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Development and validation of a Mass Spectrometry binding assay for mGlu5 receptor

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Abstract

Mass spectrometry (MS) binding assays are a label-free alternative to radioligand or fluorescence binding assays, so the readout is based on direct mass spectrometric detection of the test ligand. The study presented here describes the development and validation of a highly sensitive, rapid and robust MS binding assay for the quantification of the binding of the metabotropic glutamate 5 ($mGlu_5$) negative allosteric modulator (NAM), MPEP (2-Methyl-6-phenylethynylpyridine) at the $mGlu_5$ allosteric binding site. The LC-ESI-MS/MS (Liquid Chromatography - Electrospray Ionization - Tandem Mass Spectrometric) analytical method was established and validated with a deuterated analogue of MPEP as an internal standard. The developed MS binding assay described here allowed for the determination of MS binding affinity estimates that were in agreement with affinity estimates obtained from a tritiated MPEP radioligand saturation binding assay; indicating the suitability of this methodology for determining affinity estimates for compounds that target $mGlu_5$ allosteric binding sites.

Keywords

MPEP, mGlu5 receptor, MS binding assays, radioligand binding assays, saturation assay

Introduction

Understanding and quantifying the binding kinetics of ligand-target interactions is critical to any carefully considered drug discovery project [1-5]. To date, conventional methods for measuring drug-receptor binding interactions are based on competitive assays requiring radiolabeled [6-7] or

fluorescently labeled ligands [8] that can be quantified with high sensitivity. Despite the robustness of radioligand binding assays, they carry some inherent drawbacks in terms of safety precautions, expensive synthesis, special lab requirements and waste disposal. Alternatively, the addition of fluorescent moieties to ligands considerably modifies its chemical structure, which may affect their pharmacological properties and their synthesis can be challenging and costly. In many cases, the protein being investigated also needs to be altered to allow for the measurement of FRET (Förster resonance energy transfer) between a donor (the ligand's fluorescent scaffold) and an acceptor (the fluorophore attached to the target), which can lead to additional complications [9]. These approaches have been applied successfully to many different receptors, but assays have not been developed for all target proteins [10].

The development of mass spectrometry (MS) binding assays by several teams attempts to circumvent some of these limitations by directly measuring the binding kinetics of a ligand of interest to its target receptor in conditions that are more physiologically relevant [11-23]. In this way, the quantification of binding estimates for a ligand by the MS binding technique does offer some advantages over conventional radioligand and fluorescent binding techniques. Alike with labeled ligands though, ligands for MS binding are still required to fulfill a set of criteria to allow for accurate detection in an assay. Ligands should have high affinity and selectivity for their target and have low nonspecific binding. The compound should have appropriate physicochemical properties to allow for suitable atmospheric ionization and quantification by HPLC-MS (High Pressure Liquid Chromatography - Mass Spectrometry) with high sensitivity.

The metabotropic glutamate 5 receptor ($mGlu_5$) is one of eight mGlu receptors that belongs to the class C G protein-coupled receptor (GPCR) family, which preferentially couple to $G_{q/11}$ proteins [24]. Its essential role in regulating neuronal synaptic activity identifies the $mGlu_5$ as an important therapeutic target in neurological and psychiatric disorders such as Alzheimer's disease, pain, depression and schizophrenia [25-27]. To date, a number of allosteric modulators are available for targeting the $mGlu_5$ [27-31]. They offer greater opportunity for selectivity at a receptor subtype owing to their ability to target a topographically distinct and non-overlapping allosteric site to that of the orthosteric (endogenous) ligand binding site, which tends to be less well conserved [27-28].

In this study, we report the development and validation of a label-free MS binding assay for the $mGlu_5$ receptor, which allows for the investigation of the binding kinetics of $mGlu_5$ allosteric ligands that target the common allosteric site, without the need of a competing ligand. To allow for a straightforward validation of the MS binding assay, we employed a ligand that has already been validated as a radioligand, namely, 2-Methyl-6-(phenylethynyl)pyridine (MPEP) - a potent $mGlu_5$ negative allosteric modulator (NAM) [32-33]. This study describes the first equilibrium saturation MS binding assay for MPEP at the $mGlu_5$. To achieve this aim, MCS0455, a deuterated derivative of MPEP, was synthesized to serve as an internal standard (IS). We developed a LC-ESI-MS/MS (Liquid Chromatography - Electrospray Ionization - Tandem Mass Spectrometric) method for the quantification of MPEP, MCS0455 and VU0409106, a competing $mGlu_5$ allosteric ligand [34] required for the determination of nonspecific binding (Figure 1). This method was analytically validated and the MS binding estimates were compared to [^{3}H]MPEP saturation radioligand binding estimates.

Results and Discussion

Synthesis of MCS0455

The synthesis of MCS0455 is outlined in scheme 1. The mono Br/Li exchange reaction of 2,6-dibromopyridine (**4**) with *n*-Buthyllithium followed by reaction with deuterated iodomethane (CD_3I) provided the monoalquilated pyridine **5** [36]. Then, compound **5** reacted with the triple bond of the ethynylbenzene via a Sonogashira cross-coupling reaction to give MCS0455 (**2**).

LC-ESI-MS/MS analytical method development

A LC-ESI-MS/MS method was initially developed enabling MPEP, MCS0455 and VU0409106 quantification in the picomolar range. For the HPLC method development, various isocratic and gradient HPLC methods were evaluated to obtain suitable retention times and appropriate peak shapes. A gradient HPLC method was set as detailed in the "Materials and Methods" section. The influence of pH (3.0-10.0) of the aqueous component of the mobile phase on retention time and signal intensity of MPEP, MCS0455 and VU0409106 in LC-ESI-MS chromatograms - recording m/z 194.1, m/z 197.1 and m/z 331.1 - was studied.

Three different acidic and basic mobile phases were tested (10 mM ammonium formate, pH 3.0, 10 mM ammonium bicarbonate, pH 7.0 and 10 mM ammonium formate, pH 10.0) for solvent A in combination with acetonitrile as solvent B. Signal intensity of MPEP, MCS0455 and VU0409106 was influenced by the pH of different mobile phases (Figure 2A). MPEP and MCS0455 retention times were not modified. In contrast, the retention time of VU0409106 was reduced under basic conditions (Figure 2B). Therefore, 10 mM ammonium bicarbonate at pH 7.0 was chosen as solvent A and acetonitrile was chosen as solvent B, since the peak intensity for all the compounds was increased under these elution conditions. The effect of the injection volume was also studied, 100 μL was selected since it resulted in a larger signal to noise (SN) ratio. However, this injection volume affected retention times and resulted in a split of the VU0409106 peak; using the peak at retention time 2.06 min for quantitation. For optimized HPLC conditions, the resulting retention times of MPEP, MCS0455 and VU0409106 were 2.78 min, 2.75 min and 0.34/2.06 min, respectively (Figure 3A-C). The total run time was 9 min per sample.

Optimization of ESI-MS/MS compound-dependent parameters for precursors and product ions of MPEP, MCS0455 and VU0409106 was performed by infusion of a 0.01 mg ml^{-1} solution, dissolved in water : acetonitrile at ratio of 1:3. Our first aim was then to identify the most abundant product ions of the $[M+H]^+$ parent ion of MPEP (m/z 194.1), MCS0455 (m/z 197.1) and VU0409196 (m/z 331.1), respectively. After that, the MS/MS fragmentation was carried out. The most abundant fragments observed were m/z 152 $\pm$ 6 and 165 $\pm$ 8 for MPEP, m/z 152 $\pm$ 6 and 168 $\pm$ 8 for MCS0455 and m/z 216 $\pm$ 2 and 313 $\pm$ 2 for VU0409106, respectively (Figure 3D-F). The optimal source parameters, the compound dependent parameter, normalized collision energy (CE) and the compound dependent parameter, normalized Act Q and Act Time were established for each MS/MS transition as described in "Materials and Methods" section.

LC-ESI-MS/MS analytical method validation

The established LC-ESI-MS/MS method was validated as detailed in the "Materials and Methods" section. Since interfering peaks from matrix samples were not observed at the retention times corresponding for MPEP, MCS0455 and VU0409106 it was considered that the assays were selective. The linearity was evaluated on four separate days with two sets of calibration curves per day showing good reproducibility. The

correlation coefficients (R^2) of the plotted calibration curves were higher than 0.999. The lowest concentration of analyte in a sample which can be quantified reliably, with an acceptable accuracy and precision - the lower limit of quantification (LLOQ) - was confirmed to be 0.125 nM for MPEP and MCS0455 and 0.35 nM for VU0409106 (Table 1 and Supplementary Table 1-2). No carry over peaks were observed following the injection of the calibration curve points.

LC-ESI-MS/MS within-run and between run accuracy and precision were evaluated for each quality control (QC) sample of MPEP, MCS0455 and VU0409106 and they satisfied the indicated criteria (Table 1 and Supplementary Table 1-2). Technician within-run and between-run accuracy and precision levels were also assessed, and RE (relative error) and RSD (relative standard deviation) values were lower than ± 20 % for all the QC samples of analytes (Table 1 and Supplementary Table 1-2).

The matrix factor of MPEP and MCS0455 were obtained at 0.35 nM (Lower; L), 10 nM (Medium; M) and 20 nM (Higher; H) as explained in “Materials and Methods” section. While the matrix factor of VU0409106 was obtained at 1 nM (L), 10 nM (M) and 20 nM (H). The RSD of the IS-normalized MPEP matrix factor calculated from the 3 lots of matrix was 10.9 % for L, 5.7 % for M and 1.9 % for H. The RSD of MPEP, MCS0455 and VU0409106 matrix factor were detailed in Supplementary Table 3. The extraction recoveries of MPEP, MCS0455 and VU0409106 were consistent and reproducible for each QC (Table 2).

MS binding assay development

The protocol for the MS binding assays was designed with the following workflow: incubation, separation of target-ligand complex from unbound ligand by aspiration, liberation by protein denaturation and analysis through the validated HPLC-MS/MS methodology (Figure 4). By following the different setups established by Wanner et al, multiple parameters were studied in order to optimize assay conditions [10-19].

By using human (h)mGlu5 transiently transfected HEK 293T cells in preliminary experiments, different 96-well filter plate pre-treatment conditions (water or 0.1-1 % polyethyleneimine) were tested for their ability to reduce nonspecific binding. By selecting 0.5 % (m/v) polyethyleneimine (PEI) as the pre-treatment condition this resulted in reduced nonspecific binding and the largest window between TB (total binding) and NSB (non-specific binding) (data not shown). The effect of different elution solvents, including 100% methanol, 75% acetonitrile and 25% ammonium formate buffer (5 mM, pH 10) or 75% acetonitrile and 25% ammonium bicarbonate buffer (10 mM, pH 7), was also assessed to maximise ligand recuperation. Recovery of bound ligand and reproducibility between experiments was increased by elution with acetonitrile (3 x 100 μL/well) and 10 mM ammonium bicarbonate buffer, pH 7 (1 x 100 μL/well) (see Supplementary Figure 1). The effect of filtration or centrifugation on the last step of the standard setup was also assessed to improve compound recovery. This involved the addition of 100 μL of aqueous solvent per well to plates prior to filtration (30 s aspiration step) or centrifugation (10 min, 2000 rpm, 4ºC) before the samples were analyzed by the HPLC-MS/MS method. Since recovery techniques showed no significant differences between MPEP saturation isotherms, the filtration technique was chosen for the remainder of the experiments as it is rapid and allows for higher throughput of samples (Supplementary Figure 2).

To reduce the variability observed between membranes prepared from different batches of transiently transfected cells, a stably, inducible

hmGlu$_5$ expressing HEK293 cell line was generated. In those cells, the level of hmGlu$_5$ expression following induction with doxycycline is highly reproducible. Following optimization of conditions, the experiments were then performed with membranes prepared from this cell line. In all assays, 1.5 nM MCS0455 as IS was added to samples to correct for the loss of MPEP in the bound ligand recovery step (for more detail see method section). To prevent ligand depletion, all remaining studies used 20 μg of hmGlu$_5$ expressing membranes per well in a final incubation volume of 300 μL.

Saturation MS binding assay

The validated LC-ESI-MS/MS method was used for the quantification of MPEP, MCS0455 and VU0409106 in an hmGlu$_5$ MS saturation binding assay. We, therefore, describe the first saturation MS binding experiment with MPEP as a native marker for hmGlu$_5$. Total and nonspecific binding of MPEP at the hmGlu$_5$ was determined (Figure 5A), and by calculating the difference of these values specific MPEP binding was determined (Figure 5B). This allowed for the calculation of a K_D (equilibrium dissociation constant) value of 13.16 ± 1.24 nM and a B_{max} (maximum amount of binding sites) value of 18.53 ± 2.67 pmol/mg protein. MS binding results were in agreement with the affinity estimate (K_D = 3.65 ± 0.32 nM) obtained in [^{3}H]MPEP radioligand saturation binding assay for the same receptor (Supplementary Figure 3 and Supplementary Table 4) and to a K_D value reported in the literature for [^{3}H]MPEP at the hmGlu$_5$ receptor [37].

Materials and methods

Materials

All chemicals and solvents were from commercial suppliers and used without purification, with the exception of the anhydrous solvents such as dimethylformamide (DMF), which were treated previously through a system of solvent purification (PureSolv), degasified with inert gases and dried over alumina or molecular sieves. HEK 293T and HEK 293 cells were obtained from cell culture service (IQAC-CSIC, Barcelona, Spain) and ATCC® CRL1573™ (Molsheim, France), respectively. Dulbecco's modified Eagle medium (DMEM), Opti-MEM I reduced serum media, trypsin and fetal bovine serum were purchased from Thermo Fisher Scientific. Antibiotics were purchased from Sigma-Aldrich, except blasticidin and hygromycine B from InvivoGen. Polyethylenimine (PEI) and X-tremeGENE 9 were purchased from Sigma-Aldrich and Roche, respectively. VU0409106 were obtained from Tocris Biosciences.

Methods

Synthesis of MCS0455

Reactions were monitored by thin layer chromatography (60 F, 0.2 mm, Macherey-Nagel) by visualization under 254 and/or 365 nm lamp. Purification was made by flash column chromatography by using Panreac silica gel 60, 40-63 microns RE or by Isolera-Biotage equipment (SNAP KP-C18-HS-12g column; A: 0.05 % formic acid in water and B: 0.05 % formic acid in acetonitrile; 5 % B 3 column volume (CV), 5 % B - 100 % B 18 CV, 100 % B 5 CV). Nuclear magnetic resonance (NMR) spectrometry was performed using a Varian Mercury 400 MHz. Chemical shifts δ are reported in parts per million (ppm) against the reference compound (Chloroform δ = 7.26 ppm (^{1}H), δ = 77.16 ppm (^{13}C), and DMSOδ_6 δ = 2.50ppm (^{1}H), δ = 39.52 ppm (^{13}C). HPLC analysis was performed on a 2795 Alliance HPLC system (Waters) equipped with an 1100 diode array detector (Agilent). Chromatographic separations were performed with a Zorbax Extend C18 column (2.1 x 50 mm, 3.5 μm, Agilent) with a Zorbax Extend

C18 pre-column (2.1 x 12.5 mm, 5 μm, Agilent). The mobile phase used was a mixture of solvent A (0.05 % formic acid in water) and solvent B (0.05 % formic acid in acetonitrile) with a flow rate of 0.5 mL/min. The initial mobile phase composition was 5 % solvent B and these conditions were maintained for 0.5 min. Conditions were changed to 100 % solvent B (over 5 min) and these conditions were maintained for 2 min, before the return of the initial conditions. For all experiments, 10 μL of sample - diluted in acetonitrile - was injected and MS detection was carried out on a Quattro micro triple quadrupole mass spectrometer (Waters), using ESI source in positive ion mode. Melting points were measured with Melting Point B-545 (Büchi), ramp 0.5 ºC/min with digital temperature measurement.

2-Bromo-6-methyl-d_3-pyridine (5). 2,6-Dibromopyridine (4) (1.07 g, 4.53 mmol) was dissolved in tetrahydrofuran (THF) (1.3 mL) and the solution was cooled to -78 °C, followed by slow addition of BuLi 2.5 M in hexane (0.186 mL, 4.65 mmol). The mixture was stirred for 1 h at 78 ºC, followed by addition of iodomethane-d_3 (0.79 g, 5.44 mmol). Then, the reaction mixture was warmed to room temperature and stirred for 1 h. The reaction was then quenched with 10 mL of water, and extracted with 3 x 10 mL ethyl acetate (EtOAc). The resulting organic phases were combined, dried over anhydrous Na_2SO_4, filtered and evaporated. The residue was purified by flash column chromatography with dichloromethane; yielding 140 mg (16 %) of the desired compound.^{1}H NMR (400 MHz, $CDCl_3$) δ 7.42 td (t, *J* = 7.6, 1H), 7.29 (dd, J = 7.6, 0.9 Hz, 1H), 7.10 (dd, *J* = 7.6, 0.9 Hz, 1H) [27].

2-Methyl-d_3-6-phenylethynylpyridinehydrochloride (2). To a suspension of 2-bromo-6-methyl-d_3 pyridine (5) (140 mg, 0.80 mmol), bis(triphenylphosphine)palladium (II) dichloride (46.2 mg, 0.04 mmol) and copper iodide (7.6 mg, 0.04 mmol), in 1.5 mL of anhydrous DMF, previously purged with argon, 1-ethynylbenzene (0.097 mL, 0.80mmol) and dry triethylamine (0.33 mL, 2.4 mmol) were added, and the reaction mixture was stirred at 40 °C for 8 h. After, 40 mL of EtOAc was added to the mixture, and was washed with 40 mL of saturated solution of $NaHCO_3$ and 40 mL of brine. The organic layer was dried over Na_2SO_4, filtered and evaporated under vacuum. The remaining residue was purified via flash column chromatography with EtOAc-hexane (1:4). A pale brown solid was isolated (100 mg, 53 %). A portion of this compound was dissolved in diethyl ether and 4 M HCl/dioxane was added, the precipitate was collected by filtration to give the hydrochloride salt as a brown solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.21 (t, *J* = 8 Hz, 1H), 7.84 (d, *J* = 8 Hz, 1H), 7.72 – 7.64 (m, 3H), 7.56 – 7.43 (m, 3H). ^{13}C NMR (101 MHz, DMSO- d_6) δ156.90, 143.20, 136.30, 132.53, 131.03, 129.46, 126.92, 126.42, 120.54, 96.18, 84.13, 20.38. HRMS (m/z): $[M+H]^+$ calculated for $C_{14}D_3H_8N$, 197.1177; found, 197.1178. HPLC/DAD: purity (abs = 254 nm) = 100 %; RT = 2.44 min. m.p. 130.5 - 132.3 ºC

LC-ESI-MS/MS

Chromatographic and mass spectrometric conditions

Chromatographic separations were accomplished on a Zorbax Extend C18 column (2.1 x 50 mm, 3.5 μm, Agilent) with a Zorbax Extend C18 precolumn (2.1 x 12.5 mm, 5 μm, Agilent). HPLC analysis was performed on a Thermo Scientific Dionex UltiMate 3000 High-Performance Liquid Chromatography system equipped with a pump (LPG-3400SD), an auto-sampler (ACC-3000T) with a thermostated column compartment. Gradient HPLC method was used for the analysis of MPEP, the internal standard (IS), MCS0455, and VU0409106. The mobile phase consisted of solvent A (10 mM ammonium bicarbonate pH 7) and solvent B (acetonitrile) with

a flow rate of 0.9 mL/min. The initial mobile phase composition was 20 % solvent B, changed progressively to 100 % for 4 min. Following 2 min under these conditions, the initial conditions were reinstated within 1 min, and then were maintained to allow for column equilibration for 2 min. The column temperature was set to 40 ºC, the sampler temperature was established to 10 ºC and the injection volume was 100 μL.

Mass spectrometry detection was carried out on a LTQ XL ion trap mass spectrometer (Thermo Scientific), using ESI source in positive ion mode. Quantification was performed using single reaction monitoring (SRM) mode with the transition of m/z 194.1 → 152 ± 6, 165 ± 8 for MPEP, m/z 197.1 → 152 ± 6, 168 ± 8 for MCS0455 and m/z 331.1 → 216 ± 2, 313 ± 2 for VU0409106. The optimal source parameters were as follows: sheath gas flow at 60, aux gas flow at 10, sweep gas flow at 10, capillary temperature at 300 ºC, source voltage at 3 kV, capillary voltage at 1 V and tube lens at 35 V. The compound dependent parameter normalized collision energy (CE) was set at 65 % for MPEP and MCS0455 and 30 % for VU0409106. The compound dependent parameter normalized Act Q was set at 0.6 % for MPEP and MCS455 and 0.25 % for VU0409106. The compound dependent parameter Act Time was set at 40 ms for MPEP and MCS0455 and 30 ms for VU0409106. The wideband activation option was selected. System control and data analysis were performed by Thermo Xcalibur 2.2 software (Thermo Scientific).

Preparation of calibration curve and quality control samples

The calibration standard curves of MPEP, MCS0455 and VU0409106 with final concentrations of 0.125 - 25 nM were obtained from working solutions (1 - 50 nM) by further dilution with acetonitrile : 10 mM ammonium bicarbonate pH 7 (3:1). Working solutions were prepared from previous working standard solutions with concentrations in the range of 10 - 500 μM, by dilution of the stock solutions with acetonitrile : 10 mM ammonium bicarbonate pH 7 (3:1).

Quality control (QC) samples with final concentrations of 0.125 nM or 0.35 nM (Lower limit of quantification; LLOQ), 0.35 nM or 1 nM (Lower; L), 10 nM (Medium; M), 20 nM (Higher; H) and 25 nM (Upper limit of quantification; ULOQ) were obtained from working solutions (2 - 100 nM) by further dilution with acetonitrile: 10 mM ammonium bicarbonate, pH 7 (3:1). Working solutions were prepared in the same way as calibration standards.

Analytical method validation

The Guidance of Industry Bioanalytical Method Validation was adapted to perform a full LC-ESI-MS/MS validation process for MPEP, MCS0455 and VU0409106, recommended by FDA [38].

Selectivity of the method was assessed by processing ten blank matrix samples to investigate the potential interferences at the retention times for the analytes and IS compound. Carry over was evaluated by injecting a blank sample after the injection of every calibration curve point. Eleven-point calibration curves were established with concentrations in the range of 0.125 - 25 nM for each analyte and IS compound. Calibration curves were built by plotting the peak area of the analyte or the IS compound versus the quantities (in nmol) of the analyte or the IS compound with weighted ($1/x^2$) least-squares linear regression. The correlation coefficient (R^2) of calibration curves were more than 0.999. The back-calculated concentrations at each point were within ± 20 % of theoretical quantities, except at LLOQ and ULOQ where the calibrator should be ± 25 % of the nominal quantities at each validation run.

LC-ESI-MS/MS within-run and between-run accuracy and precision were determined by

analyzing six replicates of one sample of each QC on the same day, on three consecutive days. In contrast, technician within-run and between-run accuracy and precision were determined by analyzing five independent samples of each QC on the same day, on three consecutive days. The criteria for the data included accuracy (relative error, RE) within ± 20 % (except ± 25 % for the LLOQ and ULOQ) and a precision (relative standard deviation, RSD) not exceeding ± 20 % (± 25 % for the LLOQ and ULOQ).

For each analyte and the IS, the matrix factor (MF) was calculated for three different $hmGlu_5$ matrix samples at three QC levels (L, M and H), by calculating the ratio of the peak area in the presence of matrix (measured by analyzing blank matrix spiked with analyte after extraction), to the peak area in absence of matrix (analyte only solution). The IS normalized MF should also be calculated by dividing the MF of the analyte by the MF of the IS. The RSD MF or the RSD of the IS-normalized MF calculated from the 3 samples of matrix should be less than 15 %. The extraction recovery was determined at three QC levels (L, M and H) by comparing (in triplicate) the analytical results of extracted samples with corresponding extracts of blanks spiked with the analyte post-extraction.

Generation of HEK 293 stable hmGlu5 inducible cell line

The stably expressing, inducible $hmGlu_5$ HEK293 cell line was generated with the Flp-In-T-Rex system according to manufacturer recommendations (Invitrogen). Briefly, the cDNA encoding $hmGlu_5$ containing a Flag and SNAP tag in N terminus (for detection) was inserted into the plasmid pcDNA5-FRT-TO-GFP (Addgene), removing the GFP. This construct was co-transfected by electroporation with the recombinase plasmid pOG44 (Invitrogen) for a targeted integration of the expression vector to the same locus in Flp-In-T-Rex HEK 293 cells (Invitrogen), ensuring homogeneous levels of gene expression. Cells were grown 48 h then selected by the addition of 15 μg/mL blasticidin and 100 μg/mL of hygromycine B. Inducible expression of $hmGlu_5$ was validated following induction with 1 μg/mL doxycycline (Sigma-Aldrich) by anti-Flag ELISA.

Cell culture and transfections

The HEK 293T cells and the HEK 293 inducible, stably expressing $hmGlu_5$ cell line were cultured in DMEM supplemented with 10 % FBS and maintained at 37 °C in a humidified atmosphere with 5 % CO_2. HEK 293T cells were incubated in the presence of 1 % penicillin/streptomycin while, HEK 293 inducible, stably expressing $hmGlu_5$ cells were maintained in the presence of 15 μg/mL blasticidin and 100 μg/mL of hygromycine B. All cells were sub-cultured every two to three days in a 1:3 ratio after reaching 80 % confluence with 5 mL of trypsin to detach cells, and incubated in a humidified atmosphere at 37 °C, 5 % CO_2 for 2 - 5 min. For transient transfection, 4 million HEK 293T cells were seeded in 100 mm culture dishes one day before transfection. The next day, HEK 293T cells were transfected with 5 μg of hmGlu5 DNA using 15 μl of X-tremeGENE 9 diluted in 500 μL Opti-MEM 1X. For the inducible, stably expressing $hmGlu_5$ cells, expression of hmGlu5 was induced by the addition of doxycycline at 1 μg/mL in 150 mm culture dishes containing 15 million cells.

Membrane preparation for MS binding and [^{3}H]MPEP binding assays

Following 48 h transfection or 24 h induction, $hmGlu_5$-HEK cells were grown in 100 or 150 mm culture dishes and were detached by scraping. Cells were centrifuged at 300 g for 5 min and then resuspended in 5 mL ice-cold homogenization

buffer (25 mM HEPES, 10 mM EDTA and 2.5 mM $MgCl_2$, pH 7.5). An ultrasonic cell disruptor (SFX 150, Branson) was then used to homogenize the cell suspension with 3 x 20 s bursts, which were separated by 20 s periods on ice. Cell homogenates were then centrifuged at 600 g for 10 min at 4 °C. The supernatant was collected and transferred to a centrifuge tube, the remaining cell pellet was resuspended in homogenization buffer, re-homogenized and centrifuged as previous (this procedure was repeated 3-4 times). The obtained supernatants were combined, and membranes and the cytosolic fraction were separated by ultracentrifugation at 40 000 g, 4 ºC for 60 min. The pellet was then resuspended in MS binding storage buffer (25 mM HEPES, 1 mM EDTA and 2.5 mM $MgCl_2$, pH 7.5) or [^{3}H]MPEP binding buffer (110 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgSO_4$, 25 mM glucose, 50 mM HEPES, 58 mM sucrose; pH 7.4) to give a final concentration between 1.5 to 2.5 mg/mL and stored at -80 °C. Protein concentrations were determined by BCA (bicinchoninic acid) protein assay kit by following the instructions of the supplier.

MPEP saturation MS binding assay

$hmGlu_5$ expressing membranes (20 μg/well) were incubated with a range of concentrations of MPEP (1 - 100 nM) in binding buffer (25 mM HEPES, 100 mM NaCl and 2.5 mM $MgCl_2$, pH 7.5) for 1 h at 30 °C, while shaking at 150 rpm, in a final assay volume of 300 μL/well. The target-ligand complex was separated from the non-binding ligand by rapid vacuum filtration through 1 μm GF filter multi-well plate (AcroPrep Advance 350 μL, Pall Corporation), pre-soaked for 1 h in 0.5 % PEI, with an extraction plate manifold (Pall Corporation). Samples were washed 3 times with 150 μL of ice-cold binding buffer to eliminate excess non-binding ligand, and the filter plate was dried for 1 h at 60 ºC. The bound MPEP was then recovered by elution with acetonitrile containing 1.5 nM IS (3 x 100 μL/well) and 100 μL of ammonium bicarbonate buffer (10 mM, pH 7) was added to each well, and the solution was aspirated; the final concentration of IS in each sample was 1.125 nM. Samples were then transferred to HPLC vials and analyzed by HPLC-MS/MS methods (see *chromatographic and mass spectrometric conditions* for more detail). Nonspecific binding was determined in the presence of 10 μM VU0409106.

[^{3}H]MPEP radioligand equilibrium saturation binding assay

$hmGlu_5$ expressing membranes (10 μg/well) were incubated at 30 °C for 1 h with a range of [^{3}H]MPEP concentrations (~0.5 - 25 nM) in binding buffer in a final assay volume of 100 μL/well. All binding assays were terminated by rapid filtration through 1 μm GF multi-well plates (pre-soaked for 1 h in 0.5 % PEI), and 3 washers with ice-cold binding buffer to separate bound and free radioligand. 100 μL of scintillant was added to each well. Following 1 h of incubation, radioactivity was measured on a MicroBeta plate counter. MPEP (10 μM) was used to determine nonspecific binding in all cases.

Data analysis

For MPEP saturation MS binding studies, analysis was performed on Thermo Xcalibur 2.2 software (Qual Browser, Thermo Scientific). Obtained MPEP peak areas were transformed to nmol of bound ligand using an appropriate calibration curve, which was based on the peak area of MPEP normalized by the peak area of 1.125 nM MCS0455 (IS) versus the quantities (in nmol) of MPEP with weighted ($1/x^2$) least-squares linear regression.

GraphPad prism version 8 (San Diego, CA) was used for all curve fitting and statistical analysis. MS and radioligand saturation binding experiments,

receptor expression (B_{max}) and [^{3}H]MPEP equilibrium dissociation constants (K_A) were determined by applying the following equation to experimental datasets:

$$Y = \frac{B_{max}[A]}{[A] + K_A} + NS[A]$$

(Equation 1)

Where Y represents the bound radioligand, B_{max} is the total receptor density, [A] is the ligand concentration, K_A is the equilibrium dissociation constant of the MPEP and radioligand, and NS represents the nonspecific binding of the ligand.

Conclusions

In the present study, we describe the first protocol for a MPEP MS saturation binding assay for the $hmGlu_5$. A sensitive LC-ESI-MS/MS analytical method for detection of MPEP was developed using [$^2H_3$]MPEP as an internal standard. Its reliability was determined in accordance with FDA guidelines for bioanalytical method validation with respect to selectivity, linearity, lower limit of quantification, accuracy and precision.

A protocol for a MS binding assay was then developed that employs a filtration step for separating excess unbound MPEP from target-bound MPEP. The use of this MS saturation binding assay allowed for the calculation of an affinity estimate of MPEP for its $hmGlu_5$ binding site, which was consistent to the affinity estimate for [^{3}H]MPEP - for the same binding site - as determined by radioligand experiments. Despite the MS binding assay exhibiting lower sensitivity and throughput capabilities as compared to the radioligand binding assay, the MS assay represents a suitable alternative since it does not use toxic and expensive radiolabeled ligands and the resulting equilibrium dissociation constants between binding assays and those estimates reported in the literature were consistent. The established MS binding assay represents an appropriate and reliable test system for affinity characterization of test compounds that target the MPEP allosteric binding site of the $hmGlu_5$ receptor. The results described here clearly demonstrate the power of this approach as an alternative to conventional radioligand binding assays.

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Compliance with Ethical Standards

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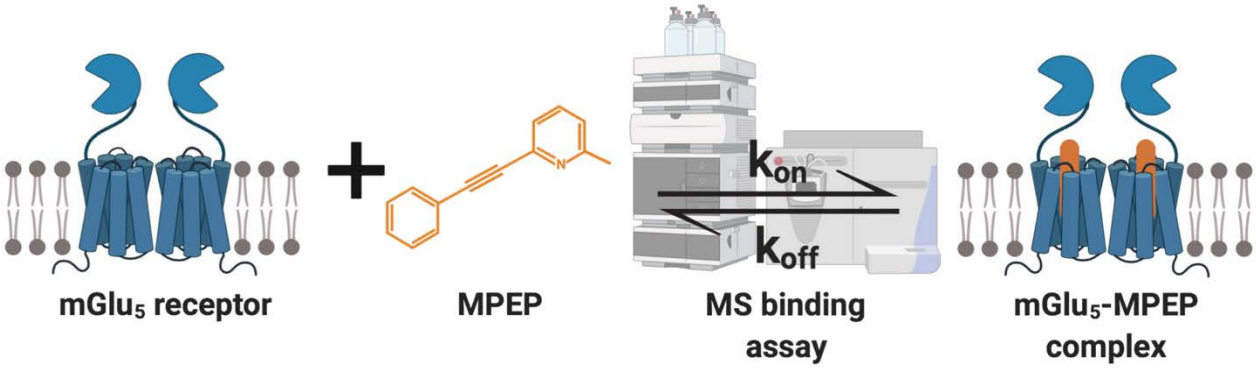
Graphical abstract



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Figures, scheme & tables

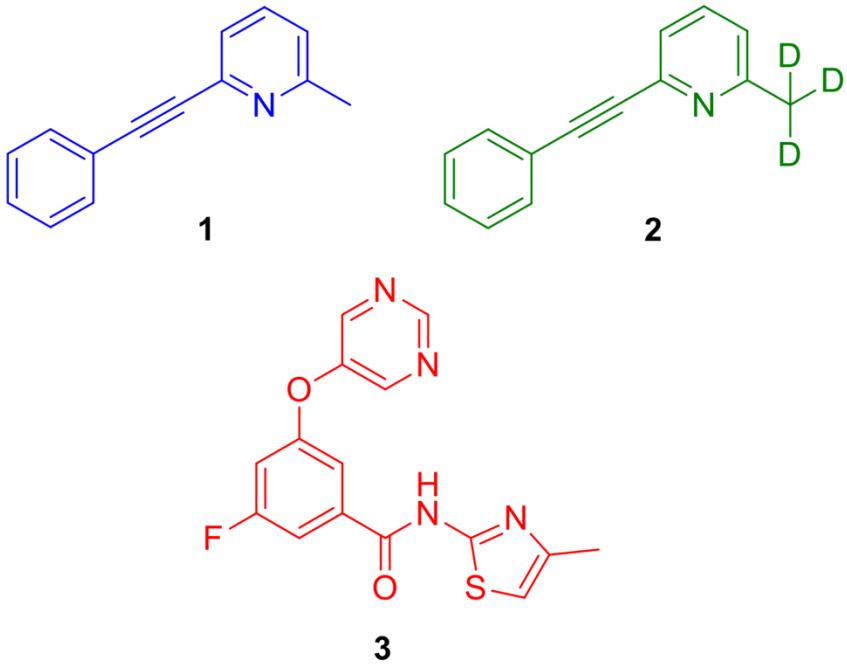
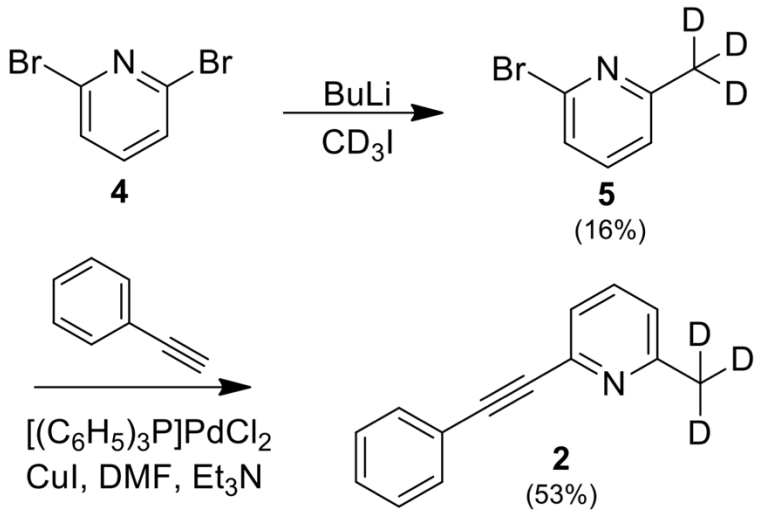


Figure 1. Chemical structures of molecules used for binding experiments. Structures of ligand marker MPEP (2-methyl-6-(phenylethynyl)pyridine) **(1)** synthesized in MCS group according to described conditions in the literature [35], deuterated MPEP (or MCS0455) **(2)** used as internal standard and VU0409106 **(3)** used for determination of nonspecific binding in MS binding assays.



Scheme 1. Synthetic route to prepare MCS0455 (2).

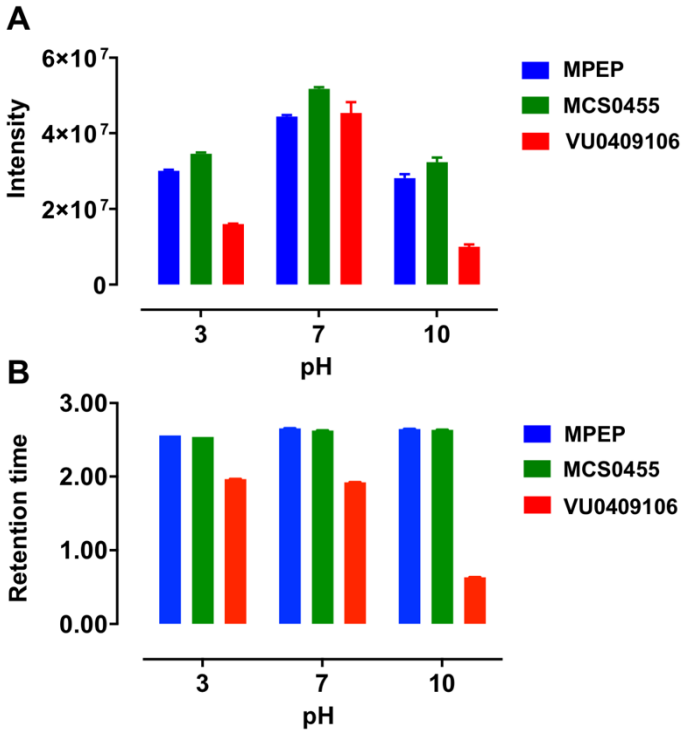


Figure 2. Influence of the aqueous phase pH on signal intensity and retention time of MPEP, MCS0455 and VU0409106. pH values are plotted against **(A)** intensity, defined as peak area (m/z 194.1 for MPEP, m/z 197.1 for MCS0455 and m/z 331.1 for VU0409106) and **(B)** retention time of 50 mM of each compound (means ± SEM, n = 4).

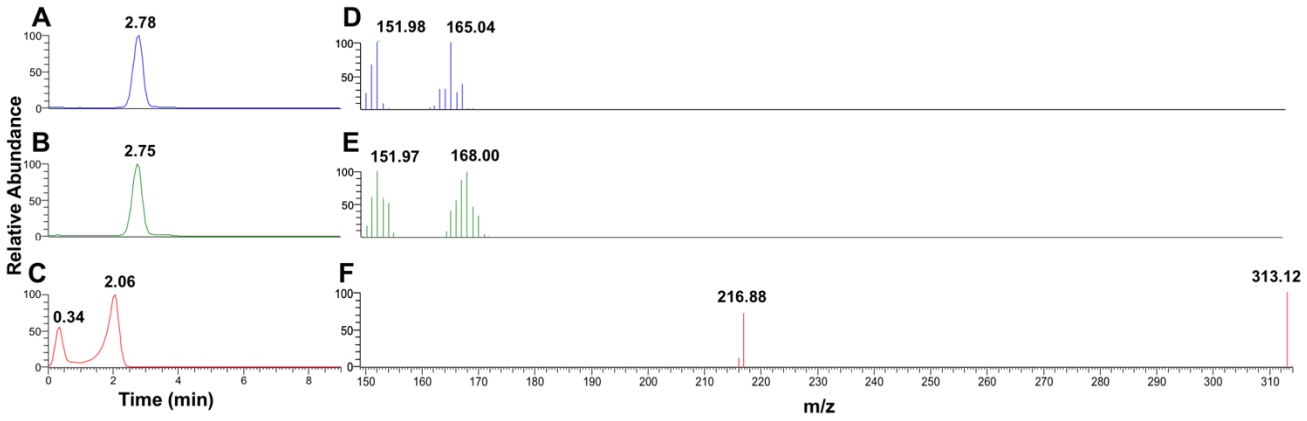


Figure 3. MRM chromatograms and production scan spectra of MPEP (blue), MCS0455 (green) and VU0409106 (red). MRM chromatogram and product ion scans in ESI (+) mode for m/z 152 ± 6 and 165 ± 8 of MPEP **(A, D)**, m/z 152 ± 6 and 168 ± 8 for MCS0455 **(B, E)** and m/z 216 ± 2 and 313 ± 2 for VU0409106 **(C, F)** at a concentration of 5 nM dissolved in 10 mM ammonium bicarbonate pH 7: acetonitrile at ratio of 1:3.

Table 1. Within- and between-run accuracy and precision of HPLC-MS/MS method to determine MPEP signal (n = 3 r, 6 replicates per run). Quality control (QC) samples with final concentrations of 0.125 nM (Lower limit of quantification; LLOQ), 0.35 nM (Lower; L), 10 nM (Medium; M), 20 nM (Higher; H) and 25 nM (Upper limit of quantification; ULOQ) were prepared in acetonitrile : 10 mM ammonium bicarbonate, pH 7 (3:1). The criteria for the data included accuracy (relative error, RE) and a precision (relative standard deviation, RSD) within ± 20 % (except ± 25 % for the LLOQ and ULOQ).

	Within-run				Between-run			
Spiked concentration (nM)	**Technician Accuracy (RE, %)**	**HPLC-MS Accuracy (RE, %)**	**Technician Precision (RSD, %)**	**HPLC-MS Precision (RSD, %)**	**Technician Accuracy (RE, %)**	**HPLC-MS Accuracy (RE, %)**	**Technician Precision (RSD, %)**	**HPLC-MS Precision (RSD, %)**
0.125	15.6	16.6	10.2	9.7	8.7	6.7	13.5	15.8
0.35	15.6	14.0	11.0	5.6	7.0	9.0	11.7	8.6
10	14.3	15.4	1.0	2.1	6.9	8.2	6.3	6.2
20	15.4	18.9	1.6	8.1	5.3	8.6	8.2	10.6
25	13.1	15.2	1.9	5.0	4.5	6.8	8.2	8.4

Table 2. Recovery of L, M and H quality control (QC) extractions for MPEP, MCS0455 and VU0409106 (n = 3 r, 3 replicates per run). Extractions were determined by comparing the analytical results of samples from corresponding extracts of blanks spiked with the analyte post-extraction. Data are expressed as a percentage of recovery range for each QC level.

	% Recovery range		
Quality control	**MPEP**	**MCS0455**	**VU0409106**
L	96.4 - 121.6	113.5 - 123.7	97.9 - 111.4
M	93.9 - 107.8	101.8 - 102.4	70.2 - 103.9
H	102.5 - 103.7	102.8 - 104.0	82.3 - 99.7

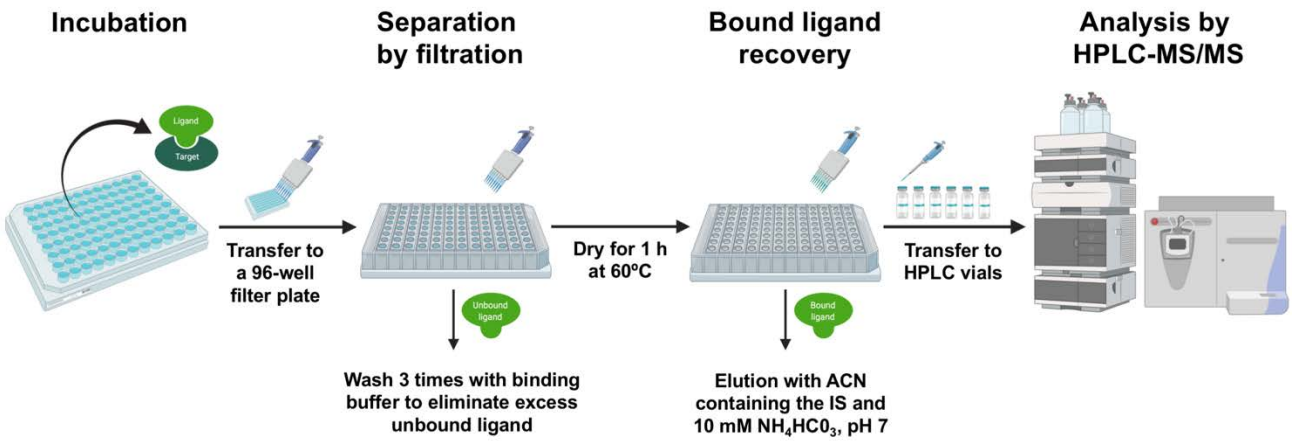


Figure 4. Flowchart of the protocol for the MS binding assay. For the saturation binding assay, $hmGlu_5$ expressing membranes (20 μg/well) were incubated with a range of concentrations of MPEP in binding buffer for 1 h at 30 °C while shaking at 150 rpm; final assay volume 300 μL/well. Then, the target-ligand complex was separated from the non-binding ligand by rapid vacuum filtration through 1 μm GF filter multi-well plate, pre-soaked for 1 h in 0.5 % PEI, with an extraction plate manifold. Samples were washed 3 times with 150 μL of ice-cold binding buffer and the filter plate was dried for 1 h at 60 ºC. The bound ligand was then recovered by filtration with acetonitrile containing the corresponding IS (3 x 100 μL/well) and 10 mM ammonium bicarbonate buffer, pH 7.0 (1 x 100 μL/well). Samples were then transferred to HPLC vials and analyzed by HPLC-MS/MS methods. Nonspecific binding was determined in the presence of 10 μM VU0409106.

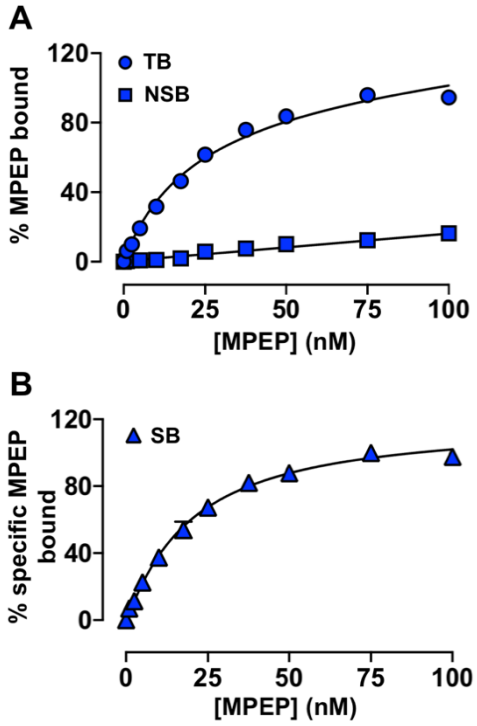


Figure 5. MPEP MS saturation binding study at $mGlu_5$. Increasing concentrations of MPEP (1 to 100 nM) were incubated with 20 μg/well of membranes prepared from $hmGlu_5$ expressing HEK cells for 1 h at 30 ºC. **(A)** Data are expressed as a percentage of bound ligand and represent the mean ± SEM of eight independent experiments performed in duplicate for total binding (TB) (○) and nonspecific binding (NSB)

(□). **(B)** Data are expressed as a percentage of bound ligand and represent the mean ± SEM of eight independent experiments performed in duplicate for specific binding (SB) (△). Nonspecific binding was determined in the presence of 10 μM VU0409106.

Analytical Bioanalytical Chemistry

Supplementary information for

Development and validation of a Mass Spectrometry binding assay for mGlu5 receptor

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Supplementary Table 1. Within- and between-run accuracy and precision of HPLC-MS/MS method to determine MCS0455 signal (n = 3 r, 6 replicates per run). Quality control (QC) samples with final concentrations of 0.125 nM (Lower limit of quantification; LLOQ), 0.35 nM (Lower; L), 10 nM (Medium; M), 20 nM (Higher; H) and 25 nM (Upper limit of quantification; ULOQ) were prepared in acetonitrile : 10 mM ammonium bicarbonate, pH 7 (3:1). The criteria for the data included accuracy (relative error, RE) and precision (relative standard deviation, RSD) within ± 20 % (except ± 25 % for the LLOQ and ULOQ).

	Within-run				Between-run			
Spiked concentration (nM)	**Technician Accuracy (RE, %)**	**HPLC-MS Accuracy (RE, %)**	**Technician Precision (RSD, %)**	**HPLC-MS Precision (RSD, %)**	**Technician Accuracy (RE, %)**	**HPLC-MS Accuracy (RE, %)**	**Technician Precision (RSD, %)**	**HPLC-MS Precision (RSD, %)**
0.125	- 16.8	- 12.8	18.7	16.6	- 7.2	- 7.6	13.5	12.4
0.35	12.7	10.9	7.2	8.0	7.1	7.4	7.5	8.5
10	- 5.6	- 4.8	3.7	1.7	- 1.2	0.1	3.4	4.2
20	- 3.7	4.1	1.1	8.8	- 0.6	2.5	2.4	6.9
25	- 5.8	- 3.2	1.8	6.7	- 4.2	- 0.7	2.0	5.4

Supplementary Table 2. Within- and between-run accuracy and precision of HPLC-MS/MS method to determine VU0409106 signal (n = 3 r, 6 replicates per run). Quality control (QC) samples with final concentrations of 0.35 nM (Lower limit of quantification; LLOQ), 1 nM (Lower; L), 10 nM (Medium; M), 20 nM (Higher; H) and 25 nM (Upper limit of quantification; ULOQ) were prepared in acetonitrile : 10 mM ammonium bicarbonate, pH 7 (3:1). The criteria for the data included accuracy (relative error, RE) and precision (relative standard deviation, RSD) within ± 20 % (except ± 25 % for the LLOQ and ULOQ).

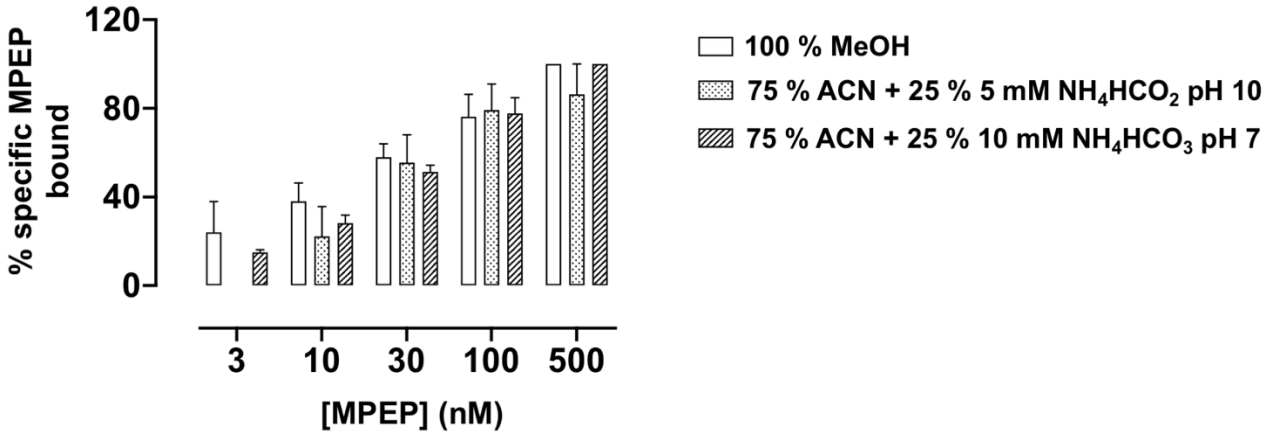
	Within-run				Between-run			
Spiked concentration (nM)	**Technician Accuracy (RE, %)**	**HPLC-MS Accuracy (RE, %)**	**Technician Precision (RSD, %)**	**HPLC-MS Precision (RSD, %)**	**Technician Accuracy (RE, %)**	**HPLC-MS Accuracy (RE, %)**	**Technician Precision (RSD, %)**	**HPLC-MS Precision (RSD, %)**
0.35	14.8	16.5	8.6	11.3	8.8	10.6	11.7	11.6
1	12.3	14.6	4.7	7.8	8.0	10.2	7.6	8.5
10	- 6.3	5.6	5.8	5.8	- 1.8	1.5	4.5	5.5
20	- 5.3	- 5.3	2.3	6.4	- 2.4	1.3	2.7	6.5
25	- 7.9	- 5.7	3.4	2.4	- 5.4	- 0.8	3.7	5.2

Supplementary Table 3. % Relative standard deviation (RSD) matrix factor of L, M and H quality controls (QCs) for MPEP, MCS0455 and VU0409106 (n = 3 r, 3 replicates per run). The matrix factor was calculated by calculating the ratio of the peak area in the presence of matrix (measured by analyzing blank matrix spiked with analyte after extraction), to the peak area in absence of matrix (analyte only solution). Data are expressed as a percentage of RSD for each QC level.

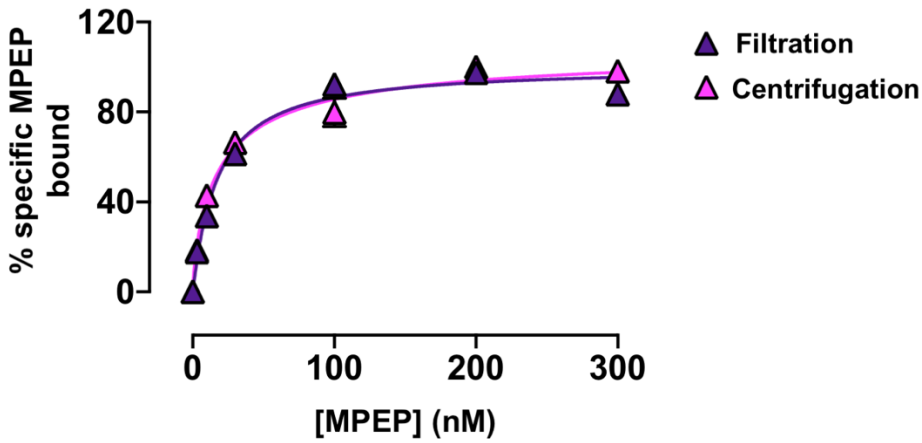
% RSD matrix factor			
Quality control	**MPEP**	**MCS0455**	**VU0409106**
L	9.4	10.7	13.1
M	4.3	2.6	8.6
H	3.4	2.6	8.8

Supplementary Table 4. Summary of [^{3}H]MPEP K_D (equilibrium dissociation constant) and B_{max} (maximum amount of binding sites) estimates as determined by [^{3}H]MPEP radioligand equilibrium saturation binding assay.

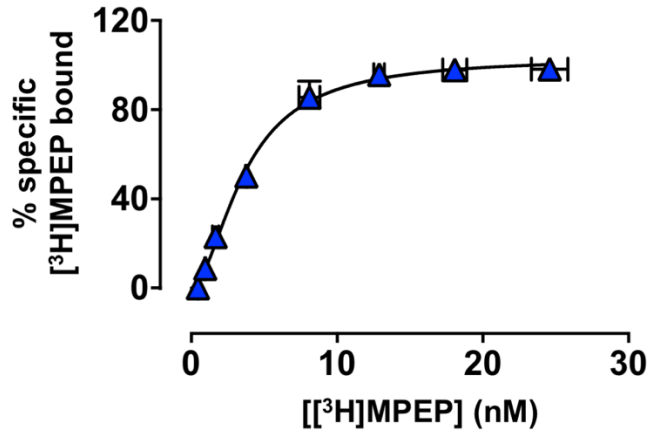
Allosteric ligand	**K_D (nM)**	**B_{max} (fmol/mg protein)**
[^{3}H]MPEP	3.65 ± 0.32	1777 ± 72.05



Supplementary Figure 1. The effect of different elution solvents on recovery of target-ligand complexes and reproducibility between experiments. Increasing concentrations of MPEP (3 to 500 nM) were incubated with $hmGlu_5$ expressing membranes (40 μg/well) for 1 h at 30 ºC. The separation of target-ligand complex from unbound ligand was performed by filtration with 96-well filter plates, pre-treated with 0.5 % PEI. The bound ligand was then recovered by elution with different solvents, including 100 % methanol, 75 % acetonitrile and 25 % ammonium formate buffer (5 mM, pH 10) or 75 % acetonitrile and 25 % ammonium bicarbonate buffer (10 mM, pH 7). Data are expressed as percentage of specific bound ligand and represent the mean ± SEM of at least three experiment performed in duplicate. Nonspecific binding was determined in the presence of 10 μM M-MPEP.



Supplementary Figure 2. The effect of filtration or centrifugation on recovery of target-ligand complexes and reproducibility between experiments. Increasing concentrations of MPEP (3 to 300 nM) were incubated with $hmGlu_5$ expressing membranes (40 μg/well) for 1 h at 30 ºC. The separation of target-ligand complex from unbound ligand was done with a 96-well filter plate, pre-treated with 0.5 % PEI. Acetonitrile was then added to each well (3 x 100 μL/well) and the bound ligand was recovered by filtration. Subsequently, 10 mM ammonium bicarbonate buffer, pH 7 was added to each well (1 x 100 μL/well) and the solution was filtered (30 s aspiration step) or centrifuged (10 min, 2000 rpm, 4ºC) before the samples were analyzed according to the HPLC-MS/MS method. Data are expressed as percentage of specific bound ligand and represent the mean ± SEM of at least two experiment performed in duplicate. Nonspecific binding was determined in the presence of 10 μM M-MPEP.



Supplementary Figure 3. [^{3}H]MPEP radioligand equilibrium saturation binding study at mGlu$_5$. 10 μg of membranes prepared from hmGlu$_5$ expressing HEK cells were incubated with increasing concentrations of [^{3}H]MPEP for 1 h at 30 ºC. Data are expressed as a percentage of specific binding and represent the mean ± SEM of three independent experiments performed in duplicate. Nonspecific binding was determined in the presence of 10 μM MPEP.