Title: Investigating mGlu5 allosteric modulator cooperativity, affinity and agonism: enriching structure-function studies and structure-activity relationships

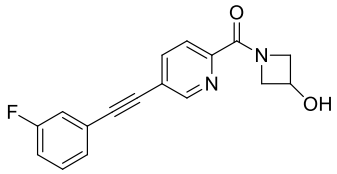
Authors: Karen J. Gregory, Meredith J. Noetzel, Jerri M. Rook, Paige N. Vinson, Shaun R. Stauffer, Alice L. Rodriguez, Kyle A. Emmitte, Ya Zhou, Aspen C. Chun, Andrew S. Felts, Brian A. Chauder, Craig W. Lindsley, Colleen M. Niswender and P. Jeffrey Conn

Journal: Molecular Pharmacology

Supporting Information

Chemistry, General. All NMR spectra were recorded on a Bruker 400 mHz instrument. ^{1}H chemical shifts are reported in δ values in ppm downfield from DMSO as the internal standard in DMSO. 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. High resolution mass spectra were recorded on a Waters Q-TOF API-US. Analytical thin layer chromatography was performed on Analtech silica gel GF 250 micron plates. Analytical HPLC was performed on an Agilent 1200 series. Preparative purification was performed on combi-flash companion (ISCO Inc.). Solvents for extraction, washing and chromatography were HPLC grade. All reagents were purchased from Aldrich Chemical Co. and were used without purification. All polymer-supported reagents were purchased from Argonaut Technologies. DIPEA (N,N-diisopropylethylamine), HATU (2-(1H-7-Azabenzotriazol-1-yl)-1,1,3,3-tetramethyl uroniumhexafluorophosphate), DMF (dimethylformamide).

Preparation of (5-((3-fluorophenyl)ethynyl)pyridin-2-yl)(3-hydroxyazetidin-1-yl)methanone VU0405398 (2).



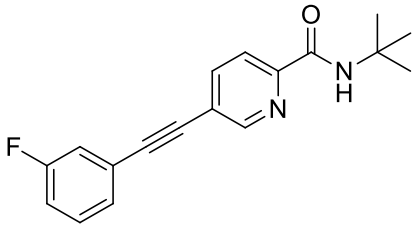
VU0405398

Step 1. (5-Bromopyridiny-2-yl)(3-hydroxyazetidin-1-yl)methanone (1). Commercially available 5-bromopicolinic acid (4.04 g, 20.0 mmol), 3-hydroxyazetidine hydrochloride (3.28 g, 30 mmol), HATU (9.89 g, 26.0 mmol), DIEA (6.98 mL, 40 mmol), were mixed in 50.0 mL of

DMF at room temperature. Analysis of the reaction at 3 h by LC-MS indicated complete consumption of the starting material**.** The mixture was quenched with H_2O (150 mL) and extracted with EtOAc (100 mL x 4). The aqueous layer was treated with aq. Na_2HCO_3 and extracted with EtOAc (100 mL x 4). The combined organic layers were dried over $MgSO_4$, and concentrated *in vacuo* to give a crude oil residue as title compound (1) which was utilized without further purification in step two (LC-MS, ESI, >95% at 215 nM).

Step 2. (5-((3-fluorophenyl)ethynyl)pyridin-2-yl)(3-hydroxyazetidin-1-yl)methanone VU0405398 (2). (5-bromopyridin-2-yl)(3-hydroxyazetidin-1-yl)methanone (**1**) (100 mg, 0.40 mmol), 1-ethynyl-3-fluorobenzene (54 mL, 0.48 mmol), $Pd(PPh_3)_4$ (23 mg, 0.02 mmol), CuI (8 mg, 0.04 mmol), and diethylamine (250 mL, 2.4 mmol) were mixed in 2.0 mL of DMF in a sealed microwave tube. The reaction was subjected to microwave irradiation at 85 ^oC for 45 min. The crude reaction was passed through a celite pad and the crude mixture was diluted with H_2O (150 mL) and extracted with EtOAc (100 mL x 4). The combined organics were dried over $MgSO_4$, and concentrated *in vacuo*. Pure product crystallized during rotary concentration. The solid was filtered and confirmed to give the desired product **VU0405398 (**2) as an off-white powder (61% yield): ^1H NMR (400 MHz, $CDCl_3$) δ 8.72 (s, 1H), 8.14 (s, 1H), 7.94 (d, J = 8.0, 1H), 7.40-7.35 (m, 2H), 7.26 (d, J = 2.4, 1H), 7.15-7.10 (m, 1H), 5.01-4.98 (m, 1H), 4.79-4.75 (m, 1H), 4.60-4.50 (m, 2H), 4.14-4.10 (dd, J = 8.0, 2.8, 1H), 2.18 (d, J = 6.0, 1H); LC-MS (ESI, >98%), m/z = 297.10 ([M+H]).

Preparation of N-(*tert*-butyl)-5-((3-fluorophenyl)ethynyl)picolinamide VU0405386 (4).



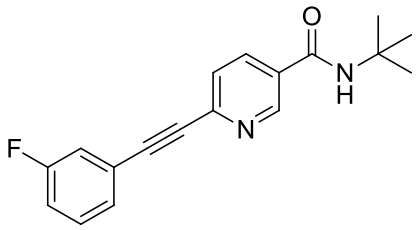
VU0405386

Step 1. 5-((3-fluorophenyl)ethynyl)picolinic acid (3). Commercially available 5-bromopicolinic acid (2.0 g, 10.0 mmol), 1-ethynyl-3-fluorobenzene (1.0 mL, 12.0 mmol), $Pd(PPh_3)_4$ (0.57 g, 0.5 mmol), CuI (0.2 g, 1.0 mmol), and diethylamine (6.2 mL, 60 mmol) were mixed in 15.0 mL of DMF in a sealed 20 mL Biotage microwave tube. The reaction was subjected to microwave irradiation at 90°C for 45 min. The crude mixture was diluted with H_2O

(60 mL). The product precipitated upon the addition of H_2O. The mixture was filtered, rinsed with cold ether and dried *in vacuo* to afford 2.37 g of the title compound **3** as an off-white solid with >98% purity by HPLC (94% yield): LC-MS (ESI, >98%) *m/z* = 242.1 ([M+H]).

Step 2. *N*-(*tert*-butyl)-5-((3-fluorophenyl)ethynyl)picolinamide VU0405386 (4). 5-((3-fluorophenyl)ethynyl)picolinic acid (**3**) (100 mg, 0.41 mmol), *tert*-butylamine (35 mg, 0.50 mmol), HATU (189 mg, 0.50 mmol) were stirred in DMF (2.0 mL) for 15 min at room temperature. To the mixture was added DIPEA (144 mL, 0.83 mmol) and the reaction mixture stirred overnight. The reaction mixture was diluted with EtOAc (15 mL) and washed with water (15 mL). The organic layer was extracted with EtOAc (20 mL), dried over $MgSO_4$, filtered, and concentrated *in vacuo*. The crude material was purified by RP-HPLC using an automated Gilson Inc. liquid handler to afford the title compound **VU0405386** (**4**) as a while solid (98% yield): ^{1}H NMR (400 MHz, MeOD) 8.74 (s, 1H), 8.08 (s, 2H), 7.45-7.40 (m, 2H), 7.33 (d, *J* = 9.6, 1H), 7.19 (t, *J* = 1.6, 1H), 1.49 (s, 9H); LC-MS (ESI, >98%) *m/z* = 297.0 ([M+H]).

Preparation of N-(*tert*-butyl)-6-((3-fluorophenyl)ethynyl)nicotinamide VU0415051 (6).



VU0415051

Step 1. 6-((3-fluorophenyl)ethynyl)nicotinic acid (5). Commercially available 6-bromonicotinic acid (4.0 g, 19.8 mmol), 1-ethynyl-3-fluorobenzene (2.75 mL, 23.8 mmol), $Pd(PPh_3)_4$ (1.14 g, 0.99 mmol), CuI (0.377 g, 1.98 mmol), and diethylamine (12.3 mL, 119 mmol) were mixed in 40.0 mL of DMF and placed into two 20 mL microwave tubes. The reaction tubes were each serially subjected to microwave irradiation at 90°C for 45 min. each. The reaction vessels were combined and basified with aq. $NaHCO_3$. To the mixture DCM (15 mL) was added to extract the triphenylphosphine oxide by-product. The resulting aqueous layer was acidified with aq. 1N HCl to pH ~ 1-2. The aqueous phase was extracted with $CHCl_3$ and the organic layer was dried over $MgSO_4$ and concentrated *in vacuo*, to afford 4.5 g of the title compound **5** (95% yield): LC-MS (ESI, >98%) *m/z* = 242.1 ([M+H]).

Step 2. N-(*tert*-butyl)-6-((3-flurophenyl)ethynyl)nicotinamide VU0415051 (6). 6-((3-fluorophenyl)ethynyl)nicotinic acid (**5**) (100 mg, 0.41 mmol), *tert*-butylamine (37 mg, 0.50 mmol), HATU (206 mg, 0.54 mmol) and DIPEA (180 mL, 1.04 mmol) were mixed in DMF (2.0 mL) at room temperature. After 2 h the reaction was complete as indicated by LC-MS and was directly purified by RP-HPLC using automated Gilson Inc. liquid handler to give 97 mg of the title product **VU0415051** (**6**) as an off-white powder (80% yield): ^{1}H NMR (400 MHz, DMSO) 8.98 (d, J = 1.6, 1H), 8.23-8.21 (m, 1H), 8.10 (s, 1H), 7.75 (d, J = 8.4, 1H), 7.56-7.49 (m, 2H), 7.40-7.35 (m, 1H), 1.40 (s, 9H); LC-MS (ESI, >98%) m/z = 297.14 ([M+H]).

Supplementary Table 1: Summary of operational model parameters for glutamate mediated intracellular Ca^{2+} mobilization in presence of positive allosteric modulators using HEK cells expressing either low or high levels of $mGlu_5$. Data represent the mean and s.e.m from a minimum of three independent determinations.

	CDPPB	VU29	CPPHA	VU0357121	VU0364289	VU0092273	VU0360172	VU0405398	VU0415051	VU0405386
HEK293A-$mGlu_5$ (low): determining composite cooperativity (αβ)										
$\log\tau_A$	0.79 ±0.05	0.75 ±0.04	0.74 ±0.12	0.61 ±0.05	0.76 ±0.06	0.77 ±0.08	0.82±0.07	0.64 ±0.08	0.71 ±0.11	0.73 ±0.08
E_m	99.8 ±2.3	101.9 ±2.2	104.0 ±2.1	102.8 ±2.6	108.1 ±6.6	99.5 ±1.2	105.2 ±3.9	103.8 ±2.8	98.8 ±1.7	98.9 ±1.9
n	1.8 ±0.2	2.2 ±0.2	2.2 ±0.3	2.7 ±0.2	2.3 ±0.3	3.1 ±0.4	3.9 ±0.5	2.5 ±0.2	2.5 ±0.5	2.7 ±0.6
basal	-1.0 ±0.2	0.7 ±0.9	0.6 ±0.4	1.9 ±0.9	1.3 ±0.6	1.6 ±0.9	1.3 ±0.3	1.0 ±0.4	0.9 ±0.4	1.4 ±0.4
HEK293A-$mGlu_5$ (low): assuming neutral affinity cooperativity (α=1)										
$\log\tau_A$	0.86 ±0.05	0.83 ±0.04	0.82 ±0.10	0.69 ±0.05	0.80 ±0.07	0.84 ±0.07	0.88 ±0.07	0.72 ±0.07	0.83 ±0.07	0.73 ±0.08
E_m	100.6 ±2.6	102.7 ±2.4	104.5 ±2.6	106.1 ±4.0	113.9 ±7.9	99.8 ±1.3	105.5 ±4.1	105.9 ±3.1	100.0 ±2.1	100.6 ±2.1
n	2.1 ±0.2	2.5 ±0.3	2.5 ±0.3	2.8 ±0.3	2.4 ±0.4	3.6 ± 0.5	4.3 ±0.5	2.6 ±0.2	2.9 ±0.8	2.3 ±0.2
basal	-0.8 ±0.1	0.7 ±0.9	0.9 ±0.0.7	1.9 ±0.9	1.1 ±0.7	1.6 ±1.0	1.3 ±0.3	0.8 ±0.3	1.3 ±0.6	1.4 ±0.4
HEK293-$mGlu_5$ (high): assuming neutral affinity cooperativity (α=1)										
$\log\tau_A$	0.41 ±0.04	0.46 ±0.07	0.49 ±0.06	0.27 ±0.04	0.25 ±0.03	0.40 ±0.06	0.42 ±0.04	0.46 ±0.16	0.30 ±0.04	0.31 ±0.04
E_m	102.2 ±3.0	108.3 ±4.3	107.0 ±1.9	124.6 ±6.0	144.1 ±10.5	115.5 ±6.2	113.9 ±3.7	122.4 ±6.6	124.0 ±8.7	130.9 ±12.7
n	3.3 ±0.2	3.5 ±0.7	3.3 ±0.8	2.1 ±0.1	2.2 ±0.2	2.5 ±0.2	2.3 ±0.2	1.9 ±0.2	2.5 ±0.3	2.1 ±0.4
basal	-0.4 ±0.5	0.2 ±0.6	0.8 ±0.3	0.0 ±0.4	0.9 ±1.3	1.8 ±1.3	-0.1 ±0.6	1.0 ±0.7	-0.4 ±1.1	0.0 ±0.6

Interactions between glutamate and allosteric modulators where quantified using equation 2 where glutamate affinity was held constant to a previously reported value ($\log K_A$ = -6.155; Mutel et al., 2000). The presence of allosteric modulators did not affect estimates of glutamate coupling efficiency ($\log\tau_A$), the transduction coefficient (n), the maximal system response (E_m) and the basal level of response; the assumption that α=1, also had no effect on these estimates (one-way ANOVA).

Supplementary Table 2: Summary of operational model parameters glutamate mediated intracellular Ca^{2+} mobilization for negative allosteric modulators using HEK293 cells expressing low and high levels of $mGlu_5$. Data represent the mean and s.e.m from a minimum of three independent determinations.

	HEK293A-$mGlu_5$ (low)					
	MPEP	M-5MPEP	VU0285683	VU0366248	VU0366249	VU0366058
$\log\tau_A$	0.72 ±0.03	0.85 ±0.05	0.82 ±0.06	0.66 ±0.05	0.81 ±0.07	0.79 ±0.06
E_m	103.9 ±2.4	102.4 ±1.7	100.6 ±1.8	101.6 ±2.3	96.8 ±1.4	100.1 ±0.8
n	1.8 ±0.2	2.3 ±0.2	3.0 ±0.5	2.5 ±0.3	3.2 ±0.8	2.6 ±0.3
basal	2.7 ±0.9	0.6 ±0.4	1.0 ±0.5	0.6 ±0.6	2.6 ±0.4	1.4 ±0.5
	HEK-$mGlu_5$ (high)					
$\log\tau_A$	0.43 ±0.03	0.31 ±0.02	0.39 ±0.03	0.32 ±0.04	0.34 ±0.03	0.28 ±0.06
E_m	101.8 ±0.9	117.6 ±2.9	104.5 ±3.7	138.1 ±23.9	109.4 ±3.1	117.9 ±10.3
n	3.9 ±0.3	2.7 ±0.1	3.3 ±0.1	2.8 ±0.5	4.4 ±0.7	3.2 ±0.7
basal	0.9 ±0.8	1.2 ±0.3	0.1 ±0.1	1.6 ±0.5	1.8 ±0.3	1.0 ±0.4

Interactions between glutamate and allosteric modulators where quantified using equation 2 where glutamate affinity was held constant to a previously reported value ($\log K_A$ = -6.155; Mutel et al., 2000). The presence of allosteric modulators did not affect estimates of glutamate coupling efficiency ($\log\tau_A$), the transduction coefficient (n), the maximal system response (E_m) and the basal level of response (one-way ANOVA).

Supplementary Table 3: Summary of operational model parameters for glu-mediated ERK1/2 phosphorylation using HEK293A cells expressing a low level of $mGlu_5$. Data represent the mean and s.e.m from a minimum of three independent determinations.

	Positive allosteric modulators									
	CDPPB	VU29	CPPHA	VU0357121	VU0364289	VU0092273	VU0360172	VU0405398	VU0415051	VU0405386
$\log\tau_A$	-0.21 ±0.06	-0.49 ±0.21	-0.15 ±0.02	-0.14 ±0.11	-0.15 ±0.04	-0.07 ±0.02	-0.09 ±0.07	-0.17 ±0.13	-0.22 ±0.07	-0.16 ±0.06
n	4.6 ±0.5	2.9 ±0.7	6.5 ±1.3	7.9 ±2.5	5.7 ±0.7	5.5 ±0.1	3.9 ±1.1	3.6 ±1.1	4.1 ±0.8	3.7 ±0.5

	Negative allosteric modulators					
	MPEP	M-5MPEP	VU0285683	VU0366248	VU0366249	VU0366058
$\log\tau_A$	-0.68 ±0.39	-0.09 ±0.08	-0.32 ±0.23	-0.19 ±0.11	-0.23 ±0.11	-1.08 ±0.61
n	2.5 ±0.8	8.1 ±1.5	5.2 ±2.2	4.7 ±2.0	4.9 ±2.2	1.7 ±0.8

For ERK1/2 phosphorylation in the low-expressing cell line, data were expressed as fold increase over basal, with the E_m defined as the response to 10% FBS (9.4 fold). The presence of allosteric modulators had no significant effect on estimates of glutamate coupling efficiency ($\log\tau_A$) or the transduction coefficient (n) (one-way ANOVA).