. Agonists were diluted in thallium buffer (125 mM sodium bicarbonate, 1 mM magnesium sulfate, 1.8 mM calcium sulfate, 5 mM glucose, 12 mM thallium sulfate, 10 mM HEPES) at 5x the final concentration to be assayed. Data were analyzed as described in Niswender et al. *Mol. Pharmacol.* 2008 (reference 45).

***In-Vitro* DMPK Methods**

Intrinsic clearance in rat liver microsomes

Rat liver microsomes (0.5 mg/mL) and 1 μM test compound were incubated in 100 mM potassium phosphate pH 7.4 buffer with 3 mM $MgCl_2$ at 37 ºC with constant shaking. After a 5 min preincubation, the reaction was initiated by addition of NADPH (1 mM). At selected time intervals (0, 3, 7, 15, 25, and 45 min), 50 μL aliquots were taken and subsequently placed into a 96-well plate containing 150 μL of cold acetonitrile with internal standard (50 ng/mL carbamazepine). Plates were then centrifuged at 3000 rcf (4 ºC) for 10 min, and the supernatant was transferred to a separate 96-well plate and diluted 1:1 with water for LC/MS/MS analysis. The *in vitro* half-life ($T_{1/2}$, min, Eq. 1), intrinsic clearance (CL_{int}, mL/min/kg, Eq. 2) and subsequent predicted hepatic clearance (CL_{hep}, mL/min/kg, Eq. 3) were determined employing the following equations:

(1) $$T_{1/2} = \frac{Ln(2)}{k}$$

where k represents the slope from linear regression analysis of the natural log percent remaining of test compound as a function of incubation time

(2) $$CL_{\text{int}} = \frac{0.693}{in\,vitro\,T_{1/2}} x \frac{mL\ incubation}{mg\ microsomes} x \frac{45\,mg\,microsomes}{gram\ liver} x \frac{45^{a}\ gram\ liver}{kg\ body\ wt}$$

[a] scale-up factor of 45 for rat

(3) $$CL_{hep} = \frac{Q_h \cdot CL\,\text{int}}{Q_h + CL\,\text{int}}$$

where Q_h (hepatic blood flow, mL/min/kg) is 70 for the rat.

Plasma Protein Binding

The protein binding of each compound was determined in rat plasma via equilibrium dialysis employing HTDialysis Teflon dialysis chamber and cellulose membranes (MWCO 12-14 K) (HTDialysis LLC, Gales Ferry, CT). Plasma was added to the 96 well plate containing test compound and mixed thoroughly for a final concentration of 5 µM. Subsequently, 150 µL of the plasma-compound mixture was transferred to the dialysis chamber, with an accompanying 150 µL of phosphate buffer (25 mM, pH 7.4) on the other side of the membrane. The device plate was sealed and incubated for 4 hours at 37 ºC with shaking. At completion, aliquots from each chamber were diluted 1:1 with either plasma (for the buffer sample) or buffer (for the plasma sample) and transferred to a new 96 well plate, at which time ice-cold acetonitrile containing internal standard (50 ng/mL carbamazepine) (2 volumes) was added to extract the matrices. The plate was centrifuged (3000 rcf, 10 min) and supernatants transferred and diluted 1:1 (supernatant: water) into a new 96 well plate, which was then sealed in preparation for LC/MS/MS analysis. Each compound was assayed in triplicate within the same 96-well plate. Fraction unbound was determined using the following equation:

$$F_u = \frac{Conc_{buffer}}{Conc_{plasma}}$$

Brain Homogenate Binding

The brain homogenate binding of each compound was determined in brain homogenate via equilibrium dialysis employing HTDialysis Teflon dialysis chamber and cellulose membranes (MWCO 12-14 K) (HTDialysis LLC, Gales Ferry, CT). Brain tissue homogenate was prepared by diluting one volume whole rat brain tissue with three volumes of phosphate buffer (25 mM, pH 7.4). The mixture was then subjected to mechanical homogenation employing a Mini-Beadbeater™ and 1.0 mm Zirconia/Silica Beads (BioSpec Products). Brain homogenate spiked with test compound and mixed thoroughly for a final concentration of 5 µM. Subsequently, 150 µL of the brain homogenate-compound mixture was transferred to the dialysis chamber with an accompanying 150 µL of phosphate buffer (25 mM, pH 7.4) on the other side of the membrane. The block was sealed and incubated for 6 hours at 37 ºC with shaking. At completion, aliquots from each side of the chamber were diluted 1:1 with either brain homogenate (to the buffer side) or buffer (to the brain homogenate side) in a new 96 well plate, at which time ice-cold acetonitrile containing internal standard (50 ng/mL carbamazepine) was added to extract the matrices. The plate was centrifuged (3000 rcf, 10 min) and supernatants transferred and diluted 1:1 (supernatant: water) into a new 96 well plate, which was then sealed in preparation for LC/MS/MS analysis. Each compound was assayed in triplicate within the same 96-well plate. Fraction unbound was determined using the following equation:

$$F_{u,tissue} = \frac{1/D_f}{(1/F_{u,\mathrm{hom}} - 1) + 1/D_f}$$

Where $F_{u,hom}$ represent the measured fraction unbound in the diluted homogenate and D_f represents dilution factor

LC/MS/MS Bioanalysis of Samples from *In Vitro* Assays

Samples were analyzed on a Thermo Electron TSQ Quantum Ultra triple quad mass spectrometer (San Jose, CA) with electrospray ionization (ESI), Shimadzu LC-10ADvp pumps (Columbia, MD), and a Leap Technologies CTC PAL autosampler (Carrboro, NC). Analytes were separated by gradient elution using Fortis C18 (3.0 x 50 mm, 3 µm) columns (Fortis Technologies Ltd, Cheshire, UK) thermostated at 40 ºC. HPLC mobile phase A was 0.1% formic acid in water and mobile phase B was 0.1% formic acid in acetonitrile. The gradient started at 30% B after a 0.2 min hold and was linearly increased to 95% B over 0.8 min; hold at 95% B for 0.2 min; returned to 30% B in 0.1 min. The total run time was 1.3 min and the HPLC flow rate was 0.5 mL/min. Compound optimization, data collection and processing was performed using Thermo Electron's QuickQuan software (v2.3) and Xcalibur (v2.0.7 SP1).

Cytochrome P450 Cocktail Inhibition Assay in Human Liver Microsomes

A cocktail of substrates for cytochrome P450 enzymes (1A2: Phenacetin, 10 µM; 2C9: Diclofenac, 5 µM; 2D6: Dextromethorphan, 5 µM; 3A4: Midazolam, 2 µM) were mixed for cocktail analysis. The positive control for pan-P450 inhibition (miconazole) was included alongside test compound in analysis. A reaction mixture of 100 mM Kpi, pH 7.4, 0.1 mg/mL human liver microsomes (HLM) and Substrate Mix is prepared and aliquoted into a 96-deepwell block. Test compound and positive control (in duplicate) were then added such that the final concentration of test compound ranged from 0.1 – 30 µM. The plate was vortexed briefly and then pre-incubated at 37 ºC while shaking for 15 minutes. The reaction was initiated with the addition of NADPH (1 mM final concentration). The incubation continued for 8 min and the

reaction quenched by 2x volume of cold acetonitrile containing internal standard (50 nM carbamazepine). The plate was centrifuged for 10 minutes (4000 rcf, 4 ºC) and the resulting supernatant diluted 1:1 with water for LC/MS/MS analysis. A 12 point standard curve of substrate metabolites over the range of 0.98 nM to 2000 nM.

Samples were analyzed via electrospray ionization (ESI) on an AB Sciex API-4000 (Foster City, CA) triple-quadrupole instrument that was coupled with Shimadzu LC-10AD pumps (Columbia, MD) and a Leap Technologies CTC PAL auto-sampler (Carrboro, NC). Analytes were separated by gradient elution using a Fortis C18 3.0 x 50 mm, 3 µm column (Fortis Technologies Ltd, Cheshire, UK) thermostated at 40 ºC. HPLC mobile phase A was 0.1% formic acid in water (pH unadjusted), mobile phase B was 0.1% formic acid in acetonitrile (pH unadjusted). The gradient started at 10% B after a 0.2 min hold and was linearly increased to 90% B over 1.2 min; held at 90% B for 0.1 min and returned to 10% B in 0.1 min followed by a re-equilibration (0.9 min). The total run time was 2.5 min and the HPLC flow rate was 0.5 mL/min. The source temperature was set at 500 ºC and mass spectral analyses were performed using multiple reaction monitoring (MRM), with transitions specific for each compound utilizing a Turbo-Ionspray® source in positive ionization mode (5.0 kV spray voltage).

The IC_{50} values for each compound were obtained for the individual CYP enzymes by quantitating the inhibition of metabolite formation for each probe substrate. A 0 µM compound condition (or control) was set to 100% enzymatic activity and the effect of increasing test compound concentrations on enzymatic activity could then be calculated from the % of control activity. Curves were fitted using XLfit 5.2.2 (four-parameter logistic model, equation 201) to determine the concentration that produces half-maximal inhibition (IC_{50}).

***In-Vivo* PK Methods**

All rodent PK experiments were conducted in accordance with the National Institute of Health regulations of animal care covered in Principles of Laboratory Animal Care (National Institutes of Health publication 85-23, revised 1985) and were approved by the Institutional Animal Care and Use Committee.

Rodent Time Course PK Studies

IV cassette PK experiments in rats were carried out according to methods described previously (Bridges et al. *Pharmacol. Res. Perspect.* 2014; reference 52). Briefly, A cassette of compounds (n = 4–5/cassette) were formulated from 10 mM solutions of compounds in DMSO. In order to reduce the absolute volume of DMSO that was administered, the compounds were combined and diluted with ethanol and PEG 400 to achieve a final concentration of 0.4–0.5 mg/mL for each compound (2 mg/mL total) administered in each cassette. The final dosing solutions consisted of approximately 10% ethanol, 40% PEG400, and 50% DMSO (v/v). Each cassette dose was administered IV via the jugular vein to two dual-cannulated (carotid artery and jugular vein) adult male Sprague–Dawley rats, each weighing between 250 and 350 g (Harlan, Indianapolis, IN) for a final dose of 0.2–0.25 mg/kg per compound. Whole blood collections via the carotid artery were performed at 0.033, 0.117, 0.25, 0.5, 1, 2, 4, 7, and 24 hours post dose.

The discrete IV PK experiment in rats (n=2) was carried out analogously using a 1.0 mg/kg solution of **15m** at 1 mg/mL in 10% EtOH, 50% PEG 400, 40% saline. The discrete IP PK experiment in rats (n=3) was carried out analogously using a 10 mg/kg fine microsuspension of **15m** at 4 mg/mL in 0.1% Tween 80 and 0.5% methyl cellulose in H_2O. Whole blood collections via the carotid artery were performed at 0.117, 0.25, 0.5, 1, 2, 4, 7, and 24 hours post dose. The

discrete PO PK experiment in rats (n=2) was carried out analogously using a 3.0 mg/kg fine microsuspension of **15m** at 0.3 mg/mL in 0.1% Tween 80 and 0.5% methyl cellulose in H_2O. Whole blood collections via the carotid artery were performed at 0.25, 0.5, 1, 2, 4, 7, and 24 hours post dose.

Rodent Tissue Distribution Studies

Single time point IP tissue distribution experiments in rodents were carried out according to methods described previously (Bridges et al. *Drug Metab. Dispos.* 2014; reference 56). Briefly, male Sprague–Dawley rats, each weighing between 250 and 350 g, or male CD-1 mice, each weighing between 20-30 g (Harlan, Indianapolis, IN) were dosed with test compound (IP). Formulations were a fine homogeneous suspension at 4.0 mg/mL in 10% EtOH and 90% PEG400 for **15m** or a fine homogeneous suspension at 4.0 mg/mL in 0.1% Tween 80. Animals were euthanized and decapitated, and the brains were removed, thoroughly washed in cold phosphate-buffered saline, and immediately frozen on dry ice. The blood (cardiac puncture) and brain were collected at 0.25 (rats) or 0.5 (mice) hours post dose.

Plasma and Brain Sample Preparation

Plasma was separated by centrifugation (4000 rcf, 4 ºC) and stored at –80 ºC until analysis. On the day of analysis, frozen whole-rat brains were weighed and diluted with 1:3 (w/w) parts of 70:30 isopropanol:water. The mixture was then subjected to mechanical homogenation employing a Mini-Beadbeater™ and 1.0 mm Zirconia/Silica Beads (BioSpec Products) followed by centrifugation. The sample extraction of plasma (20 μL) or brain homogenate (20 μL) was performed by a method based on protein precipitation using three volumes of ice-cold

acetonitrile containing an internal standard (50 ng/mL carbamazepine). The samples were centrifuged (3000 rcf, 5 min) and supernatants transferred and diluted 1:1 (supernatant: water) into a new 96 well plate, which was then sealed in preparation for LC/MS/MS analysis.

LC/MS/MS Bioanalysis of Samples from *In Vivo* Assays

In vivo samples were analyzed via electrospray ionization (ESI) on an AB Sciex API-4000 (Foster City, CA) triple-quadrupole instrument that was coupled with Shimadzu LC-10AD pumps (Columbia, MD) and a Leap Technologies CTC PAL auto-sampler (Carrboro, NC). Analytes were separated by gradient elution using a Fortis C18 3.0 x 50 mm, 3 µm column (Fortis Technologies Ltd, Cheshire, UK) thermostated at 40 ºC. HPLC mobile phase A was 0.1% formic acid in water (pH unadjusted), mobile phase B was 0.1% formic acid in acetonitrile (pH unadjusted). The source temperature was set at 500 ºC and mass spectral analyses were performed using multiple reaction monitoring (MRM), with transitions specific for each compound utilizing a Turbo-Ionspray® source in positive ionization mode (5.0 kV spray voltage). The calibration curves were constructed, and linear response was obtained by spiking known amounts of test compound in blank brain homogenate or plasma. All data were analyzed using AB Sciex Analyst software v1.5.1. The final PK parameters were calculated by noncompartmental analysis using Phoenix (version 6.2) (Pharsight Inc., Mountain View, CA).

Behavioral Pharmacology Methods

Ketamine was purchased from Patterson Veterinary Supply Inc. Ketamine was dissolved in sterile saline. Ketamine was administered SC.

Mouse Tail Suspension.

Adult male CD-1 mice with a body weight of 25 – 30 g were obtained from Envigo (Indianapolis, IN). Mice were house under a 12 hr/12 hr light/dark cycle with food and water available *ad libitum*. Experiments were approved by the Institutional Animal Care and Use Committee of Vanderbilt University and followed the *Guide for the Care and Use of Laboratory Animals*.

VU6010572 and VU6001966 were formulated in 10% Tween 80 in sterile water and placed in a sonicator bath at 39 °C until in solution or microsuspension. Mice were injected intraperitoneally (i.p.) with vehicle (10% Tween 80), VU6010572 (1 – 3 mg/kg), VU6001966 (3 – 30 mg/kg), or the positive comparator ketamine (30 mg/kg) and 15 min later the tail suspension test was performed as follows (Crowley et al. 2005):

Mice were suspended by taping their tail to a vertical metal bar connected to the strain gauge of an automated tail suspension apparatus (Med Associates, St. Albans, VT). The animals' movements were digitally recorded using standard settings (lower threshold = 7, gain = 16, resolution = 10 ms). Test data are presented as the mean (± S.E.M.) duration of immobility during the 6-min test session. Statistical comparisons were made by one-way ANOVA followed by Dunnett's test using (GraphPad Prism V5.04).

Data Analysis. The data for the dose-response studies were analyzed by a between-group analysis of variance. If there was a main effect of dose, then each dose group was compared with the vehicle control group using a Dunnett's comparison. The calculations were performed using JMP IN 8 (SAS Institute, Cary, NC) statistical software and graphed using SigmaPlot9 (Sasgua, MA).

Ancillary Pharmacology Profile of Compound 15m (VU6001966)

LeadProfilingScreen®, Eurofins Panlabs, Inc. (http://www.eurofinspanlabs.com)

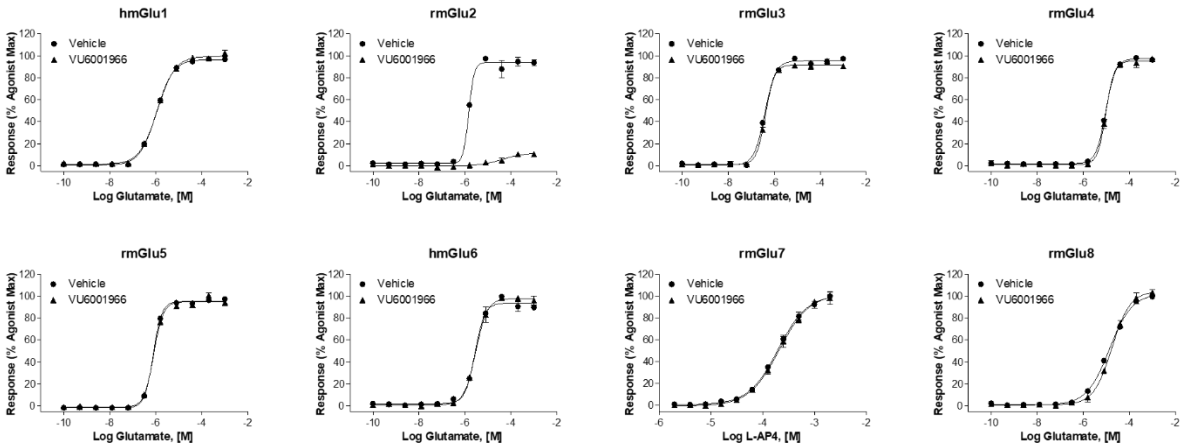
Compound tested at 10 µM

Target	Sp	% Inh
Adenosine A_1	hum	42
Adenosine A_{2A}	hum	29
Adenosine A_3	hum	48
Adrenergic α_{1A}	rat	0
Adrenergic α_{1B}	rat	14
Adrenergic α_{1D}	hum	11
Adrenergic α_{2A}	hum	3
Adrenergic β_1	Hum	15

Target	Sp	% Inh
Histamine H_3	hum	-3
Imidazoline I_2, central	rat	28
Interleukin IL-1	mouse	7
Leukotriene, cysteinyl $CysLT_1$	hum	-12
Melatonin MT_1	hum	24
Muscarinic M_1	hum	0
Muscarinic M_2	hum	4
Muscarinic M_3	hum	6

Adrenergic β_2	hum	8	Neuropeptide Y Y_1	hum	8
Androgen AR	rat	-1	Neuropeptide Y Y_2	hum	-21
Bradykinin B_1	hum	-9	Nicotinic acetylcholine	hum	3
Bradykinin B_2	hum	7	Nicotinic acetylcholine α1, bungarotoxin	hum	12
Ca^{2+} channel L-type, benzothiazepine	rat	0	Opiate δ_1 (OP1, DOP)	hum	-3
Ca^{2+} channel L-type, dihydropyridine	rat	4	Opiate κ(OP2, KOP)	hum	23
Ca^{2+} channel, N-type	rat	6	Opiate μ(OP3, MOP)	hum	3
Cannabinoid CB_1	hum	2	Phorbol ester	mouse	-3
Dopamine D_1	hum	2	Platelet activating factor (PAF)	hum	-3
Dopamine D_{2S}	hum	19	Potassium Channel [K_{ATP}]	ham	9
Dopamine D_3	hum	13	Potassium Channel hERG	hum	3
Dopamine $D_{4.2}$	hum	4	Prostanoid EP_4	hum	7

Target	Sp	% Inh	Target	Sp	% Inh
Endothelin ET_A	hum	22	Purinergic P_{2X}	rabbit	-8
Endothelin ET_B	hum	-5	Purinergic P_{2Y}	rat	0
Epidermal Growth Factor (EGF)	hum	-5	Rolipram	rat	12
Estrogen ERα	hum	3	Serotonin 5-HT_{1A}	hum	-1
$GABA_A$, flunitrazepam, central	rat	14	Serotonin 5-HT_{2B}	hum	23
$GABA_A$, muscimol, central	rat	4	Serotonin 5-HT_3	hum	3
$GABA_{B1A}$	hum	13	Sigma σ_1	hum	11
Glucocorticoid	hum	8	Sodium channel, site 2	rat	-21
Glutamate, kainate	rat	1	Tachykinin NK_1	hum	24
Glutamate, NMDA, agonism	rat	11	Thyroid hormone	rat	8
Glutamate, NMDA, glycine	rat	-15	Transporter, dopamine (DAT)	hum	-2
Glutamate, NMDA, phencyclidine	rat	2	Transporter, GABA	rat	-3
Histamine H_1	hum	8	Transporter, norepinephrine (NET)	hum	6
Histamine H_2	hum	1	Transporter, serotonin (SERT)	hum	-2



Supporting Figure 1. mGluR selectivity for VU6001966 (**15m**) in standard glutamate fold-shift assay at 10 μM.