SUPPORTING INFORMATION

Discovery of N-(5-fluoropyridin-2-yl)-6-methyl-4-(pyrimidin-5-yloxy)picolinamide (VU0424238): A novel negative allosteric modulator of metabotropic glutamate receptor subtype 5 selected for clinical evaluation

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EXPERIMENTAL SECTION

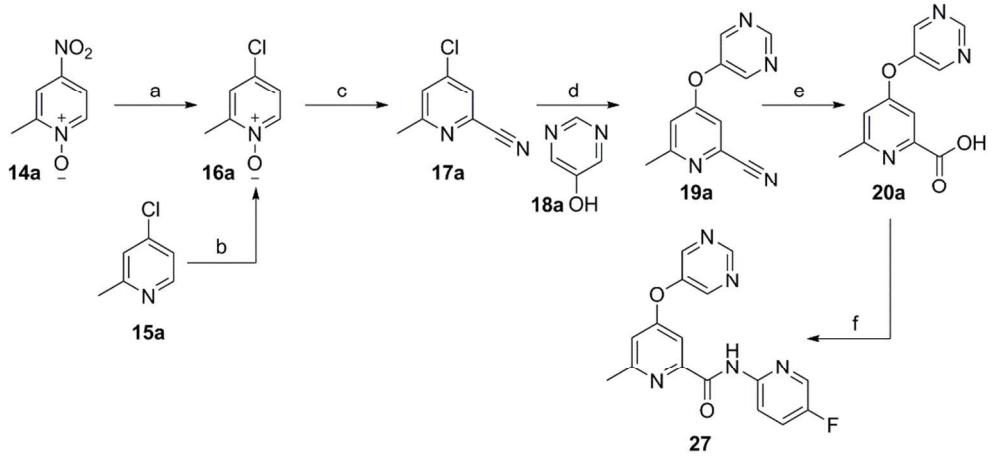
Synthetic Procedures and Characterization Data

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). 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.

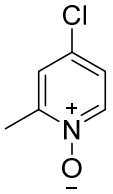
Purity. Low resolution reverse phase LCMS analysis was used to assess compound purity at two wavelengths: 215 and 254 nm. All analogs were at least 95% pure according to this analysis. Low resolution reverse phase LCMS analysis was performed using an Agilent 1200 system comprising a binary pump with degasser, high-performance autosampler, thermostatted column compartment, diode-array detector (DAD) and a C18 column. Flow from the column was split to a 6130 SQ mass spectrometer and Polymer Labs ELSD. The MS detector was configured with an electrospray ionization source. Data acquisition was performed with Agilent Chemstation and Analytical Studio Reviewer software. Method 1: Samples were separated on a ThermoFisher Accucore C18 column (2.6 um, 2.1 x 30 mm) at 1.5 mL/min, with column and solvent

temperatures maintained at 45 ºC. The gradient conditions were 7% to 95% acetonitrile in water (0.1% TFA) over 1.4 min. Low-resolution mass spectra were acquired by scanning from 135 to 700 atomic mass units (AMU) in 0.25 s with a step size of 0.1 AMU and peak width of 0.03 min. Drying gas flow was 11 L/min at a temperature of 350 ºC and a nebulizer pressure of 40 psi. The capillary needle voltage was 3000 V, and the fragmentor voltage was 100V. Method 2: Samples were separated on a ThermoFisher Accucore C18 column (2.6 um, 2.1 x 30 mm) at 1.5 mL/min, with column and solvent temperatures maintained at 45 ºC. The gradient conditions were 7% to 95% acetonitrile in water (0.1% TFA) over 1.1 min. Low-resolution mass spectra were acquired by scanning from 135 to 700 AMU in 0.25 s with a step size of 0.1 AMU and peak width of 0.03 min. Drying gas flow was 11 L/min at a temperature of 350 ºC and a nebulizer pressure of 40 psi. The capillary needle voltage was 3000 V, and the fragmentor voltage was 100V. Method 3: Samples were separated on a Restek Aqueous C18 column (3 um, 3.2 x 30 mm) at 1.25 mL/min, with column and solvent temperatures maintained at 45 ºC. The gradient conditions were 10% to 100% acetonitrile in water (0.1% TFA) over 2 min. Low-resolution mass spectra were acquired by scanning from 100 to 1500 AMU in 0.25 s with a step size of 0.1 AMU and peak width of 0.03 min. Drying gas flow was 11 L/min at a temperature of 350 ºC and a nebulizer pressure of 40 psi. The capillary needle voltage was 3000 V, and the fragmentor voltage was 70V.

Preparation of Compound 27. Compound **27** was prepared via the route pictured immediately below.

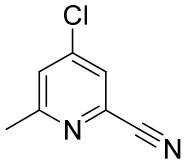


Reagents and conditions: (a) con. HCl, reflux, *75%*; (b) H_2O_2·urea, $(F_3CCO)_2O$, THF, 0 ºC to r.t., *99%*; (c) Me_3SiCN, CH_2Cl_2, Me_2NCOCl, *76%*; (d) **18a**, K_2CO_3, DMF, 80 ºC, *77%*; (e) 2N aq. NaOH, dioxane, reflux *99%*; (f) 5-fluoro-2-aminopyridine, $POCl_3$, pyridine, -15 ºC, *60%*.



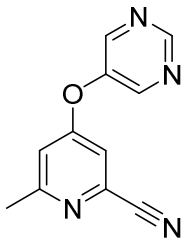
4-Chloro-2-methylpyridine-*N*-oxide (16a). 2-methyl-4-nitropyridine 1-oxide **14a** (5.0 g, 32 mmol, 1.0 eq) was dissolved in concentrated HCl (80 mL) and refluxed for 3 days. The reaction was cooled, and the excess concentrated HCl was removed in vacuo. The viscous oil was neutralized with 10% K_2CO_3 and extracted with CH_2Cl_2 (5x). The combined organics were dried ($MgSO_4$), filtered, and concentrated *in vacuo*. Purification by flash chromatography on silica gel afforded 3.49 g (75%) of the title compound as a yellow solid.

(*Alternative Procedure*) 4-Chloro-2-methylpyridine **15a** (5.0 g, 39 mmol, 1.0 eq) and hydrogen peroxide-urea adduct (7.37 g, 78.4 mmol, 2.0 eq) were dissolved in THF (196 mL) and cooled to 0 ºC. Trifluoroacetic anhydride (12 mL, 86.3 mmol, 2.2 eq) was added dropwise over 15 min, and the reaction was allowed to warm to room temperature. After determination of the completion of the reaction by LCMS (approximately 45 min), the reaction was cooled to 0 ºC and quenched with a 10% aqueous solution of sodium thiosulfate. The reaction was extracted with EtOAc (3x), dried ($MgSO_4$), filtered and concentrated *in vacuo*. Purification by flash chromatography on silica gel afforded 5.63 g (99%) of the title compound as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.23 (d, J = 7.0 Hz, 1H), 7.66 (d, J = 3.0 Hz, 1H), 7.40 (dd, J = 7.0, 3.0 Hz, 1H), 2.32 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 149.53, 139.78, 128.73, 126.41, 124.17, 17.09 ppm; LCMS (Method 1): R_T = 0.302 min, m/z = 144.2 $[M+H]^+$; HRMS, calc'd for C_6H_6ClNO [M], 143.0138; found 143.0139.

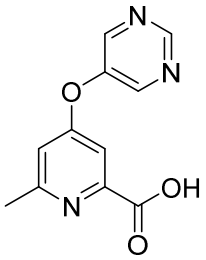


4-Chloro-6-methylpicolinonitrile (17a). Compound **16a** (8.97 g, 62.5 mmol, 1.0 eq) was dissolved in CH_2Cl_2 and dried over $MgSO_4$. The solution was added to a flame-dried round-bottom flask and CH_2Cl_2 was added to give a total volume of 188 mL. Trimethylsilyl cyanide (10 mL, 75 mmol, 1.2 eq) was added, and the reaction stirred for 15 min. Dimethylcarbamyl chloride (6.9 mL, 75 mmol, 1.2 eq) was added dropwise over 20 min, and the reaction was stirred for 24 h. An additional one equivalent each of trimethylsilylcyanide and dimethylcarbamyl chloride were added, and the reaction was stirred for another 72 h. The reaction was made basic with 10% K_2CO_3 and extracted with CH_2Cl_2 (3x). The combined

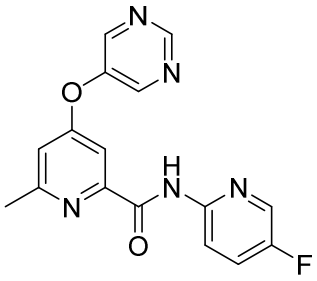
organics were dried ($MgSO_4$), filtered and concentrated *in vacuo*. Purification by flash chromatography on silica gel afforded 7.2 g (76%) of the title compound as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.13 (d, J = 1.6 Hz, 1H), 7.82 (d, J = 1.7 Hz, 1H), 2.52 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ = 161.99, 144.13, 133.01, 127.65, 126.40, 116.60, 23.57 ppm; LCMS (Method 1): R_T = 0.715 min, *m/z* = 153.2 $[M+H]^+$; HRMS, calc'd for $C_7H_5ClN_2$ [M], 152.0141; found 152.0139.



6-Methyl-4-(pyrimidin-5-yloxy)picolinonitrile (19a). Compound **17a** (4.0 g, 26 mmol, 1.0 eq), 5-hydroxypyrimidine **18a** (5.56 g, 57.9 mmol, 2.2 eq), K_2CO_3 (7.24 mg, 52.4 mmol, 2.0 eq) and DMF (66 mL) were added to a reaction vessel and heated at 80 °C for 16 h. The reaction was filtered and concentrated on silica gel (25 g). The silica gel was loaded on top of a fresh bed of silica gel and washed with 50% EtOAc/hexane. The solvents were removed in vacuo and the crude solid was purified by flash chromatography on silica gel to afford 4.31 g (77%) of the title compound as a pale-yellow solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.17 (s, 1H), 8.85 (s, 2H), 7.74 (d, J = 2.4 Hz, 1H), 7.32 (d, J = 2.3 Hz, 1H), 2.48 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ = 164.04, 162.28, 155.51, 150.02, 148.75, 133.54, 117.05, 115.59, 115.04, 23.82 ppm; LCMS (Method 1): R_T = 0.535 min, *m/z* = 213.2 $[M+H]^+$; HRMS, calc'd for $C_{11}H_8N_4O$ [M], 212.0698; found 212.0697.



6-Methyl-4-(pyrimidin-5-yloxy)picolinic acid (20a). Compound **19a** (4.31 g, 20.3 mmol, 1.0 eq) was dissolved in dioxane (90 mL), and 2N NaOH (45 mL) was added. The mixture was refluxed for 18 h and after cooling the reaction was neutralized with 2N HCl (45 mL). The water and solvent were removed *in vacuo*, and the crude reaction was dissolved in 10% MeOH/CH_2Cl_2. The undissolved salt was filtered off and the solvents were removed *in vacuo* to afford 4.65 g (99%) of the title compound as a white solid which was used without further purification.

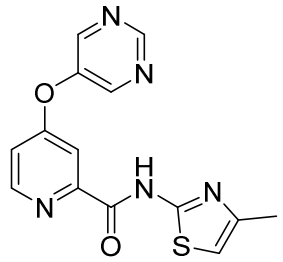


***N*-(5-Fluoropyridin-2-yl)-6-methyl-4-(pyrimidin-5-yloxy)picolinamide (27)**. Compound **20a** (16.84 g, 72.83 mmol, 1.0 eq) and 5-fluoro-2-aminopyridine (16.33 g, 0.15 mol, 2.0 eq) were dissolved in pyridine (485 mL) in a flame-dried round-bottom flask. The reaction was cooled to -15 ºC, and phosphorus oxychloride (7.47 mL, 80.14 mmol, 1.1 eq) was added dropwise while keeping the temperature below -15 ºC. After stirring for 30 min at -15 ºC, the reaction was quenched with ice-water and neutralized with 10% K_2CO_3. The mixture was extracted with EtOAc (3x), and the combined organics were dried ($MgSO_4$), filtered, and concentrated *in vacuo*.

Purification by flash chromatography on silica gel afforded 14.3 g (60%) of the title compound as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.48 (s, 1H), 9.18 (s, 1H), 8.88 (s, 2H), 8.40 (d, J = 3.0 Hz, 1H), 8.27 (dd, J = 9.2 Hz, 4.10 Hz, 1H), 7.86 (td, J = 8.6, 3.1 Hz, 1H), 7.55 (d, J = 2.3 Hz, 1H), 7.29 (d, J = 2.3 Hz, 1H), 2.58 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ = 165.15, 161.11, 160.57, 156.12 (d, J(C,F) = 249.0 Hz), 155.41, 150.29, 150.07, 149.01, 146.92 (d, J(C,F) = 2.1 Hz), 135.94 (d, J(C,F) = 25.5 Hz), 125.83 (d, J(C,F) = 19.9 Hz), 114.56, 114.27 (d, J(C,F) = 4.7 Hz), 108.01, 23.84 ppm; LCMS (Method 2): R_T = 0.727 min, m/z = 326.2 $[M+H]^+$; HRMS, calc'd for $C_{16}H_{12}FN_5O_2$ [M], 325.0975; found 325.0979.

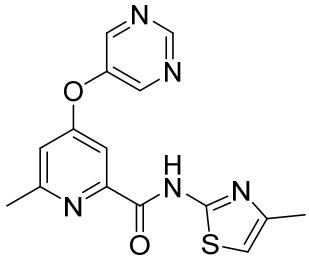
*The following compounds were prepared analogous to compound **27***

_N_-(4-Methylthiazol-2-yl)-4-(pyrimidin-5-yloxy)picolinamide (10)



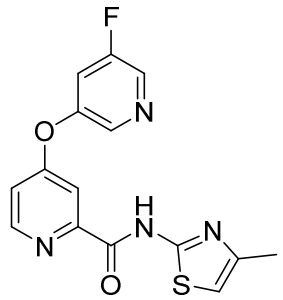
LCMS (Method 3): R_T = 1.147 min, m/z = 314.2 $[M+H]^+$.

6-Methyl-_N_-(4-methylthiazol-2-yl)-4-(pyrimidin-5-yloxy)picolinamide (11)



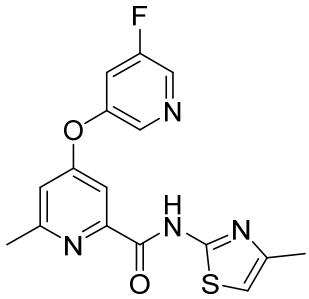
LCMS (Method 2): R_T = 0.670 min, m/z = 328.2 $[M+H]^+$.

4-((5-Fluoropyridin-3-yl)oxy)-*N*-(4-methylthiazol-2-yl)picolinamide (12)



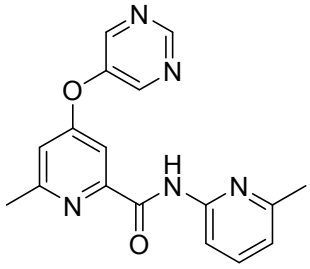
LCMS (Method 2): R_T = 0.732 min, *m/z* = 331.1 $[M+H]^+$.

4-((5-Fluoropyridin-3-yl)oxy)-6-methyl-*N*-(4-methylthiazol-2-yl)picolinamide (13)



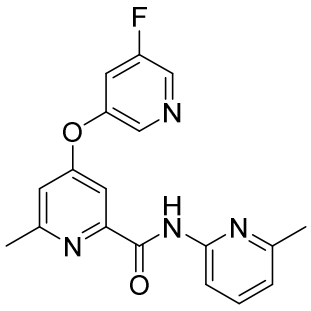
LCMS (Method 2): R_T = 0.740 min, *m/z* = 345.1 $[M+H]^+$.

6-Methyl-*N*-(6-methylpyridin-2-yl)-4-(pyrimidin-5-yloxy)picolinamide (21)



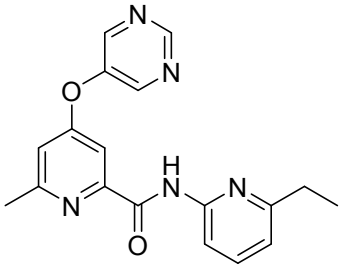
LCMS (Method 2): R_T = 0.653 min, *m/z* = 322.2 $[M+H]^+$.

4-((5-Fluoropyridin-3-yl)oxy)-6-methyl-*N*-(6-methylpyridin-2-yl)picolinamide (22)



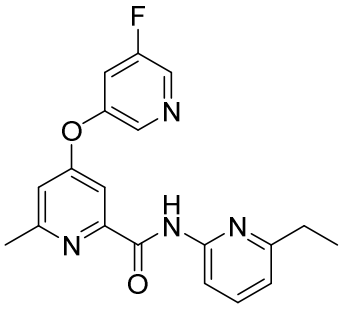
LCMS (Method 2): R_T = 0.730 min, *m/z* = 339.2 $[M+H]^+$.

***N*-(6-Ethylpyridin-2-yl)-6-methyl-4-(pyrimidin-5-yloxy)picolinamide (23)**



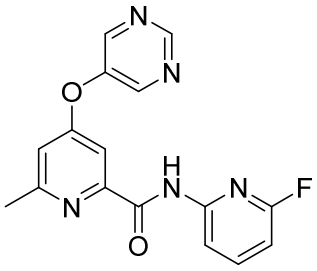
LCMS (Method 2): R_T = 0.624 min, *m/z* = 336.1 $[M+H]^+$.

***N*-(6-Ethylpyridin-2-yl)-4-((5-fluoropyridin-3-yl)oxy)-6-methylpicolinamide (24)**



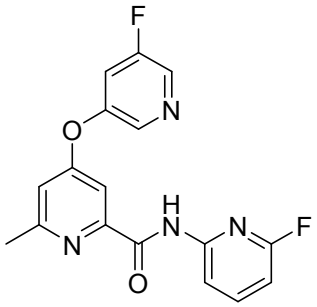
LCMS (Method 2): R_T = 0.777 min, *m/z* = 353.2 $[M+H]^+$.

***N*-(6-Fluoropyridin-2-yl)-6-methyl-4-(pyrimidin-5-yloxy)picolinamide (25)**



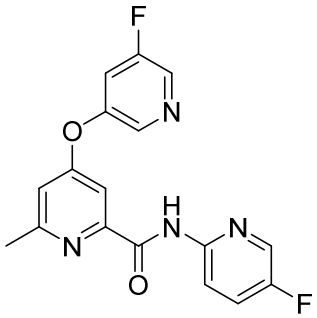
LCMS (Method 2): R_T = 0.780 min, *m/z* = 326.1 $[M+H]^+$.

_N_-(6-Fluoropyridin-2-yl)-4-((5-fluoropyridin-3-yl)oxy)-6-methylpicolinamide (26)



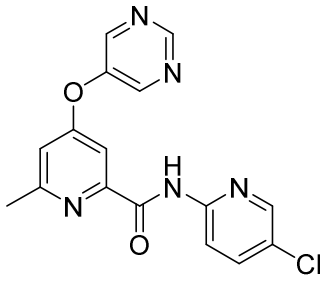
LCMS (Method 2): R_T = 0.840 min, *m/z* = 343.2 $[M+H]^+$.

_N_-(5-Fluoropyridin-2-yl)-4-((5-fluoropyridin-3-yl)oxy)-6-methylpicolinamide (28)



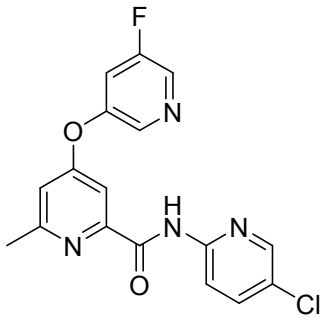
LCMS (Method 2): R_T = 0.822 min, *m/z* = 343.1 $[M+H]^+$.

_N_-(5-Chloropyridin-2-yl)-6-methyl-4-(pyrimidin-5-yloxy)picolinamide (29)



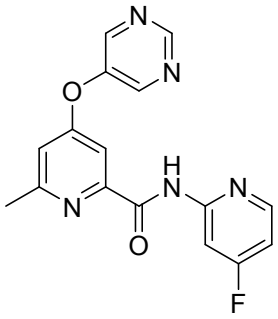
LCMS (Method 2): R_T = 0.803 min, *m/z* = 342.1 $[M+H]^+$.

_N_-(5-Chloropyridin-2-yl)-4-((5-fluoropyridin-3-yl)oxy)-6-methylpicolinamide (30)



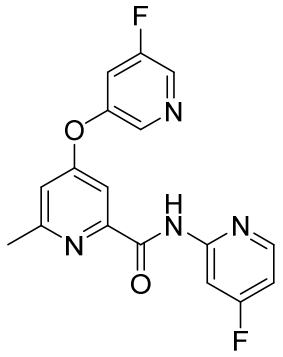
LCMS (Method 2): R_T = 0.874 min, *m/z* = 359.1 $[M+H]^+$.

_N_-(4-Fluoropyridin-2-yl)-6-methyl-4-(pyrimidin-5-yloxy)picolinamide (31)



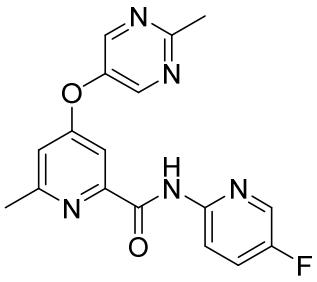
LCMS (Method 2): R_T = 0.744 min, *m/z* = 326.2 $[M+H]^+$.

_N_-(4-Fluoropyridin-2-yl)-4-((5-fluoropyridin-3-yl)oxy)-6-methylpicolinamide (32)



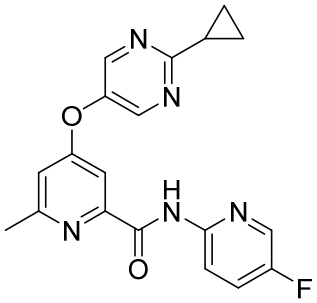
LCMS (Method 2): R_T = 0.822 min, *m/z* = 343.2 $[M+H]^+$.

_N_-(5-Fluoropyridin-2-yl)-6-methyl-4-((2-methylpyrimidin-5-yl)oxy)picolinamide (33)



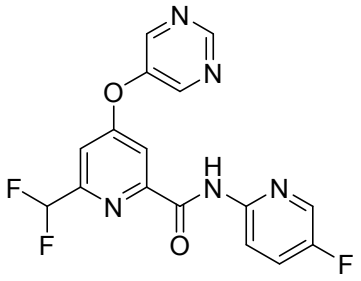
LCMS (Method 2): R_T = 0.776 min, *m/z* = 340.2 $[M+H]^+$.

4-((2-Cyclopropylpyrimidin-5-yl)oxy)-_N_-(5-fluoropyridin-2-yl)-6-methylpicolinamide (34)



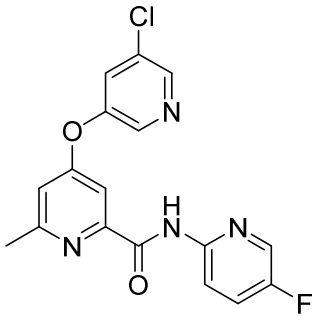
LCMS (Method 1): R_T = 0.937 min, *m/z* = 366.2 $[M+H]^+$.

6-(Difluoromethyl)-_N_-(5-fluoropyridin-2-yl)-4-(pyrimidin-5-yloxy)picolinamide (35)



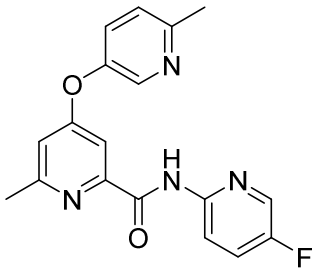
LCMS (Method 2): R_T = 0.765 min, *m/z* = 362.1 $[M+H]^+$.

4-((5-Chloropyridin-3-yl)oxy)-*N*-(5-fluoropyridin-2-yl)-6-methylpicolinamide (36)



LCMS (Method 1): R_T = 0.942 min, *m/z* = 359.1 $[M+H]^+$.

***N*-(5-Fluoropyridin-2-yl)-6-methyl-4-((6-methylpyridin-3-yl)oxy)picolinamide (37)**



LCMS (Method 1): R_T = 0.639 min, *m/z* = 339.1 $[M+H]^+$.

<u>Molecular Pharmacology Methods</u>

$mGlu_5$ Ca^{2+} flux assays (CRC format)

HEK 293 cells expressing rat or human $mGlu_5$ were plated in black-walled, clear-bottomed, poly-D-lysine coated 384-well plates in 20 μL of assay medium (DMEM containing 10% dialyzed FBS, 20 mM HEPES, and 1 mM sodium pyruvate) at a density of 20,000 cells/well. The cells were grown overnight at 37 ºC in the presence of 5% CO_2. The next day, medium was removed using a BioTek ELx washer and the cells incubated with 20 μL of 2 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 (HBSS), 20 mM HEPES, and 2.5 mM probenecid) for 45 min at 37 ºC. Dye was removed, 20 μL of assay buffer was added, and the plate was incubated for 10 min at room temperature. Compounds were serially diluted 1:3 in DMSO into 10 point concentration response curves and transferred to daughter plates using the Echo acoustic plate reformatter (Labcyte, Sunnyvale, CA) followed by further dilution into assay buffer to a 2x stock using a Thermo Fisher Combi (Thermo Fisher, Waltham, MA). Ca^{2+} flux was measured using the Functional Drug Screening System (FDSS7000, Hamamatsu, Japan). After establishment of a fluorescence baseline for about 3 sec, compound was added to the cells, and the response in cells was measured. 2.3 min later an EC_{20} concentration of the $mGlu_5$ receptor agonist glutamate was added to the cells, and the response of the cells was measured for 1.9 min; an EC_{80} concentration of agonist was added and readings taken for an additional 1.7 min. Data were collected at 1 Hz. Data were analyzed as described in Rodriguez, A. L., et al. *Mol. Pharmacol.* **2010**, *78*, 1105-1123. Concentration response curves were generated using a four point logistical equation with XLfit curve fitting software for Excel (IDBS, Guildford, U.K.), GraphPad Prism (GraphPad Software, Inc., La Jolla, CA), or the Dotmatics software platform (Dotmatics, Bishop's Stortford, UK).

mGlu Selectivity Assays

The mGlu selectivity assays have been described in detail previously (Noetzel, M. J., et al. *Mol. Pharmacol.* **2012**, *81*, 120-133).

$mGlu_1$ Fold-shift Selectivity Assay

HEK293 cells stably expressing rat $mGlu_1$ were plated in 384-well, poly-D-lysine-coated, black-walled, clear-bottomed plates in 20 μL of assay medium (DMEM containing 10% dialyzed FBS, 20 mM HEPES, and 1 mM sodium pyruvate) at a density of 20,000 cells/well. The cells were grown overnight at 37 °C in the presence of 5% CO_2. The following day, medium was removed and replaced with assay buffer (HBSS, 20 mM HEPES, pH 7.4, and 2.5 mM probenecid) containing 2 μM Fluo4-AM using a BioTek ELx washer. The cells were incubated for 45 min at 37 ºC, 5% CO_2, followed by a second ELx wash with assay buffer containing no dye. After a 10 min equilibration period at room temperature, cell plates were introduced into the Functional Drug Screening System 6000 (Hamamatsu, Hamamatsu City, Japan) for calcium flux measurements. To assess the effect of the modulator, either vehicle or a fixed concentration of test compound (10 μM) was added to the cells at 2x final concentration and the resulting calcium mobilization in cells was measured. The appropriate concentration of glutamate was added 2.3 min later at 5x the final concentration and readings were taken for an additional 2.6 min. Data were analyzed as described in Rodriguez, A. L., et al. *Mol. Pharmacol.* **2010**, *78*, 1105-1123.

$mGlu_{2/3/4/6/7/8}$ Fold-shift Selectivity Assays

Compound 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, C.M., et al. *Mol. Pharmacol.* **2008**, *73,* 1213-1224). 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 $mGlu_2$, rat $mGlu_3$, 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 (DMEM, 10% dialyzed FBS, 20 mM HEPES, 1mM Na Pyruvate) 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 (HBSS, 20mM HEPES, 4.16 mM Na Bicarbonate) was added. Cells were incubated for 1 h at room temperature and the dye was replaced with 20 μL/well of assay buffer. After establishment of a fluorescence baseline for about 3 sec, 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 min. 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, C.M., et al. *Mol. Pharmacol.* **2008**, *73,* 1213-1224.

Ca^{2+} Mobilization Progressive Fold-Shift in Rat Cortical Astrocytes

Primary rat cortical astrocytes were received from Lonza (Basel, Switzerland) and stored in liquid nitrogen until use. Astrocytes were thawed following the protocol provided by Lonza and plated on BD Falcon Primaria dishes in assay growth medium (AGM; assay basal medium

supplemented with AGM Singlequots from Lonza). Astrocytes were fed with AGM 4-5 h after initial plating and then every 3-4 days until confluent. Astrocytes were plated in 384-well, poly-D-lysine coated, black-walled, clear-bottomed plates in 100 μL of AGM at a density of approximately 20,000 cells/well. The next day astrocytes were supplemented with G5 diluted 1:100 in AGM. Ca^{2+} flux was measured using the Functional Drug Screening System (FDSS6000, Hamamatsu, Japan). After the establishment of a fluorescence baseline for about 3 sec, compound **27** was added to the cells at 2x final concentration and the resulting calcium mobilization in cells was measured. The appropriate concentration of glutamate was added 2.3 min later at 5x the final concentration and readings were taken for an additional 2.6 min. Data were collected at 1 Hz. Data were analyzed as described in Rodriguez, A. L., et al. *Mol. Pharmacol.* **2010**, *78*, 1105-1123. 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).

Equilibrium Binding Assays

The analog of MPEP (**1**), [^{3}H]-3-methoxy-5-(pyridin-2-ylethynyl)pyridine (**39**) was used to evaluate the ability of compound **27** to interact with the common allosteric site on $mGlu_5$. Membranes were prepared from rat $mGlu_5$ HEK293 cells as previously described (Rodriguez, A. L., et al. *Mol. Pharmacol.* **2005**, *68*, 1793-1802). Compound **27** was serially diluted in DMSO then added to assay buffer (50 mM Tris/0.9% NaCl, pH 7.4) to reach a 5x stock and 50 μL test compound was added to each well of a 96 deep-well assay plate. 150 μL aliquots of membranes diluted in assay buffer (20 μg/well) were added to each well. 50 μL [^{3}H]-**39** (2 nM final concentration for competition assays) was added, and the reaction was incubated at room

temperature for 1 h with shaking. After the incubation period, the membrane-bound ligand was separated from free ligand by filtration through glass-fiber 96 well filter plates (Unifilter-96, GF/B, PerkinElmer Life and Analytical Sciences, Boston, MA). The contents of each well were transferred simultaneously to the filter plate and washed 3-4 times with assay buffer using a cell harvester (Brandel Cell Harvester, Brandel Inc., Gaithersburg, MD). 40 µL scintillation fluid was added to each well and the membrane-bound radioactivity determined by scintillation counting (TopCount, PerkinElmer Life and Analytical Sciences). Non-specific binding was estimated using 5 µM of compound **1**. Concentration response curves were generated using a four parameter logistical equation in GraphPad Prism (GraphPad Software, Inc., La Jolla, CA). Competition binding studies were performed with eleven concentrations of **27** ranging from 5.1 x 10^{-10} M to 3.0 x 10^{-5} M. Saturation binding experiments were performed with increasing concentrations of [^{3}H]-**39** ranging from 0.5 to 60 nM in the presence and absence of multiple concentrations of **27** (1, 3, and 10 nM).

Kinetic Binding Assays

Rat $mGlu_5$ membranes (20 µg) were equilibrated with [^{3}H]-**39** (2 nM) at room temperature for 1 h in assay buffer (400 µL). Non-specific binding was estimated using 10 µM MPEP (**1**), which was added prior to the incubation. Equivalent volumes of 10x compound **1** was combined with 10x compound **27** (300 nM or 100 µM) or vehicle. 100 µL treatment solutions of **27** or vehicle were added at desired time points in reverse time course order (i.e., 30 min group added first, 2 min group added last) such that t = 0 was the same for all treatment groups. At t = 0, the contents of each well were transferred simultaneously to the filter plate and washed 3-4 times with assay buffer using a cell harvester. 40 µL scintillation fluid was added to each well and the

membrane-bound radioactivity determined by scintillation counting. Dissociation curves were generated using a one-phase exponential decay equation in GraphPad Prism.

5-Methyl-6-(phenylethynyl)-pyridine (40) Ca^{2+} Assays

HEK 293A cells stably expressing rat $mGlu_5$ were plated in black-walled, clear-bottomed, poly-D-lysine coated 384-well plates in 20 μL of assay medium (DMEM containing 10% dialyzed FBS, 20 mM HEPES, and 1 mM sodium pyruvate) at a density of 20K cells/well. The cells were grown overnight at 37 °C in the presence of 5% CO_2. The next day, medium was removed and the cells incubated with 20 μL of 2 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, pH 7.4) for 45 min at 37 ºC. Dye was removed, 20 μL of assay buffer was added, and the plate was incubated for 10 min at room temperature. Ca^{2+} flux was measured using the Functional Drug Screening System (FDSS7000, Hamamatsu, Japan). Equivalent volumes of 4x compound **27** were combined with 4x compound **40** or vehicle. After establishment of a fluorescence baseline for about 3 sec, the **29**/**40** solution was added to the cells at 2x final concentration, and the response in cells was measured. 2.3 min later an EC_{80} concentration of glutamate was added at 5x the final concentration and readings taken for an additional 2.6 min. Data were analyzed as described in Rodriguez, A. L., et al. *Mol. Pharmacol.* **2010**, *78*, 1105-1123. EC_{50} values for the effect of **27** on the $mGlu_5$ response to glutamate in the presence of **40** or vehicle were generated using a 4 parameter logistic equation in GraphPad Prism.

Ancillary Pharmacology and Safety Screening

LeadProfilingScreen® (catalog #68), Eurofins Panlabs, Inc. (http://www.eurofinspanlabs.com)

Compounds **27** and **28** tested at 10 μM in a panel of radioligand binding assays. Data expressed as percent inhibition.

Target	Sp	27	28	Target	Sp	27	28
Adenosine A_1	hum	13	26	Histamine H_3	hum	-2	-12
Adenosine A_{2A}	hum	13	-1	Imidazoline I_2, central	rat	26	10
Adenosine A_3	hum	11	33	Interleukin IL-1	mouse	5	2
Adrenergic α_{1A}	rat	13	10	Leukotriene, cysteinyl $CysLT_1$	hum	1	2
Adrenergic α_{1B}	rat	-1	6	Melatonin MT_1	hum	13	12
Adrenergic α_{1D}	hum	-5	15	Muscarinic M_1	hum	-2	-3
Adrenergic α_{2A}	hum	16	-7	Muscarinic M_2	hum	-7	-1
Adrenergic β_1	hum	3	2	Muscarinic M_3	hum	-2	5
Adrenergic β_2	hum	1	-6	Neuropeptide Y Y_1	hum	-2	2
Androgen AR	rat	7	12	Neuropeptide Y Y_2	hum	-1	6
Bradykinin B_1	hum	14	-2	Nicotinic acetylcholine	hum	-1	-1
Bradykinin B_2	hum	-3	5	Nicotinic acetylcholine α1, bungarotoxin	hum	-2	4
Ca^{2+} channel L-type, benzothiazepine	rat	5	12	Opiate δ_1 (OP1, DOP)	hum	4	12
Ca^{2+} channel L-type, dihydropyridine	rat	1	9	Opiate κ(OP2, KOP)	hum	-7	4
Ca^{2+} channel, N-type	rat	3	-5	Opiate μ(OP3, MOP)	hum	-3	5
Cannabinoid CB_1	hum	11	16	Phorbol ester	mouse	-9	17
Dopamine D_1	hum	3	7	Platelet activating factor (PAF)	hum	5	31
Dopamine D_{2S}	hum	8	2	Potassium Channel [K_{ATP}]	ham	3	7
Dopamine D_3	hum	11	13	Potassium Channel hERG	hum	16	3
Dopamine $D_{4.2}$	hum	12	9	Prostanoid EP_4	hum	7	8
Endothelin ET_A	hum	2	4	Purinergic P_{2X}	rabbit	-2	2
Endothelin ET_B	hum	-2	-1	Purinergic P_{2Y}	rat	4	4
Epidermal Growth Factor (EGF)	hum	7	14	Rolipram	rat	8	12
Estrogen ERα	hum	-1	4	Serotonin 5-HT_{1A}	hum	4	7
$GABA_A$, flunitrazepam, central	rat	1	6	Serotonin 5-HT_{2B}	hum	14	19
$GABA_A$, muscimol, central	rat	7	-1	Serotonin 5-HT_3	hum	-6	1
$GABA_{B1A}$	hum	11	-2	Sigma σ_1	hum	5	6
Glucocorticoid	hum	-4	-11	Sodium channel, site 2	rat	8	-3
Glutamate, kainate	rat	15	5	Tachykinin NK_1	hum	-3	3
Glutamate, NMDA, agonism	rat	-7	1	Thyroid hormone	rat	-3	22
Glutamate, NMDA, glycine	rat	6	-4	Transporter, dopamine (DAT)	hum	34	86
Glutamate, NMDA, phencyclidine	rat	-3	-6	Transporter, GABA	rat	23	-2
Histamine H_1	hum	-5	-8	Transporter, norepinephrine (NET)	hum	8	48
Histamine H_2	hum	-7	2	Transporter, serotonin (SERT)	hum	6	17

ChanTest Cardiac Ion Chanel Panel

http://www.criver.com/products-services/drug-discovery/therapeutic-areas/oncology/safety-assessment/cardiac-risk/cardiac-ion-channel-panel

Compound **27** was tested at eight concentrations ranging from 0.03 to 100 μM using Ion Works™ Quattro automated patch clamp system (MDS-AT) versus cloned cardiac ion channels expressed in CHO cells. Details regarding the cell lines and targets are summarized below.

1. Cloned hERG potassium channels (expressed in CHO cells), responsible for I_{Kr}
2. Cloned hNav1.5 sodium channel (expressed in CHO cells), responsible for I_{Na}, fast sodium current
3. Cloned hKvLQT1/hminK potassium channels (coexpressed in CHO cells), responsible for I_{Ks}, slow delayed rectifier potassium current.
4. Cloned hKv4.3/hKChIP2.2 potassium channels (expressed in CHO cells), responsible for I_{to}, transient outward potassium current
5. Cloned hKv1.5 potassium channels (expressed in CHO cells), responsible for I_{Kur}, ultra-rapid delayed rectifier potassium current
6. Cloned L-type calcium channels (hCav1.2/β2/α2δ1 expressed in CHO cells), responsible for $I_{Ca,L}$, high threshold calcium current.
7. Cloned T-type calcium channels (hCav3.2 expressed in CHO cells), responsible for $I_{Ca,T}$, low threshold calcium current.
8. Cloned hKir2.1 potassium channels (expressed in CHO cells), responsible for I_{K1}, inwardly rectifying potassium current.
9. Cloned hHCN2 potassium channels (expressed in CHO cells), responsible for I_f potassium current.
10. Cloned hHCN4 potassium channels (expressed in CHO cells), responsible for I_f potassium current.

Bacterial Reverse Mutation Assay

The potential mutagenic activity of compound **27** was evaluated at IIT Research Institute in Chicago, IL (http://iitri.org/genetic-toxicology). The plate incorporation method was employed using four Salmonella typhimurium tester strains (TA98, TA100, TA1535, and TA1537) and one Escherichia coli strain (WP2 uvrA) in the presence and absence of an external metabolic

activation system (Aroclor-1254-induced rat liver S9 fraction mixture). Positive controls were employed for each strain and metabolic activation system. Compound **27** was tested at 7 concentrations ranging from 0.05 to 5.0 mg/plate.

In-Vitro DMPK Methods

Plasma Protein Binding

The protein binding of each compound was determined in rat or human 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 h 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 h 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 Analysis 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°C. 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 Pooled 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 min. 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 min (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}).

Stability in Human Cryproserved Hepatocytes

Materials: Compound **27** (10mM DMSO stock); midazolam (10mM DMSO stock), Sigma Catalog #UC430; cryopreserved hepatocytes, Celsis In Vitro Technologies, 1mL/vial, human (19 Donor; Pooled Mixed Gender) 5 million cells/vial: Product #X008000, Lot: OOO; InVitroGRO HT (Thawing) Medium, Celsis In Vitro Technologies: Product #Z99019; InVitroGRO KHB, Celsis In Vitro Technologies: Product #Z99074. Compound preparation was as follows: dilution of 3 μL of 10 mM stocks of midazolam (positive control) and compound **27** into 3 mL of 37 ºC warmed KHB for a final concentration of 10 μM in the KHB.

Thawing: (1) InVitroGRO HT (Thawing) Medium and KHB bottles were sterilized in the TC hood; (2) 50 mL of the medium was transferred to a separate sterile tube (50 mL conical) and placed into the 37 ºC water bath to warm (~ 30 min); (3) the hepatocyes were removed from LN2 storage and quickly thawed (about 1.5 min) and poured into the 50 mL conical tube of medium; (4) the cells were mixed by inversion and centrifuged at 50xg for 5 min at room temperature; (5) the supernatant was poured off and the cells were re-suspended in 2 mL of room temperature InVitroGRO KHB buffer for counting/viability assessment.

Experimental protocol: (1) Quench plate was prepared by adding 150 μL of cold 50 nM carbamezapine in acetonitrile to the appropriate wells; (2) incubation plate was prepared by adding 250 μL of the warm, KHB-incubated compound to the appropriate wells; (3) step 2 was immediately followed by the addition of 250 μL of the cell mixture to initiate the reaction. A 50 μL aliquot, $t = 0$ time point, was immediately removed and placed into the appropriate $t = 0$ row of the quench plate; quench plates were stored at 4 ºC during the study; the final concentration of the test compounds was 5 μM; (4) the incubation plate was shaken at 37 ºC and 900 rpm for the remainder of the study with 50 μL aliquots quenched at the appropriate times (15, 30, 60, and

120 min); (5) once completed, the quench plate was vortexed and centrifuged at 4000 rpm for 10 min; (6) the supernatant from the samples was diluted 1:1 in water for LC/MS/MS analysis; a specific method was developed for each compound to monitor % parent remaining based on compound-specific fragmentation patterns.

P-gp Substrate Assessment Using MDR1-MDCK or Caco-2 Cells

Materials: Compound **27** (10 mM DMSO stock); controls – propranolol (Sigma catalog #P0884-16), loperamide (Sigma catalog #L4762-5G), mitoxantrone (Tocris catalog #4250), digoxin (Sigma catalog #D6003); all controls were made in 10mM DMSO stock; Hank's Balanced Salt Solution (HBSS) Buffer 1X (Cell Gro catalog #21-023CV); D-glucose (Sigma catalog # G7528-1KG), 3.5 g/L in HBSS buffer; HEPES (Sigma catalog # H4034-100G), 5.96 g/L in HBSS buffer.

Preparation work: (1) HBSS buffer (pH 7.4) was prepared by adding D-glucose 1.75 g and HEPES 2.98 g to 500 mL bottle of HBSS buffer; the contents were mixed thoroughly and the pH adjusted to 7.4 using 1 N NaOH; (2) test compound solutions were prepared: **27** (2 μM), propranolol (2 μM), loperamide (5 μM) and mitoxantrone (10 μM) in HBSS buffer from 10 mM DMSO stock; propranolol is a high permeability compound and was used as a positive control for passive diffusion; both loperamide and digoxin were used as positive controls for P-glycoprotein (P-gp) substrates; mitoxantrone is used as a positive control for breast cancer resistance protein (BCRP) efflux transporter; (3) the HBSS buffer and test compound solutions were maintained at 37 ºC by water bath; (4) the MIDSCI mini incubator shaker was set at 50 RPM speed with a 37 ºC temperature control at least 30 min before the experiment began; (5) a

24 well plate with collagen coated inserts was prepared; this plate was used to measure a blank TransEpithelial Electrical Resistance (TEER) value.

Experimental Protocol: (1) the cell culture plate was brought out of the incubator and the media was removed from the plate using a glass Pasteur pipette; (2) to the plate was added 37 ºC HBSS buffer; for 24 well plates, the volume of the buffer added on the apical side was 300 µL using repeater pipette and basolateral side was 1 mL using 10 mL serological pipette; care was taken to add the buffer on the wall of the insert and not directly on the monolayer as direct pipetting of the buffer on the cells may cause damage to the monolayer and invalidate the experiment (3) to the blank plate (with no cells) equal amount of HBSS buffer was added on both sides of the insert; the blank plate was used to measure the TEER reading for the plate with no cells which is considered a blank reading (usual value for the blank 24 well plate was around 200Ω; (4) to calibrate the Millicell Volt-Ohm Meter, the electrodes were disconnected from the meter and another cable with no electrodes was connected to the meter; a reading of 1000Ω was adjusted using the available screw on the meter, and the cable was disconnected and electrodes reconnected to the meter; (5) after calibration, the blank plate TEER value reading was taken by dipping the electrodes in the buffer in the plate; one electrode was placed on each side of the insert; care was made to make sure that the electrode did not touch the monolayer but was completely immersed in the buffer solution while taking the reading. (6) after measuring blank plate reading, TEER value measurements are taken for 24 well-plates containing Caco-2 or MDCK cells taking the same precautions; a TEER value of 200-400Ω for MDCK and 1500-2200Ω for Caco-2 cells as compared to blank 24 well-plates was typical; (7) after the TEER value measurement, the HBSS buffer was removed from the apical and basolateral sides of the plate and new HBSS buffer was added to the plate; for 24 well plates, 300 µL of HBSS buffer

was added on apical and 1 mL of HBSS buffer was added on basolateral side of the plate; the plate was placed on 37 ºC shaker (50 RPM speed) and allowed to incubate for one h; (8) after 1 h incubation, the HBSS buffer was removed from the apical and basolateral sides of the plate and test compound was added to the donor side and HBSS buffer was added to the receiver side; for 24 well plates, the solution volume for the apical side was 320 µL and the basolateral side was 1.02 mL; (9) for the 24 well plate transport assay at 0 and 2-h time points, 20 µL and 50 uL of sample was collected from donor and receiver side, respectively; thirty microliters of HBSS was then added to the wells with 20 µL of samples in 96-well plate; (10) stands were prepared in HBSS buffer in a series of concentrations (5, 2.5, 1.25, 0.625, 0.313, 0.165, 0.078, 0.039, 0.019, 0.0098, 0.0049, and 0.0024 µM); 50 µL was added to 96-well plate; (11) 150 µL of acetonitrile (with 50 nM carbamazepine in 100% acetonitrile for acidic compounds and 500 nM tolbutamide in 100% acetonitrile for basic compounds) was added to the 96-well plate to precipitate all the proteins in the sample in the 96 well plate; (12) the above mixture was centrifuged at 3999 RCF for 10 min at 20 ºC; after centrifugation, 50 µL of supernatant was diluted with 150 µL of double distilled water in another 96 well plate; the plate was sealed and samples were analyzed by LC-MS/MS (AB Sciex 4000).

Quality control criteria: (1) the acceptable range for A-B permeability (P_{app}A-B) of 2 µM Propranolol is 2−10 × 10^{-6} cm/sec and 5 µM Propranolol is 5−15 × 10^{-6} cm/sec.; (2) the acceptable efflux ratio of loperamide should be greater than 2 for the P-gp assay; (3) the acceptable efflux ratio of digoxin should be greater than 4 for the P-gp assay; (4) the acceptable efflux ratio of mitoxantrone should be greater than 2 for BCRP assay.

$$P_{app} = \frac{dQ}{dT \cdot A \cdot C_0} = \frac{VdC}{dT \cdot A \cdot C_0}$$

Equation 1

where V = sample volume (ml), dC = concentration variations, dQ = quantity variations, dT = time variations, C_0 = the initial concentration in the donor compartment, and A = exposed surface (cellular monolayer in cm^2). In the above formula, $dQ/(dT \cdot A)$ represents the mass transfer per unit time and unit surface across the monolayer.

Definitive Cytochrome P450 Inhibition Assays in Pooled Human Liver Microsomes

The ability of compound **27** to reversibly inhibit the metabolism of form-selective probe substrates of cytochromes P450 (CYP) 1A2, 2B6, 2C8, 2C9, 2C19, 2D6, and 3A4 was examined externally at Q^2 Solutions in Indianapolis, IN (http://www.q2labsolutions.com/bioanalytical-adme-laboratories/in-vitro-adme-and-metabolite-identification-services). Seven concentrations of **27** and a solvent only control, were incubated in triplicate with pooled human liver microsomes (HLM), a K_m concentration of CYP probe substrate, and 1 mM NADPH for the required incubation time in 100 mM sodium phosphate buffer, pH 7.4. Incubation times and HLM concentrations used reflect linear rate incubation conditions for the various probe substrates. Probe substrates were prepared in methanol at a concentration 100-fold greater than the target concentration to allow for direct addition into the incubation mixtures. Positive controls for reversible inhibition and for time-plus NADPH-dependent inhibition were incubated in triplicate for the appropriate CYP. The ability of **27** to inhibit the above CYPs in a time-dependent (NADPH-independent) manner (samples pre-incubated for 30 min without NADPH) or time-plus NADPH-dependent manner (samples pre-incubated for 30 min with NADPH) was also examined in addition to reversible inhibition. Compound **27** was prepared in methanol at a

concentration 100-fold greater than the target concentration to allow for direct addition into the incubation mixtures.

Reversible inhibition: Inhibitor (1 μL) was added to 88 μL of HLM in 100 mM sodium phosphate buffer, pH 7.4, followed by addition of the probe substrate (1 μL) to the incubation mixture. The incubation plate was pre-incubated for 3 min at 37 °C. 10 mM NADPH (10 μL) was added to initiate the activity reaction for a final concentration of 1 mM.

Time-dependent (NADPH-independent) inhibition: Inhibitor (1 μL) was added to 88 μL of HLM in 100 mM sodium phosphate buffer, pH 7.4. The plate was pre-incubated for 30 min at 37 °C. The probe substrate (1 μL) was added to the incubation mixture, followed by addition of 10 μL of 10 mM NADPH (final concentration of 1 mM) to initiate the activity reaction.

Time- plus NADPH-dependent inhibition: Inhibitor (1 μL) was added to 88 μL of HLM in 100 mM sodium phosphate buffer, pH 7.4, followed by addition of 10 μL of 10 mM NADPH (final concentration of 1 mM). The plate was pre-incubated for 30 min at 37 °C. The probe substrate (1 μL) was added to the incubation mixture to initiate the activity reaction.

Reactions were quenched with the addition of 100 μL of acetonitrile containing the appropriate internal standard, mixed, and centrifuged to precipitate the protein. These samples were prepped for analysis by LC/MS/MS.

***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 and Tissue Distribution PK Studies

Compounds were formulated for IV dosing in 10% ethanol, 50-70% PEG 400, and 20-40% saline. Each IV PK experiment in rats (n=2) dose was conducted via IV administration in the jugular vein of 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 1.0 mg/kg. Whole blood collections via the carotid artery were performed at 0.033, 0.117, 0.25, 0.5, 1, 2, 4, 7, and 24 h post dose. Compounds **27**, **28**, and **33** were formulated for PO dosing in 10% polysorbate 80 in 0.5% methyl cellulose. Each PO time course PK experiment in rats (n=2) was conducted with two cannulated (carotid artery) adult male Sprague–Dawley rats, each weighing between 250 and 350 g (Harlan, Indianapolis, IN) for a final dose of 10 mg/kg. Each PO tissue distribution study experiment in rats (n=2) was conducted with two adult male Sprague–Dawley rats, each weighing between 250 and 350 g (Harlan, Indianapolis, IN) for a final dose of 10 mg/kg.

In the IV time course experiments, whole blood collections via the carotid artery were performed at 0.117, 0.25, 0.5, 1, 2, 4, 7, and 24 h post dose. In the PO time course experiments, whole blood collections via the carotid artery were performed at 0.25, 0.5, 1, 2, 4, 7, and 24 h post dose. In the PO tissue distribution studies, at one h post dose, animals were euthanized and decapitated, blood was collected via cardiac puncture and the brains were removed, thoroughly washed in cold phosphate-buffered saline, and immediately frozen on dry ice.

In the IP tissue distribution study with compound **27** in mice, **27** was formulated in 10% polysorbate 80 and dosed IP in male CD-1 mice (2-3 per time point). At 0.25, 0.5, 1, 3, and 6 h post dose, animals were euthanized and decapitated, blood was collected via cardiac puncture and the brains were removed, thoroughly washed in cold phosphate-buffered saline, and immediately frozen on dry ice.

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 Analysis 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).

NHP IV and PO Time Course PK Studies

The in-life portion of the NHP PK studies was conducted at Ricerca Biosciences, LLC in Concord, OH. Cynomolgus monkeys were obtained from the stock colony at Ricerca Biosciences. Animals were housed individually in stainless steel cages. Individual group housing enclosures were as specified in the *USDA Animal Welfare Act* (9 CFR, Parts 1, 2, and 3) and described in *Guide for the Care and Use of Laboratory Animals* (ILAR publication, 1996, National Academy Press, Washington DC, 1996). The location of the room housing animals that were assigned to study is maintained as part of the study record. Cleaning of the animal room and cages was performed according to testing facility SOPs. The environmental conditions of the animal room were maintained per Guide for the Care and Use of Laboratory Animals and were continuously monitored and documented. Treatment of the animals was in accordance with regulations outlined in the USDA Animal Welfare Act (9 CFR Parts 1, 2, and 3) and the conditions specified in *Guide for the Care and Use of Laboratory Animals* (National Academy

Press, Washington DC, 1996). Four (4) male monkeys were used in this study. Their ages ranged from 3.8 to 4.3 years old, and the weight range for the animals at the time of allocation to groups was 3.10-4.60 kg.

Compounds **27** and **28** were formulated together for an IV cassette in 10% ethanol, 70% PEG400, and 20% saline for a final concentration of 1 mg/mL for each compound, which enabled a 0.5 mg/kg dose per compound and a total dose of 1 mg/kg. Compounds **27** and **28** were formulated for discrete oral doses in 10% polysorbate 80 in 0.5% methyl cellulose and dosed at 5.0 mg/kg. IV administration was done by injection into the saphenous vein. Oral dosing was made via nasogastric gavage. Blood samples were collected from the femoral vein at the following time points: 0.0333 h (IV only), 0.083 h, 0.25 h, 0.50 h, 0.75 h (PO only), 1 h, 2 h, 4 h, 8 h, 12 h, and 24 h. Blood samples were placed on ice and centrifuged within approximately 30 min of collection under refrigeration. Plasma samples were harvested into a single 96-well plate storage tube and frozen at approximately -70 ºC until they were shipped to Vanderbilt. LC/MS/MS bioanalysis at Vanderbilt was carried out as described above for plasma samples from rodent PK studies.

Behavioral Pharmacology

All rodent behavioral pharmacology 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.

Compounds. The $mGlu_5$ NAM **27** was prepared according to methods outlined herein. The $mGlu_5$ NAM MTEP (**2**) was prepared in house according to methods described in the literature (Cosford, N. D., et al. *J. Med. Chem.* **2003**, *46*, 204-206). Ketamine was purchased from Patterson Veterinary Supply Inc. Doses of **27** and **2** were dissolved in 10% polysorbate 80 in H_2O, vortexed vigorously, heated gently with a Master Heat Gun (Master Appliance Corp., Racine, WI), and sonicated at 37 ºC for 30 min. The pH was checked using 0-14 EMD strips and adjusted to approximately 7. Compound **27** and **2** were administered IP. Ketamine was dissolved in sterile saline. Ketamine was administered SC.

Marble burying dose groups. vehicle, 15 mg/kg **2** (positive control), 0.1 mg/kg **27**, 0.3 mg/kg **27**, 1.0 mg/kg **27**, 3.0 mg/kg **27**, 10 mg/kg **27**, and 30 mg/kg **27**.

Marble burying subjects. This study was conducted using male Harlan CD-1 mice (Indianapolis, IN), weighing 30 to 35 grams. Subjects were housed in a large colony room under a 12-h light/dark cycle (lights on at 6:00 a.m.) with food and water provided ad libitum. Test sessions were performed between 10:00 a.m. and 4:00 p.m. All dose groups consisted of 10 mice. All 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.

Marble burying procedure. Eight small Plexiglass cages (32 x 17 x 14 cm) were arranged in two rows of four cages on top of a large, round table. Mice were transported from the colony

room to the testing room and allowed to habituate for 30 min. Mice were pretreated with vehicle, a dose of **27**, or a dose of **2** for 15 min and individually placed in the cages in which 12 black glass marbles (14 mm diameter) had been evenly distributed (spaced 6.4 cm vertically and 4.25 cm horizontally from each other and the walls of the cage) on top of 2.5 cm Diamond Soft Bedding (Harlan Teklad, Madison, WI). The compound and comparator were evaluated in a counterbalanced design, in which all doses of compounds were tested in each session. Mice receiving the same dose were placed in cages on opposite sides of the table to control for effects of lighting and context. Clear, perforated plastic lids were set on top of each cage and the amount of marble burying was recorded over a 30 min interval. The mice were then removed from the cages and the number of buried marbles was counted using the criteria of greater than two-thirds covered by bedding. Each session was videotaped with a Sony MiniDV camcorder equipped with a Sony wide-angle lens mounted on a 1.5 m tripod. Effects that could be characterized as overtly sedating or impairing motor function were monitored visually and were not observed with compound **27**.

Forced swim test (FST) dose groups. vehicle, 10 mg/kg ketamine (positive control), 1.0 mg/kg **27**, 3.0 mg/kg **27**, 10 mg/kg **27**, and 30 mg/kg **27**.

FST subjects. This study was conducted using male Harlan Sprague-Dawley rats (Indianapolis, IN), weighing 275 to 325 grams. Subjects were housed in a large colony room under a 12-h light/dark cycle (lights on at 6:00 a.m.) with food and water provided *ad libitum*. Test sessions were performed between 10:00 a.m. and 4:00 p.m. All dose groups consisted of 8-10 rats. All 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.

FST procedure. Rats were exposed to 2 swim sessions, a habituation session and 24 h later a 6 min testing session. In the habituation session rats were placed in plexiglass cylinders (45 x 20cm) containing 23-25 ºC water approximately 30 cm deep for 15 min. On the testing day compound or vehicle was administered thirty min prior to the test. After the pre-treatment period the animals were placed in swim tanks as described above and then underwent a 6 min testing session which was recorded. Following both swim sessions, rats were removed from the cylinder, dried with a paper towel, then transferred to a drying environment for 15 min or until the rat is sufficiently dry to return to its home cage. Each session was videotaped with a Sony MiniDV camcorder equipped with a Sony wide-angle lens mounted on a 1.5 m tripod. Videos were manually scored for the time the animal exhibited immobility.

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).

µPET Imaging Study of Compound 27 in Rats Using [^{18}F]-41

All rodent μPET imaging 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.

Receptor occupancy of **27** in rats utilizing positron emission tomography (PET) was determined as previously described (Rook, J. M., et al. *Neuropsychopharmacology* **2015**, *40*, 755). Male Sprague-Dawley (~300 g) rats were implanted with carotid and jugular catheters and allowed 5 - 7 days for recovery. Rats were administered **27** orally (10% polysorbate 80, 0.5% methyl cellulose in sterile water, 3 ml/kg, 30 min). Rats were then anesthetized using 1.5% isoflurane and positioned in the microPET Focus 220 (Siemens, Knoxville, TN). Approximately ~15MBq/0.4 mL [^{18}F]-**41** ([^{18}F]-FPEB) was injected via jugular catheter while simultaneously initiating a 60 min dynamic acquisition. Blood samples were drawn via arterial catheter every 10 sec for the first min, then 1.5, 2, 4, 6, 8, 12, 20, 30, 45, and 60 min thereafter. Samples were centrifuged and plasma activity was measured using a well counter. Through acetonitrile extraction and thin layer chromatography, metabolite corrections were carried out on selected blood samples and an arterial plasma input function was constructed. Attenuation corrections of images were carried out using a ^{57}Co transmission scan. Images were reconstructed using OSEM2D. Using an MRI rat brain template (Rubins, D. J., et al. *Neuroimage* **2003**, *20*, 2100), PET images were co-registered. Regions-of-interest (ROIs) were drawn around the cerebellum and striatum of the template and superimposed over all PET dynamic frames. Time-activity curves (TACs) were established for these regions over the duration of the scan. The metabolite-corrected plasma TAC was used as the input function to estimate striatal volume of distribution (VT) using graphical analysis (Logan, J. *J. Cereb. Blood Flow Metab.* **1996**, *16*, 834). Percent

occupancy was calculated using the following equation, percent occupancy = [($V_{Tvehicle}$ − $V_{Ttreatment}$)/ $V_{Tvehicle}$] x 100.

PET Imaging Study of Compound 27 in Female Baboons Using [^{18}F]-41

Two female baboons (Papio anubis) were used as research subjects for [^{18}F]-**41** PET brain imaging studies and were housed at the Yale University School of Medicine (New Haven, CT). The baboons were imaged following identical procedures with a Siemens Biograph mCT scanner. PET imaging studies were carried out under institutional animal care protocols complying with Federal regulations. Animal care approval and oversight for this study was provided by the Yale University Institutional Animal Care and Use Committee. Animals were fasted for 18 to 24 h before the study. At 2 to 2.5 h before radiotracer injection, the animal was anesthetized with ketamine (10 mg/kg) and glycopyrrolate 0.01 mg/kg intramuscular, transferred to the Biograph mCT camera; and immediately intubated with an endotracheal tube for continued anesthesia with 2.5% isoflurane administered through a rebreathing circuit. An intravenous line was placed and used for injection of the radiolabeled compound. Body temperature was kept at 37 °C using a heated water blanket. Vital signs, including heart rate, blood pressure, respiration rate, oxygen saturation and body temperature, were monitored at least every 2 to 15 min during the study.

PET imaging with [^{18}F]-**41** (3 min bolus, intravenous (IV) was conducted in 2 female baboons. Both animals completed 1 baseline scan. The animals were scanned again with [^{18}F]-**41** after intravenous (IV) administration of compound **27** to determine the relationship between dose levels of **27**, plasma levels, and $mGlu_5$ occupancy. Compound **27** was administered over 20 min,

beginning 30 min before [^{18}F]-**41** injection, with the exception of the initial study where **27** was administered over 2 min starting 50 min before the radiotracer.

Compound **27** solution was formulated in 10% ethanol, 30% polyethylene glycol 400 (PEG 400) and 20% 2-hydroxypropyl-β-cyclodextrin (HP-β-CD) in water for injection (WFI). An excess of **27** was first dissolved in PEG 400 overnight. A 28.6% HP-β-CD solution in WFI was prepared, to which the required amount of ethanol was added. Finally, the **27**-PEG400 solution was slowly added to the HP-β-CD and ethanol solution under stirring. The resulting solution was filtered through a 0.2 μm sterile filter into a 30 mL sterile empty vial. Due to only partial dissolution, final concentration was determined by high performance liquid chromatography (HPLC), using an XBridge C18 (5 μm, 4.6 x 250 mm) column eluted with MeOH/H_2O 75/25 at 1 mL/min. The vial was visually inspected for clarity, color and particulates.

Following [^{18}F]-**41** injection, arterial samples were drawn at 0.75, 1.5, 2.25, 3, 3.75, 4.5, 5.25, 6, 8, 10, 15, 20, 25, 30, 45, 60, 90,120, 150 and 180 min (1.0 mL, except 3.5 mL at 6, 15, 30, 60, 90, 120 and 180 min) post-injection. Samples were counted to determine whole blood and plasma radioactivity concentration over time. The larger samples were used to measure the [^{18}F]-**41** parent fraction in plasma over time. All blood data were used to generate a metabolite-corrected arterial input function needed for kinetic modeling. Arterial samples (1 mL) were collected at -5, 5, 10, 20, 30, 45, 75, 120 and 210 min, relative to **27** administration to measure the plasma levels of **27**. Plasma samples were subsequently shipped to the Vanderbilt Center for Neuroscience Drug Discovery for bioanalysis and determination of plasma concentrations using a previously established LC-MS/MS method for **27**.

An IV line was placed and used for injection of the radiotracer [^{18}F]-**41** and to administer **27**. Compound **27** was administered over 20 min starting at 30 min before tracer administration for

all but one scan. For the initial study with **27**, 0.5 mg/kg was to be administered over 5 min starting 30 min before [^{18}F]-**41** injection, but the administration was stopped after 2 min with an effective injected dose of 0.18 mg/kg because of an observed increase of heart rate, blood pressure and respiratory rate. The scan for this study started 50 min post-injection to ensure that vital levels remained stable before scanning. For all subsequent scans, **27** was administered over 20 min with no noticeable effect on vital signs. All brain imaging data were acquired on the mCT scanner post-injection of [^{18}F]-**41** over 180 min. PET data were reconstructed into a series of 45 frames as follows: 6 x 0.5 min, 3 x 1 min, 2 x 2 min and 34 x 5 min. The 0.18 mg/kg study was shortened to 150 min of acquisition because the animal started to wake, and the scan data was reconstructed into 39 frames with the same frame timing, less 6 frames. Before radiotracer injection, a computed tomography (CT) scan was acquired and used to correct for attenuation in the emission data. All scans were reconstructed with standard corrections for normalization, random, scatter and attenuation.

Reconstructed dynamic brain PET images were transferred and analyzed using the image processing PMOD software package (PMOD Technologies, Zurich, Switzerland). All PET images were normalized to an magnetic resonance (MR) baboon template. A volume of interest (VOI) template was applied including the following regions: frontal, temporal, parietal and occipital cortices, thalamus, caudate, putamen, globus pallidus, anterior and posterior cingulate, hippocampus, brain stem, midbrain, corpus callosum, vermis, cerebellar white matter and cerebellum. Average activity concentration (kBq/cc) within each VOI was determined and time activity curves (TACs) representing the regional brain activity concentration over time were generated. Brain TACs and images were expressed in standard uptake value units (SUV, g/mL) by normalizing by the weight of the animal and the injected dose.

The 2-tissue compartmental model, requiring the arterial plasma input function, was applied to the regional TACs to determine the total volume of distribution, V_T. The occupancy of [^{18}F]-**41** from **27** was determined using the Lassen plot. Briefly, $V_T^{Baseline} - V_T^{\mathbf{27}}$ (y-axis) was plotted against $V_T^{Baseline}$ (x-axis) for the regions examined and the slope of the regression was used to determine the occupancy at each dose. [^{18}F]-**41** occupancy was plotted against dose of **27** and plasma levels, fit with a one site-specific binding model. To plot occupancy versus plasma levels, the maximum **27** plasma concentration at a single time point was used.