Supporting Information

# Discovery, Synthesis, and SAR Development of a Series of *N*-(4-acetamido)-phenylpicolinamides as Positive Allosteric Modulators of Metabotropic Glutamate Receptor 4 ($mGlu_4$) with CNS Exposure in Rats

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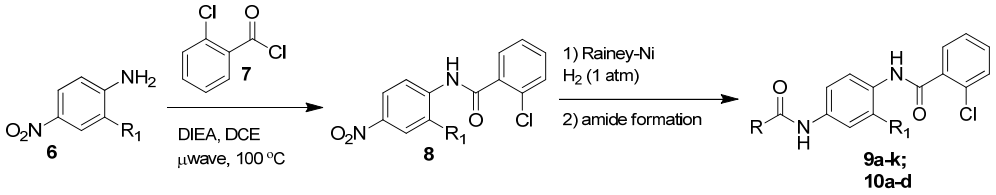
**General.** All NMR spectra were recorded on a 400 MHz AMX Bruker NMR spectrometer. $^1$H chemical shifts are reported in δ values in ppm downfield with the deuterated solvent as the internal standard. Data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, br = broad, m = multiplet), integration, coupling constant (Hz). Low resolution mass spectra were obtained on an Agilent 1200 series 6130 mass spectrometer with electrospray ionization. High resolution mass spectra (HRMS) were recorded on a Waters Q-TOF API-US plus Acquity system with electrospray ionization. Analytical thin layer chromatography was performed on EM Reagent 0.25 mm silica gel 60-F plates. Analytical HPLC was performed on an Agilent 1200 series with UV detection at 214 nm and 254 nm along with ELSD detection. LC/MS: Method 1 = (J-Sphere80-C18, 3.0 x 50 mm, 4.1 min gradient, 5%[0.05%TFA/$CH_3CN$]:95%[0.05%TFA/$H_2O$] to 100%[0.05%TFA/$CH_3CN$]; Method 2 = (Phenomenex-C18, 2.1 X 30 mm, 2 min gradient, 7%[0.1%TFA/$CH_3CN$]:93%[0.1%TFA/$H_2O$] to 100%[0.1%TFA/$CH_3CN$]; Method 3 = (Phenomenex-C18, 2.1 X 30 mm, 1 min gradient, 7%[0.1%TFA/$CH_3CN$]:93%[0.1%TFA/$H_2O$] to 95%[0.1%TFA/$CH_3CN$]. Preparative purification was performed on a custom HP1100 purification system with collection triggered by mass detection.[1] Solvents for extraction, washing and chromatography were HPLC grade. All reagents were purchased from Aldrich Chemical Co. and were used without purification. Chemical solvent abbreviations: DIEA: diisopropyl ethylamine; DCE: dichloroethane; DCM: dichloromethane; EDCI: 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide); HOBt: hydroxybenzotriazole; DMF: dimethylformamide.



**General Procedure for Library Amide Synthesis (9a-k and 10a-d):**

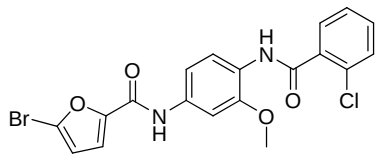
**2-Chloro-*N*-(4-nitrophenyl)benzamide 8:** Into a microwave vial was charged the appropriate 4-nitroaniline, **6**, (1.0 equivalent), DIEA (2.2 equivalents) and DCE (0.2M). After the addition of 2-chlorobenzoyl chloride, **7**, (1.1 equivalent), the rxn was subjected to microwave conditions (100 °C, 30 min). The rxn was added to DCM:$NaHCO_3$ (aq) (1:1) and the organic layer was separated, washed with brine (saturated aqueous NaCl), dried ($MgSO_4$) and filtered. Concentration afforded compound **8**, a yellow solid, which was carried through to the next step.

**Synthesis of compound 9a-k and 10a-d:**

To a solution of compound **8** in EtOH:EtOAc (1:1) was added Rainey-Ni (~0.1 equivalent). An $H_2$ atmosphere (1 atm) was applied to the rxn mixture. After 16 h, the rxn was filtered through Celite and concentrated. The crude residue was deemed >85% pure by LCMS and carried through without further purification (85 – 94%, 2 steps).

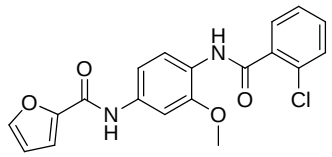
Amide formation (Method A): A solution of the appropriate carboxylic acid (1.0 equivalent) in dry 1,4-dioxane (1.0M) at room temperature was charged with HOBt (1.0 equivalent), DIEA (2.0 equivalents) and EDCI (1.5 equivalents). After 15 min, the appropriate aniline (1.0 equivalent) was added and the heterogeneous mixture was heated to 50 °C. After 12 h, the rxn was transferred to DCM:water (1:1). The organic layer was separated and sequentially washed with water (2 x), and passed through a Phase separator. After concentration, the desired analogs were purified by mass-directed preparative HPLC.

Amide formation (Method B): To a solution of the appropriate aniline in DMF:DIEA (4:1) (0.1 M) . The appropriate acid chloride (1.0 equivalent) was added and after 12 h at rt, the desired analogs were directly purified by mass-guided preparative HPLC.



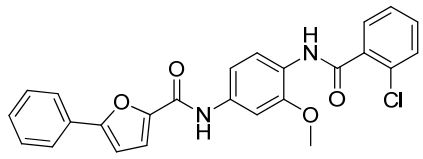
**5**

**5-bromo-*N*-(4-(2-chlorobenzamido)-3-methoxyphenyl)furan-2-carboxamide (5):** Following the general procedure above (Method A), compound (**5**) was obtained: Analytical LCMS (Method 3): single peak (220 nm); $R_T$ = 0.845 min; MS ($ESI^+$) *m/z* 451.0 $[M + H]^+$.



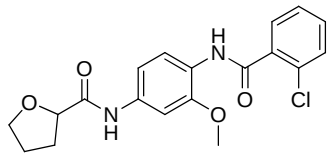
**9a**

***N*-(4-(2-chlorobenzamido)-3-methoxyphenyl)furan-2-carboxamide (9a):** Following the general procedure above (Method B), compound (**9a**) was obtained: Analytical LCMS (Method 3): single peak (220 nm); $R_T$ = 0.788 min; MS ($ESI^+$) *m/z* 371.0 $[M + H]^+$.



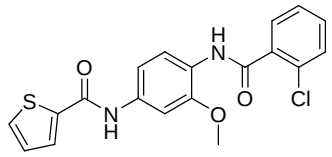
**9b**

***N*-(4-(2-chlorobenzamido)-3-methoxyphenyl)-5-phenylfuran-2-carboxamide (9b):** Following the general procedure above (Method A), compound (**9b**) was obtained: Analytical LCMS (Method 3): single peak (220 nm); $R_T$ = 0.906 min; MS ($ESI^+$) *m/z* 447.0 $[M + H]^+$.



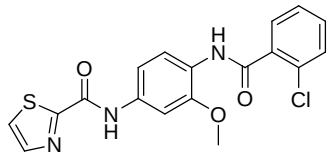
**9c**

***N*-(4-(2-chlorobenzamido)-3-methoxyphenyl)tetrahydrofuran-2-carboxamide (9c):** Following the general procedure above (Method A), compound (**9c**) was obtained: Analytical LCMS (Method 3): single peak (220 nm); $R_T$ = 0.784 min; MS ($ESI^+$) *m/z* 375.2 $[M + H]^+$.



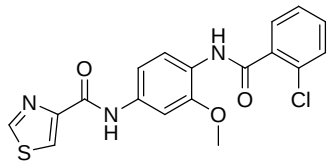
**9d**

***N*-(4-(2-chlorobenzamido)-3-methoxyphenyl)thiophene-2-carboxamide (9d):** Following the general procedure above (Method B), compound (**9d**) was obtained: Analytical LCMS (Method 3): single peak (220 nm); $R_T$ = 0.822 min; MS ($ESI^+$) *m/z* 387.0 $[M + H]^+$.



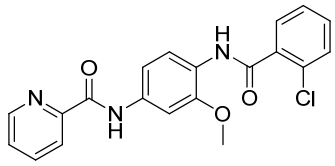
**9e**

***N*-(4-(2-chlorobenzamido)-3-methoxyphenyl)thiazole-2-carboxamide (9e):** Following the general procedure above (Method A), compound (**9e**) was obtained: Analytical LCMS (Method 3): single peak (220 nm); $R_T$ = 0.814 min; MS ($ESI^+$) *m/z* 388.2 $[M + H]^+$.



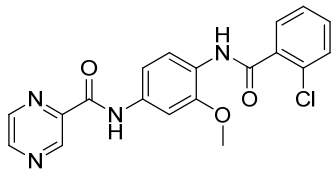
**9f**

***N*-(4-(2-chlorobenzamido)-3-methoxyphenyl)thiazole-4-carboxamide (9f):** Following the general procedure above (Method A), compound (**9f**) was obtained: Analytical LCMS (Method 3): single peak (220 nm); $R_T$ = 0.787 min; MS ($ESI^+$) *m/z* 388.2 $[M + H]^+$.



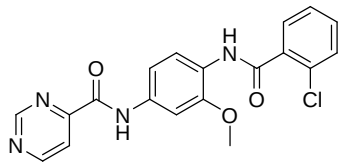
**9g**

***N*-(4-(2-chlorobenzamido)-3-methoxyphenyl)picolinamide (9g):** Following the general procedure above (Method B), compound (**9g**) was obtained: Analytical LCMS (Method 2): single peak (220 nm), $R_T$ = 1.167 min, *m/z* 382.0 $[M + H]^+$; $^1H$ NMR (400 MHz, DMSO-$d_6$): δ 10.64 (s, 1H), 9.58 (s, 1H), 8.74 (d, J = 4.4 Hz, 1H), 8.16 (d, J = 8.0 Hz, 1H), 8.07 (ddd, J = 8.0, 8.0, 1.6 Hz, 1H), 7.83 (d, J = 8.8 Hz, 1H), 7.75 (d, J = 2.0 Hz, 1H), 7.68 (dd, J = 6.4, 4.8 Hz, 1H), 7.58-7.46 (m, 5H), 3.82 (s, 3H); HRMS, calc'd for $C_{20}H_{17}N_3O_3Cl$ ($M+H^+$), 382.0958; found 382.0958.



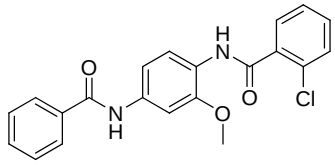
**9h**

***N*-(4-(2-chlorobenzamido)-3-methoxyphenyl)pyrazine-2-carboxamide (9h):** Following the general procedure above (Method A), compound (**9h**) was obtained: Analytical LCMS (Method 3): single peak (220 nm); $R_T$ = 0.781 min; MS ($ESI^+$) *m/z* 383.2 $[M + H]^+$.



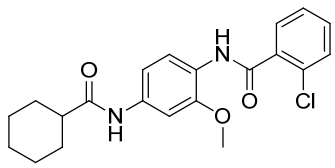
**9i**

***N*-(4-(2-chlorobenzamido)-3-methoxyphenyl)pyrimidine-4-carboxamide (9i):** Following the general procedure above (Method A), compound (**9i**) was obtained: Analytical LCMS (Method 3): single peak (220 nm); $R_T$ = 0.773 min; MS ($ESI^+$) *m/z* 383.2 $[M + H]^+$.



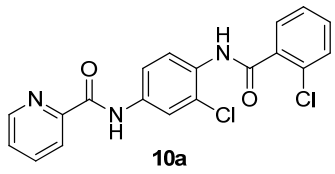
**9j**

***N*-(4-benzamido-2-methoxyphenyl)-2-chlorobenzamide (9j):** Following the general procedure above (Method B), compound (**9j**) was obtained: Analytical LCMS (Method 3): single peak (220 nm); $R_T$ = 0.829 min; MS ($ESI^+$) *m/z* 381.2 $[M + H]^+$.



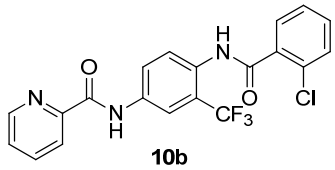
**9k**

**2-chloro-*N*-(4-(cyclohexanecarboxamido)-2-methoxyphenyl)benzamide (9k):** Following the general procedure above (Method B), compound (**9k**) was obtained: Analytical LCMS (Method 3): single peak (220 nm); $R_T$ = 0.855 min; MS ($ESI^+$) *m/z* 387.2 $[M + H]^+$.



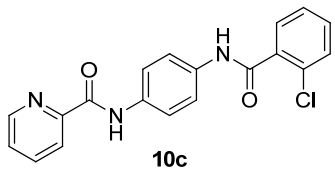
**10a**

***N*-(3-chloro-4-(2-chlorobenzamido)phenyl)picolinamide (10a):** Following the general procedure above (Method B), compound (**10a**) was obtained as a tan solid (81%): Analytical LCMS (Method 2): single peak (214 nm); $R_T$ = 1.496 min; $^1H$ NMR (400 MHz, DMSO-$d_6$): δ 10.91 (s, 1H), 10.18 (s, 1H), 8.77 (d, *J* = 4.8 Hz, 1H), 8.23 (d, *J* = 2.0 Hz, 1H), 8.19 (d, *J* = 7.6 Hz, 1H), 8.10 (ddd, *J* = 8.0, 1.6, 1.6 Hz, 1H), 7.93 (dd, *J* = 8.8, 2.0 Hz, 1H), 7.73-7.70 (m, 1H), 7.63-7.46 (m, 5H); HRMS, calc'd for $C_{19}H_{14}N_2O_2Cl_2$ $[M + H]^+$, 386.0463; found 386.0467.



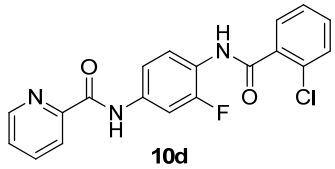
**10b**

***N*-(4-(2-chlorobenzamido)-3-(trifluoromethyl)phenyl)picolinamide (10b):** Following the general procedure above (Method B), compound (**10b**) was obtained: Analytical LCMS (Method 2): single peak (220 nm); $R_T$ = 1.453 min; MS ($ESI^+$) *m/z* 420.0 $[M + H]^+$.



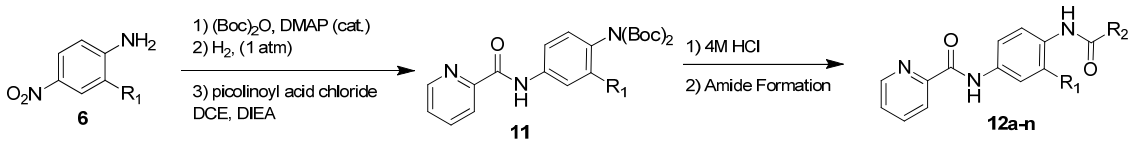
**10c**

***N*-(4-(2-chlorobenzamido)phenyl)picolinamide (10c):** Following the general procedure above (Method B), compound (**10c**) was obtained: Analytical LCMS: single peak (220 nm); $R_T$ = 1.404 min; MS ($ESI^+$) *m/z* 352.2 $[M + H]^+$.




**10d**

***N*-(4-(2-chlorobenzamido)-3-fluorophenyl)picolinamide (10d):** Following the general procedure above (Method B), compound (**10d**) was obtained: Analytical LCMS (Method 1): single peak (220 nm); $R_T$ = 3.015 min; MS ($ESI^+$) *m/z* 370.1 $[M + H]^+$.




**General Procedure for Library Amide Synthesis (12a-n):**

**Synthesis of picolinamide 11:** To a solution of **6**, and DMAP (0.1 equivalent) in THF (0.2M) at rt was added $(Boc)_2O$ (2.2 equivalents). After 16 h, the rxn was added to EtOAc:water (1:1). The organic layer was separated and washed with brine, dried ($MgSO_4$), filtered and concentrated. To a solution of the crude residue in EtOAc (0.5M) was added Rainey-Ni (~0.1 equivalent) and an $H_2$ atmosphere (1 atm) was applied. After 16 h, LCMS confirmed the loss of starting material, the reaction mixture (rxn) was filtered through a Celite filter, concentrated, and the product was carried without purification (76 – 98%, 2 steps).

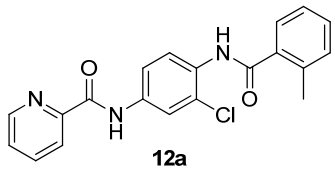
To a solution of the crude residue in DCE (0.5 M) was added DIEA (2.5 equivalents), followed by picolinoyl acid chloride hydrochloride (1.1 equivalents). After 16 h, the rxn was added to DCM:$NaHCO_3$ (aq) (1:1) and the organic layer was separated. The organic layer was washed with brine, dried ($MgSO_4$), filtered and concentrated to afford amide **11** which was carried on without purification (90 – 98% yield).

**Synthesis of final compounds, 12a-n:** To a solution of compound **11**, (1.0 equivalent) in DCM (0.2 M) at 0 °C was added 4M HCl in 1,4-dioxane (10 equivalents). After 15 min, the ice bath was removed and the rxn was monitored by LCMS. The solvent was removed and the residue was redissolved in DCM:$NaHCO_3$ (aq) (1:1). The organic layer was separated, washed with brine, dried ($MgSO_4$), filtered and concentrated to afford the desired aniline (90 – 95% yield).

Amide formation (Method A): A solution of the appropriate carboxylic acid (1.0 equivalent) in dry 1,4-dioxane (1.0M) at room temperature was charged with HOBt (1.0 equivalent), DIEA (2.0 equivalents) and EDCI (1.5 equivalents). After 15 min, the appropriate aniline (1.0 equivalent) was added and the heterogeneous mixture was heated to 50 °C. After 12 h, the rxn was transferred to DCM:water (1:1). The organic layer was separated and sequentially washed with water (2 x), and passed through a Phase separator. After concentration, the desired analogs were purified by mass-directed preparative HPLC.

Amide formation (Method B): To a solution of the appropriate aniline in DMF:DIEA (4:1) (0.1 M). The appropriate acid chloride (1.0 equivalent) was added and after 12 h at rt, the desired analogs were directly purified by mass-guided preparative HPLC.

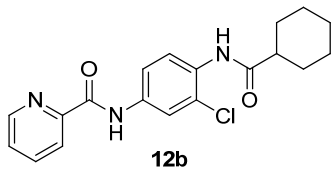
**HCl Salt Formation for in vivo studies:** To a solution of the amide in DCM (0.2M) at 0 °C was added 4M HCl in 1,4-dioxane (5 eq.) dropwise. After 15 min, the ice bath was removed. After an additional 30 min, the solvent was removed to provide a pure HCl salt of the appropriate amide.




**12a**

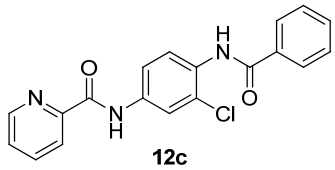
***N*-(3-chloro-4-(2-methylbenzamido)phenyl)picolinamide (12a):** Following the general procedure above (Method B), compound (**12a**) was obtained as a tan solid (81%): Analytical LCMS (Method 2): single peak (214 nm); $R_T$ = 1.482 min; $^1$H NMR (400 MHz, DMSO-$d_6$): δ 10.90 (s, 1H), 9.94 (s, 1H), 8.77 (d, *J* = 4.0 Hz, 1H), 8.22 (d, *J* = 2.0 Hz, 1H), 8.19 (d, *J* = 8.0 Hz, 1H), 8.10 (ddd, *J* = 8.0, 2.0, 2.0 Hz, 1H), 7.91 (d, *J* = 8.4 Hz, 1H), 7.71 (ddd, *J* = 7.6, 4.8, 1.6 Hz, 1H), 7.58 (d, *J* = 8.8 Hz, 5H), 7.54 (d,

$J$ = 7.6 Hz, 1H), 7.40 (dd, $J$ = 7.2, 7.2 Hz, 1H), 7.33-7.30 (m, 2H), 2.46 (s, 3H); HRMS, calc'd for $C_{20}H_{17}N_3O_2Cl$ $[M + H]^+$, 366.1009; found 366.1009.



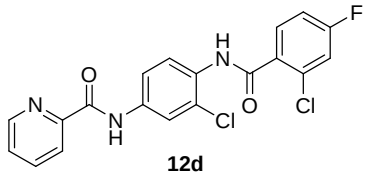
**12b**

***N*-(3-chloro-4-(cyclohexanecarboxamido)phenyl)picolinamide (12b):** Following the general procedure above (Method B), compound (**12b**) was obtained: Analytical LCMS (Method 1): single peak (220 nm); $R_T$ = 3.321 min; MS ($ESI^+$) *m/z* 358.2 $[M + H]^+$.



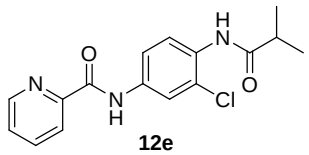
**12c**

***N*-(4-benzamido-3-chlorophenyl)picolinamide (12c):** Following the general procedure above (Method B), compound (**12c**) was obtained: Analytical LCMS (Method 1): single peak (220 nm); $R_T$ = 3.126 min; MS ($ESI^+$) *m/z* 352.1 $[M + H]^+$.



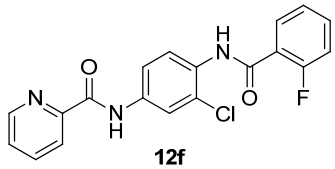
**12d**

***N*-(3-chloro-4-(2-chloro-4-fluorobenzamido)phenyl)picolinamide (12d):** Following the general procedure above (Method B), compound (**12d**) was obtained: Analytical LCMS (Method 1): single peak (220 nm); $R_T$ = 3.267 min; MS ($ESI^+$) *m/z* 404.1 $[M + H]^+$.



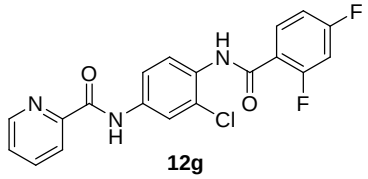
**12e**

***N*-(3-chloro-4-isobutyramidophenyl)picolinamide (12e):** Following the general procedure above (Method B), compound (**12e**) was obtained: Analytical LCMS (Method 1): single peak (220 nm); $R_T$ = 2.871 min; MS ($ESI^+$) *m/z* 318.1 $[M + H]^+$.



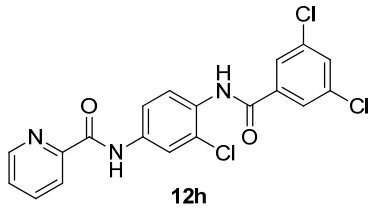
**12f**

***N*-(3-chloro-4-(2-fluorobenzamido)phenyl)picolinamide (12f):** Following the general procedure above (Method B), compound (**12f**) was obtained: Analytical LCMS (Method 1): single peak (220 nm); $R_T$ = 3.415 min; MS ($ESI^+$) *m/z* 370.1 $[M + H]^+$.



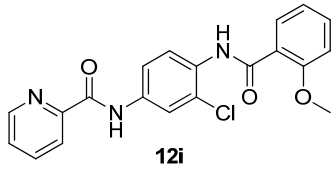
**12g**

***N*-(3-chloro-4-(2,4-difluorobenzamido)phenyl)picolinamide (12g):** Following the general procedure above (Method B), compound (**12g**) was obtained: Analytical LCMS (Method 1): single peak (220 nm); $R_T$ = 3.453 min; MS ($ESI^+$) *m/z* 388.1 $[M + H]^+$.



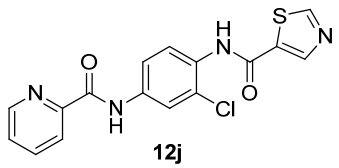

**12h**

***N*-(3-chloro-4-(3,5-dichlorobenzamido)phenyl)picolinamide (12h):** Following the general procedure above (Method B), compound (**12h**) was obtained: Analytical LCMS (Method 3): single peak (220 nm)$R_T$ = 0.918 min; MS ($ESI^+$) *m/z* 422.0 $[M + H]^+$.



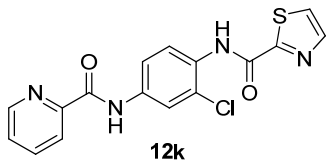

**12i**

***N*-(3-chloro-4-(2-methoxybenzamido)phenyl)picolinamide (12i):** Following the general procedure above (Method B), compound (**12i**) was obtained: Analytical LCMS (Method 1): single peak (220 nm); $R_T$ = 3.511 min; MS ($ESI^+$) *m/z* 382.1 $[M + H]^+$.



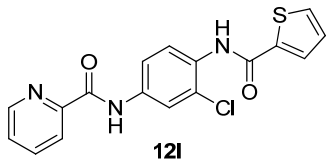

**12j**

***N*-(2-chloro-4-(picolinamido)phenyl)thiazole-5-carboxamide (12j):** Following the general procedure above (Method A), compound (**12j**) was obtained: Analytical LCMS (Method 1): single peak (220 nm); $R_T$ = 2.722 min; MS ($ESI^+$) *m/z* 359.0 $[M + H]^+$.



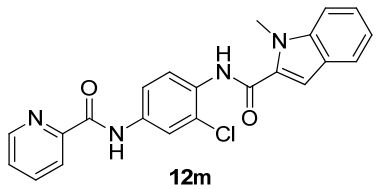
**12k**

***N*-(2-chloro-4-(picolinamido)phenyl)thiazole-2-carboxamide (12k):** Following the general procedure above (Method A), compound (**12k**) was obtained: Analytical LCMS (Method 1): single peak (220 nm); $R_T$ = 2.885 min; MS ($ESI^+$) *m/z* 359.0 $[M + H]^+$.



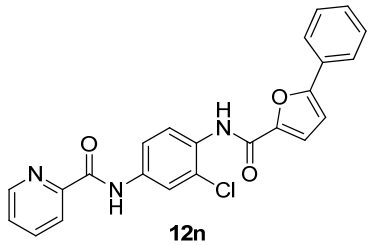
**12l**

***N*-(3-chloro-4-(thiophene-2-carboxamido)phenyl)picolinamide (12l):** Following the general procedure above (Method B), compound (**12l**) was obtained: Analytical LCMS (Method 1): single peak (220 nm); $R_T$ = 2.579 min; MS ($ESI^+$) *m/z* 358.0 $[M + H]^+$.



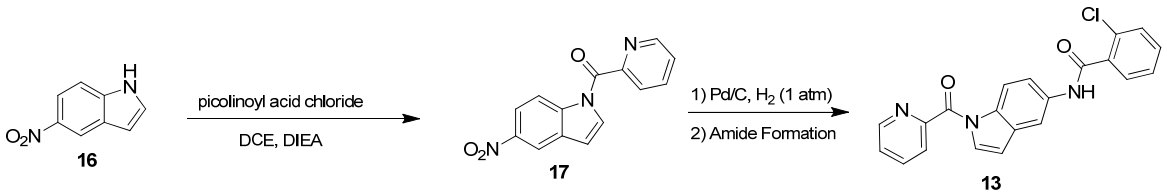
**12m**

***N*-(2-chloro-4-(picolinamido)phenyl)-1-methyl-1H-indole-2-carboxamide (12m):** Following the general procedure above (Method A), compound (**12m**) was obtained: Analytical LCMS (Method 1): single peak (220 nm); $R_T$ = 3.127 min; MS ($ESI^+$) *m/z* = 405.0 $[M + H]^+$.

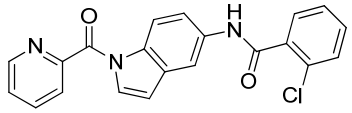



**12n**

***N*-(3-chloro-4-(5-phenylfuran-2-carboxamido)phenyl)picolinamide (12n):** Following the general procedure above (Method A), compound (**12n**) was obtained: Analytical LCMS (Method 1): single peak (220 nm); $R_T$ = 4.080 min; MS ($ESI^+$) *m/z* 418.0 $[M + H]^+$.




16 17 13



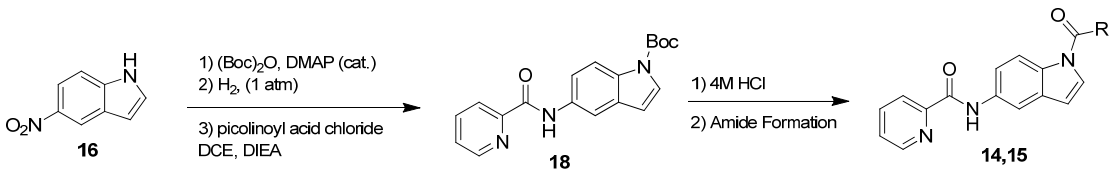
13

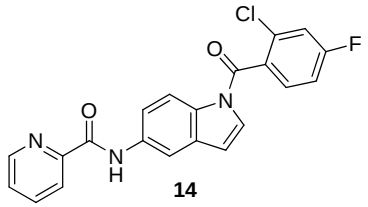
**2-chloro-*N*-(1-picolinoyl-1*H*-indol-5-yl)benzamide (13):** To a solution of 5-nitroindole, **16**, (1.0 equivalent), DMAP (0.1 equivalent) and $Et_3N$ (2.0 equivalents) in DCM at 0°C was added picolinoyl chloride hydrochloride (1.13 equivalent). After 15 min, the ice bath was removed and the rxn was stirred for 18 h at rt. Afterwards, the rxn was added to DCM:water (1:1) and the organic layer was separated, dried ($MgSO_4$), filtered and concentrated. The crude residue, **17**, was taken through without further purification.

To a solution of the crude residue in EtOAc:MeOH (2:1; 0.03M) was added 10% Pd/C (~0.1 equivalent) and an $H_2$ atmosphere (balloon, 1atm) was applied to the system. After 16 h, the rxn was filtered through a pad of Celite and the solvent was removed under vacuo.

To a solution of the above residue in DMF:DIEA (4:1) (0.1 M) was added 2-chlorobenzoyl chloride (1.0 equivalent) and after 12 h at rt, the desired compound, **13**, was directly purified by mass-guided preparative HPLC.

LCMS (Method 2): $R_T$ = 1.44 min., single peak (220 nm), *m/z* 375.8 $[M + H]^+$.



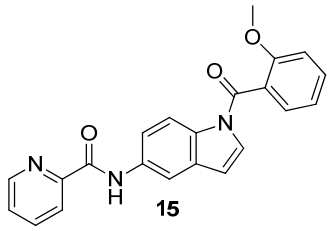




***N*-(1-(2-chloro-4-fluorobenzoyl)-1*H*-indol-5-yl)picolinamide (14):** To a solution 5-nitroindole, **16**, (1.0 equivalent) and DMAP (0.1 equivalent) in DCM (0.12M) was added $(Boc)_2O$ (1.2 equivalent). After 16 h, the rxn was added to DCM:$NaHCO_3$ (aq) (1:1) and the organic layer was separated. The organic layer was washed with brine, dried ($MgSO_4$), filtered and concentrated. The residue was redissolved in EtOAc:MeOH (2:1, 0.1M) and 10% Pd/C (~0.1 equivalent) was added. An $H_2$ atmosphere (1 atm) was applied via balloon. After 12 h, the rxn was filtered through a pad of Celite and the solvent was removed under vacuo.

To a solution of the above residue (1.0 equivalent), DMAP (0.2 equivalent) and $Et_3N$ (2.3 equivalents) in DMF at 0°C was added picolinoyl chloride hydrochloride (1.13 equivalent). After 15 min, the ice bath was removed and the rxn was stirred for 18 h at rt. Afterwards, the rxn was added to EtOAc:water (1:1) and the organic layer was separated, dried ($MgSO_4$), filtered and concentrated to afford compound **18**. The crude residue was taken through without further purification.

Compound **18** was dissolved in DCM (0.2M) and 4M HCl in 1,4-dioxane (3.0 equivalents) was added. After 3h, the solvent was removed. To a solution of the crude residue (1.0 equivalent), DMAP (0.2 equivalent) and $Et_3N$ (2.0 equivalent) in DCM (0.5M) at 0°C was added 2-chloro-4-fluorobenzoyl chloride (1.0 equivalent). After 15 min, the ice bath was removed. After 16 h, the solvent was removed and compound (**14**) was purified by mass-guided preparative HPLC.

LCMS (Method 2): $R_T$ = 1.59 min., single peak (220 nm), *m/z* 394.0 $[M + H]^+$.




**15**

***N*-(1-(2-methoxybenzoyl)-1*H*-indol-5-yl)picolinamide (15):** Following a similar procedure as compound **14**, compound (**15**) was obtained.

LCMS (Method 2): $R_T$ = 1.54 min., single peak (220 nm), *m/z* 372.1 $[M + H]^+$.

**In Vitro Pharmacology:**

**Cell culture.** Human $mGlu_4$ ($hmGlu_4$)/CHO cells stably transfected expressing the chimeric G protein $G_{qi5}$ 2 in pIRESneo3 (Invitrogen, Carlsbad, CA) were cultured in 90% Dulbecco's Modified Eagle Media (DMEM), 10% dialyzed fetal bovine serum (FBS), 100 units/ml penicillin/streptomycin, 20 mM HEPES (pH 7.3), 1 mM sodium pyruvate, 2 mM glutamine, 400 μg/ml G418 sufate (Mediatech, Inc., Herndon, VA) and 5 nM methotrexate (Calbiochem, EMD Chemicals, Gibbstown, NJ). Human Embryonic Kidney (HEK-293) cell lines co-expressing rat mGluR 2, 3, 4, 7 or 8 and GIRK potassium channels3 were grown in Growth Media containing 45% DMEM, 45% F-12, 10% FBS, 20 mM HEPES, 2 mM Lglutamine, antibiotic/antimycotic non-essential amino acids, 700 μg/ml G418, and 0.6 μg/ml puromycin at 37ºC in the presence of 5% $CO_2$. Rat $mGlu_{1,5}$ cells were cultured as described in Hempstapat et al., 2007. All cell culture reagents were purchased from Invitrogen Corp. (Carlsbad, CA) unless otherwise noted.

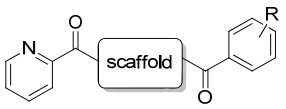
**Calcium mobilization assays.** Assays were performed within Vanderbilt University's High-Throughput Screening Center and the primary $mGlu_4$ HTS has been described in detail. Human $mGlu_4/G_{qi5}$/CHO cells (30,000 cells/20 •l/well) were plated in blackwalled, clear-bottomed, TC treated, 384 well plates (Greiner Bio-One, Monroe, North Carolina) in DMEM containing 10% dialyzed FBS, 20 mM HEPES, 100 units/ml penicillin/streptomycin, and 1 mM sodium pyruvate (Plating Medium). The cells were grown overnight at 37 °C in the presence of 5% CO2. During the day of assay, the medium was replaced with 20 μL of 1 μM Fluo-4, AM (Invitrogen, Carlsbad, CA) 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 (Sigma-Aldrich, St. Louis, MO)) for 45 minutes at 37 °C. Dye was removed and replaced with 20 μL of Assay Buffer. Test compounds were transferred to daughter plates using an Echo acoustic plate reformatter (Labcyte, Sunnyvale, CA) and then diluted into Assay Buffer. $Ca^{2+}$ flux was measured using the Functional Drug

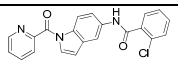
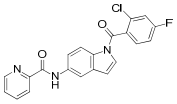
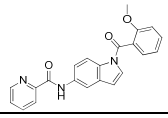
Screening System 6000 (FDSS6000, Hamamatsu, Japan). Baseline readings were taken (10 images at 1 Hz, excitation, 470±20 nm, emission, 540±30 nm) and then 20 •l/well test compounds were 14 added using the FDSS's integrated pipettor. Cells were incubated with compounds for approximately 2.5 minutes and then an $EC_{20}$ concentration of glutamate was applied; 2 min. later an $EC_{80}$ concentration of glutamate was added. For concentration-response curve experiments, compounds were serially diluted 1:3 into 10 point concentration response curves and were transferred to daughter plates using the Echo. Test compounds were again applied and followed by $EC_{20}$ concentrations of glutamate. For fold shift experiments, compounds were added at 2X their final concentration and then increasing concentrations of glutamate were added in the presence of vehicle or the appropriate concentration of test compound. Curves were fitted using a four point logistical equation using Microsoft XLfit (IDBS, Bridgewater, NJ). Subsequent confirmations of concentration response parameters were performed using independent serial dilutions of source compounds and data from multiple days experiments were integrated and fit using a four point logistical equation in GraphPad Prism (GraphPad Software, Inc., La Jolla, CA). Calcium assays were used to assess activity of compounds at mGlu's 1, 4 and 5.

**Thallium flux assays.** Compound activity at the rat group II and group III mGluRs was assessed using thallium flux through GIRK channels, a method that has been described in detail.[2] Briefly, cells were plated into 384 well, black-walled, clear-bottom poly-D-lysine coated plates at a density of 15,000 cells/20 •l/well in Plating Medium and incubated overnight at 37°C 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 h at room temperature and the dye was replaced with 20 µl/well of Assay Buffer. For these assays, compounds were added at 2x final concentration and then 2.5 min later the appropriate concentration of agonist was added using the FDSS 6000. 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. Five frames of data were collected (excitation,

470±20 nm emission, 540±30 nm) at ½ Hz prior to compound addition. Data collection continued at ½ Hz until 10 seconds prior to agonist addition, when the rate was increased to 1 Hz for 2 min after agonist addition. Data were analyzed as described. For the concentration-response curve experiments, compounds were serially diluted 1:3 into 10 point concentration response curves and were transferred to 15 daughter plates using the Echo. Test compounds were applied and followed by $EC_{20}$ concentrations of glutamate. For fold shift experiments, compounds were added at 2X their final concentration and then increasing concentrations of glutamate were added in the presence of vehicle or the appropriate concentration of test compound.

**Table 1.** Human and Rat mGlu4 potency and %Glu Max response (as normalized to standard PHCCC) for selected core scaffold analogs (**13-15**).




| Compd | | $hEC_{50}$ (nM)[a] | GluMax (%Cmpd **1**)[b] | $rEC_{50}$ (nM)[a] | GluMax (%Cmpd **1**)[b] |
|---|---|---|---|---|---|
| **13** |  | inactive[c] | 21.1 ± 1.1 | inactive[c] | 17.6 ± 3.6 |
| **14** |  | 1420 ± 70 | 71.7 ± 1.4 | 1660 ± 270 | 104.3 ± 4.1 |
| **15** |  | 1410 ± 80 | 91.3 ± 1.4 | 1340 ± 200 | 107.1 ± 3.2 |

[a] $EC_{50}$ and GluMax, are the average of at least three independent determinations performed in triplicate (Mean ± SEM shown in table). [b] Cmpd **1** is run as a control compound each day, and the maximal response generated in $mGlu_4$ CHO cells in the presence of $mGlu_4$ PAMs varies slightly in each experiment. Therefore, data were further normalized to the relative **1** response obtained in each day's run. [c] Inactive compounds are defined as %GluMax did not surpass 2X the $EC_{20}$ value for that day's run at the highest concentration (30 μM).

**Table 2.** mGlu Selectivity of **10a**.

| | Selectivity (hmGlu, FS @ 10 μM)[a] | | | | | | |
|---|---|---|---|---|---|---|---|
| Compd | 1 | 2 | 3 | 5 | 6 | 7 | 8 |
| **10a** | inactive[b] | inactive[b] | inactive[b] | inactive[b] | inactive[b] | 5.3 FS | 3.8 FS |

[a] Selectivity was assessed by incubating cells with either DMSO-matched vehicle or a 10 μM final concentration of compound in the presence of a full glutamate concentration-response. This allows for the determination of potentiator (shift in the glutamate concentration-response curve to the left) or antagonist (shift of the curve to the right with a possible decrease in the maximal response) activity within a single experiment. FS = fold shift of the glutamate concentration-response curve. Cmpd **10a** showed weak potentiator activity on $mGlu_7$ and $mGlu_8$ (leftward shift of the glutamate concentration-response). [b]Inactive compounds showed no ability to left-shift the glutamate response curve at 10 μM. No antagonist activity was noted for **10a** at any receptor tested.

**Table 3.** Fold shift, intrinsic clearance and plasma protein binding (PPB, rat) and CYP450 enzyme inhibition data for selected compounds.

| Compd | $rEC_{50}$ (nM)[a] | GluMax (%Cmpd 1)[b] | rFS | Rat Clint ($Cl_{HEP}$), (mL/min/kg) | PPB (rat, %fu) | P450 (μM) 1A2 | 2C9 | 3A4 | 2D6 |
|---|---|---|---|---|---|---|---|---|---|
| **9e** | 377 ± 101 | 133.9 ± 6.6 | 32.2 ± 2.4 | 185.3 (50.8) | 0.56 | ND | ND | ND | ND |
| **9g** | 106 ± 28 | 147.0 ± 4.3 | 72.0 ± 4.5 | 42.6 (26.5) | 0.33 | >30 | 14.3 | >30 | 12.8 |
| **10a** | 570 ± 131 | 128.2 ± 5.6 | 18.3 ± 1.3 | 44.9 (27.3) | 0.29 | >30 | 11.8 | >30 | 13.1 |
| **10d** | 739 ± 92 | 91.4 ± 2.9 | 4.6 ± 0.1 | 31.2 (21.6) | 0.58 | ND | ND | ND | ND |
| **12a** | 915 ± 161 | 123.1 ± 2.9 | 9.6 ± 0.6 | 46.2 (27.8) | 0.35 | >10 | 14.1 | >30 | 12.0 |
| **12d** | 169 ± 28 | 118.8 ± 7.3 | 6.7 ± 2.0 | 54.0 (30.5) | ND | ND | ND | ND | ND |
| **12k** | 533 ± 145 | 105.5 ± 2.4 | 6.0 ± 0.6 | ND | 0.65 | ND | ND | ND | ND |
| **15** | 1340 ± 200 | 107.1 ± 3.2 | 5.9 ± 1.1 | 605.2 (62.7) | 0.16 | ND | ND | ND | ND |

[a] $EC_{50}$ and GluMax, are the average of at least three independent determinations performed in triplicate (Mean ± SEM shown in table). [b] Cmpd **1** is run as a control compound each day, and the maximal response generated in $mGlu_4$ CHO cells in the presence of $mGlu_4$ PAMs varies slightly in each experiment. Therefore, data were further normalized to the relative **1** response obtained in each day's run. ND = not determined.

**Table 4.** Rat Pharmacokinetic Data for Compounds VU0415374 (**9g**), VU0366037 (**10a**), and VU0366038 (**12a**) following SC dosing (10 mg/kg; 10% tween 80 microsuspension). AUC=area under the curve as assessed from 0-6 hours.

| parameters | VU0415374, **9g** | VU0366037, **10a** | VU0366038, **12a** |
|---|---|---|---|
| Plasma AUC (ng·hr/mL) | 938 | 657.8 | 766.1 |
| Plasma $C_{max}$ (ng/mL) | 265 | 198.1 | 219.2 |
| Brain AUC (ng·hr/g) | 312 | 681.0 | 155.4 |
| Brain $C_{max}$ (ng/g) | 69.1 | 163.2 | 59.4 |
| $T_{max}$ (h) | 1 | 0.5 | 0.5 |
| $AUC_{0\text{-}6h,\ brain}/AUC_{0\text{-}6h,plasma}$ | 0.33 | 1.04 | 0.2 |

**In vitro PK Analysis:**

**Microsomal stability:** The metabolic stability of each compound was investigated in rat hepatic microsomes (BD Biosciences, Billerica, MA) using substrate depletion methodology (% parent compound remaining). In separate 96-well plates for each time point, a mixture of 0.1M potassium phosphate-buffered (pH 7.4), 1μM test compound, 0.5 mg/mL microsomes, and 1mM NADPH (t=3, 7, 15, 25, or 45min) or buffer (t=0) were continually incubated at 37°C under ambient oxygenation. At the respective time, each plate's reaction was precipitated by the addition of 2 volumes of ice-cold acetonitrile containing glyburide as an internal standard (50 ng/mL). The plates were centrifuged at 3000 rpm (4°C) for 10 min. The resulting supernatants were transferred and diluted 1:1 (supernatant: water) into new 96-well plates in preparation for LC/MS/MS analysis. Each compound was assayed in triplicate within the same 96-well plate. The in vitro half-life ($t_{1/2}$, min, Eq. 1), intrinsic clearance (*CLint*, mL/min/kg, Eq. 2) and subsequent predicted hepatic clearance (*CLhep*, mL/min/kg, Eq. 3) was determined employing the following equations:

*1)* $t_{1/2}$ = Ln(2) / $k$ ; where $k$ represents the slope from linear regression analysis (% test compound remaining)

*2)* *CLint* = (0.693 / $t_{1/2}$) (rxn volume / mg of microsomes) (45 mg microsomes / gram of liver) ($20^a$ gm of liver / kg body weight); $^a$scale-up factors of 20 (human) and 45 (rat)

*3)* $$CLhep = \frac{Q \cdot CL\,\mathrm{int}}{Q + CL\,\mathrm{int}}$$

**Plasma Protein Binding**. The protein binding of each compound was determined in rat plasma via equilibrium dialysis employing Single-Use RED Plates with inserts (ThermoFisher Scientific, Rochester, NY). Plasma (220 μL) was added to the 96 well plate containing test compound (5 μL) and mixed thoroughly. Subsequently, 200 μL of the plasma-compound mixture was transferred to the *cis*

chamber (red) of the RED plate, with an accompanying 350 µL of phosphate buffer (25 mM, pH 7.4) in the *trans* chamber. The RED plate was sealed and incubated for 4 hours at 37°C with shaking. At completion, 50 µL aliquots from each chamber were diluted 1:1 (50 µL) with either plasma (*cis*) or buffer (*trans*) and transferred to a new 96 well plate, at which time ice-cold acetonitrile (2 volumes) was added to extract the matrices. The plate was centrifuged (3000 rpm, 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.

Microsomal stability and plasma protein binding samples were analyzed on a Thermo Electron TSQ Quantum Ultra triple quad detector via electrospray ionization (ESI) with two Themo Electron Accella pumps (San Jose, CA), and a Leap Technologies CTC PAL autosampler. Analytes were separated by gradient elution on a dual column system with two Waters Acquity BEH C18, 2.1x50mm, 1.7µm columns (Milford, MA) heated at 50°C. HPLC mobile phase A was 95:5:0.1 Water:Acetonitrile:Formic Acid, while mobile phase B was 95:5:0.1 Acetonitrile:Water:Formic Acid. Pump 1 runs the gradient: 95:5 (A:B) at 800µL/min hold 0 to 0.5min, linear ramp to 5:95 (A:B) 0.5 to 1.0min, 5:95 (A:B) hold 1.0 to 1.9min, return to 95:5 (A:B) at 1.9 min. While pump 1 runs the gradient method, pump 2 equilibrates the other column isocratically with 95:5 (A:B). The total run time is 2.0 minutes per injection. All compounds are optimized using Thermo Electron's QuickQuan software.

**Cytochrome P450 inhibition:** A four-in-one, 96-well plate assay for determining $IC_{50}$ values against human P450s 1A2, 2C9, 2D6 and 3A4 was developed based on previous reports (1,2). Human liver microsomes (final concentration of 0.1 mg/mL) and a substrate mixture containing the P450 probe substrates phenacetin (10µM), diclofenac (5µM), dextromethorphan (5µM) and midazolam (2µM) were added to a potassium phosphate buffered solution (0.1M, pH 7.4) and warmed to 37$^{o}$C. The reaction mixture was divided evenly into the 96-well plate and various dilutions of each inhibitor/compound of

interest (in duplicate) were then added to this reaction mixture such that the final concentration of each compound ranged from 100nM to 30μM. This mixture was allowed to pre-incubate for 15 minutes while shaking at 37°C. Buffer or NADPH (1mM) was added and the reaction mixture was incubated for an additional 8 minutes at 37°C prior to quenching with 2 volumes of ice-cold acetonitrile containing 50ng/mL of carbamazepine as internal standard. The plates were centrifuged at 4000 rpm (4°C) for 10 minutes and the supernatant was removed and diluted with water (1:4, v/v) in preparation for LC/MS/MS analysis. The $IC_{50}$ values for each compound were obtained for the individual P450 enzymes by quantitating the inhibition of metabolite formation for each probe; acetaminophen (1A2), 4-hydroxydiclofenac (2C9), dextrorphan tartrate (2D6) and 1-hydroxymidazolam (3A4). Miconazole was included as a positive control broad spectrum P450 inhibiton (REF). For discrete 2C19 inhibition experiments, a similar assay design was employed with the following exceptions: the probe substrate was S-mephenytoin (40μM), the NADPH incubation with the reaction mixture went for 30 minutes, the supernatant was reconstituted 1:1 with water for analysis, and the metabolite used for quantitation was 4-hydroxymephenytoin.

Cytochrome P450 inhibition 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 2.1 x 50 mm, 3 μm column (Fortis Technologies Ltd, Cheshire, UK) thermostated at 40°C. 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 1.3 min; 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°C 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). All data were analyzed using AB Sciex Analyst 1.4.2 software.

**In vivo PK Analysis:**

**In vivo pharmacokinetics:** Male Sprague-Dawley rats (n=2) weighing around 250-300g were purchased from Harlan Laboratories (Indianapolis, IN) and implanted with catheters in the carotid artery and jugular vein. The cannulated animals were acclimated to their surroundings for approximately one week before dosing and provided food and water *ad libitum*. Parenteral administration of compounds to rats was achieved via a jugular vein catheter at a dose of 1 mg/kg (20% DMSO/80% saline) and a dose volume of 1 mL/kg. Blood collections via the carotid artery were performed at pre-dose, and at 2 min, 7 min, 15 min, 30 min, and 1, 2, 4, 7 and 24 hrs post dose. Samples were collected into chilled, EDTA-fortified tubes, centrifuged for 10 minutes at 3000 rpm (4°C), and resulting plasma aliquoted into 96-well plates for LC/MS/MS analysis. Pharmacokinetic parameters were obtained from noncompartmental analysis (WinNonLin, V5.3, Pharsight Corp., Mountain View, CA) of individual concentration-time profiles following the parenteral administration of a test article. For oral exposure studies, measuring both systemic plasma and CNS tissue exposure, compounds were administered (oral gavage) to fasted rats (n=2) as suspensions in 10% tween 80/0.5% methylcellulose at a dose of 10 mg/kg and in a dosing volume of 10 mL/kg; blood and whole brain samples were collected at 1.5 hours post-dose. Whole blood was collected into chilled, EDTA-fortified tubes, centrifuged for 10 minutes at 3000 rpm (4°C) and stored at -80°C until LC/MS/MS analysis. The brain samples were rinsed in PBS, snap frozen and stored at -80°C. Prior to LC/MS/MS analysis, brain samples were thawed to room temperature and subjected to mechanical homogenation employing a Mini-Beadbeater™ and 1.0 mm Zirconia/Silica Beads (BioSpec Products). All animal studies were approved by the Vanderbilt University Medical Center Institutional Animal Care and Use Committee. The animal care and use program is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International.

**Liquid chromatography/mass spectrometry analysis.** 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 2.1 x 50 mm, 3 μm column (Fortis Technologies Ltd, Cheshire, UK) thermostated at 40°C. 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 30% B after a 0.2 min hold and was linearly increased to 90% B over 0.8 min; held at 90% B for 0.5 min and returned to 30% 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°C 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). All data were analyzed using AB Sciex Analyst 1.4.2 software.

## References and Notes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