Title: VU0477573: Partial negative allosteric modulator of the subtype 5 metabotropic glutamate receptor with *in vivo* efficacy

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Abbreviations: 5MPEP, 5-methyl-2-phenylethynyl-pyridine; Br-5MPEPy, 3-(6-methylpyridin-2-ylethynyl)-5-bromopyridine; CNS, central nervous system; CRC, concentration-response curve; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethylsulfoxide; FBS, fetal bovine serum; GPCR, G protein-coupled receptor; HBSS, Hanks buffered saline solution; L-AP4, (L-(+)-2-Amino-4-phosphaonobutyric acid); M-5MPEP, 2-(3-methoxyphenylethynyl)-5-methylpyridine; mGlu, metabotropic glutamate receptor; methoxy-PEPy, 3-methoxy-5-(2-pyridinylethynyl)pyridine; MPEP, 2-methyl-6-(phenylethyl)pyridine; MTEP, 3-[(2-Methyl-1,3-

thiazol-4-yl)ethynyl]pyridine; NAM, negative allosteric modulator; PAM, positive allosteric

modulator; VU0477573, *N,N*-diethyl-5-((3-fluorophenyl)ethynyl)picolinamide.

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ABSTRACT

Negative allosteric modulators (NAMs) of metabotropic glutamate receptor subtype 5 (mGlu$_5$) have potential applications in the treatment of Fragile X syndrome, levodopa-induced dyskinesia in Parkinson's disease, Alzheimer's disease, addiction and anxiety. However, clinical and preclinical studies raise concerns that complete blockade of mGlu$_5$ and inverse agonist activity of current mGlu$_5$ NAMs contribute to adverse effects that limit therapeutic utility of these compounds. We report discovery and characterization of a novel mGlu$_5$ NAM, VU0477573, that binds to the same allosteric site as the prototypical mGlu$_5$ NAM MPEP but displays weak negative cooperativity. Due to this weak cooperativity, VU0477573 acts as a "partial NAM" so that full occupancy of the MPEP site does not completely inhibit maximal effects of mGlu$_5$ agonists on intracellular calcium mobilization, inositol phosphate (IP) accumulation, or inhibition of synaptic transmission at the hippocampal Schaffer collateral-CA1 synapse. Unlike previous mGlu$_5$ NAMs, VU0477573 displays no inverse agonist activity assessed using measures of effects on basal [^{3}H]IP accumulation. VU0477573 acts as a full NAM when measuring effects on mGlu$_5$-mediated extracellular signal-related kinases 1/2 phosphorylation, which may indicate functional bias. VU0477573 exhibits an excellent pharmacokinetic profile and good brain penetration in rodents and provides dose-dependent full mGlu$_5$ occupancy in the CNS with systemic administration. Interestingly, VU0477573 shows robust efficacy, comparable to the mGlu$_5$ NAM MTEP, in models of anxiolytic activity at doses that provide full CNS occupancy of mGlu$_5$ and demonstrate an excellent CNS occupancy/efficacy relationship. VU0477573 provides an exciting new tool to investigate the efficacy of partial NAMs in animal models.

INTRODUCTION

Glutamate signaling in the CNS is mediated by activation of both ionotropic and metabotropic glutamate receptors. Metabotropic glutamate (mGlu) receptors are G protein-coupled receptors (GPCRs) that belong to the GPCR class C/family 3 group. The mGlu receptor family contains eight members: $mGlu_1$- $mGlu_8$ (Niswender and Conn, 2010). The receptors are grouped according to their structure, signaling partners and pharmacology. Group I receptors ($mGlu_1$, $mGlu_5$) signal through the G protein $G_{q/11}$ and mediate inositol phosphate (IP_3)/calcium (Ca^{2+}) signal transduction (Abe et al., 1992). Group I receptors and activate additional signaling pathways, including extracellular signal-related kinases (Mao et al., 2005). $mGlu_5$ receptors are widely expressed in the CNS and also expressed in the periphery (Julio-Pieper et al., 2011). In neurons, $mGlu_5$ is primarily expressed postsynaptically, modulating cell excitability and post-synaptic efficacy (Conn and Pin, 1997). Within the CNS, $mGlu_5$ receptor expression levels are high in the hippocampus, nucleus accumbens, striatum, globus pallidus, and substantia nigra pars reticulata (Ferraguti and Shigemoto, 2006). CNS therapeutic indications for which $mGlu_5$ receptors are potential targets include Parkinson's disease, Fragile X syndrome/autism spectrum disorders, schizophrenia, Alzheimer's disease, addiction, depression, anxiety and pain (Nickols and Conn, 2014). In addition, $mGlu_5$ NAMs have potential in the treatment of gastroesophageal reflux disease (GERD) (Niswender and Conn, 2010; Zerbib et al., 2011).

GPCRs contain seven transmembrane regions, with class C GPCRs possessing a large extracellular *N*-terminal region that contains the endogenous ligand (orthosteric) binding site. Receptor subgroups show abundant sequence homology at the orthosteric binding site, making the synthesis of subtype selective agents challenging. Allosteric ligands bind to a topographically distinct site within the seven transmembrane-spanning domains, which contains a higher level of

sequence diversity between receptor subtypes as compared to the orthosteric site (Conn et al., 2009). This sequence diversity allows allosteric ligands to exhibit greater subtype selectivity than can be achieved with orthosteric ligands of mGlu receptors (Christopoulos and Kenakin, 2002; Conn et al., 2009). Allosteric modulators act in concert with the endogenous orthosteric ligand by changing the affinity and/or efficacy of the orthosteric ligand. The alteration of the orthosteric ligand affinity and/or efficacy is defined as cooperativity. Positive allosteric modulators (PAMs) display positive cooperativity and potentiate responses to orthosteric agonists, whereas negative allosteric modulators (NAMs) display negative cooperativity and block orthosteric agonist responses. In addition, neutral allosteric ligands (NAL) bind to the allosteric site and show neutral cooperativity with orthosteric agonists (Christopoulos, 2014). In traditional allosteric models, the cooperativity of allosteric ligands is a saturable effect, with allosteric ligands acting in the presence of the orthosteric agonist, thereby preserving the temporal and spatial activation of the receptor.

The prototypical mGlu$_5$ NAM is MPEP, with others including MTEP and fenobam (Gasparini et al., 1999; Gasparini et al., 2002; Cosford et al., 2003b). Preclinical and clinical studies suggest that mGlu$_5$ NAMs have efficacy in animal models of anxiety disorders (Porter et al., 2005; Swanson et al., 2005; Felts et al., 2013), Fragile X syndrome (Bear et al., 2004; Gasparini et al., 2008), Parkinson's disease (Marino et al., 2002; Marino and Conn, 2002), Parkinson's disease L-DOPA-induced dyskinesia (Dekundy et al., 2006; Mela et al., 2007; Levandis et al., 2008; Rylander et al., 2009; Morin et al., 2010; Rylander et al., 2010; Morin et al., 2013), depression (Pecknold et al., 1982; Porter et al., 2005; Li et al., 2006; Chaki et al., 2013; Hughes et al., 2013; Pilc et al., 2013), neuroprotection (Bruno et al., 2000; Bao et al., 2001), addictive disorders (Bird and Lawrence, 2009; Lindsley et al., 2011; Achat-Mendes et al.,

2012; Amato et al., 2013), and GERD (Frisby et al., 2005; Jensen et al., 2005). mGlu$_5$ NAMs

have reached or are now in active clinical studies to evaluate clinical efficacy for these disorders

(Campbell et al., 2004; McGeehan et al., 2004; Pietraszek et al., 2004; Rodriguez et al., 2010;

Zerbib et al., 2011). Despite these encouraging results, both clinical and preclinical studies

suggest that mGlu$_5$ NAMs may induce on-target adverse effects, including cognitive and

memory impairments and psychotomimetic effects (Campbell et al., 2004; Porter et al., 2005;

Rodriguez et al., 2010; Abou Farha et al., 2014). This raises the question whether complete

blockade of mGlu$_5$ may contribute to these deleterious effects thereby limiting the therapeutic

utility of mGlu$_5$ NAMs.

The degree of cooperativity of allosteric modulators varies from strong positive

cooperativity (PAMs) to strong negative cooperativity (NAMs) with a range of values between

these extremes (Conn et al., 2009; Sams et al., 2011; Nickols and Conn, 2014; Gregory KJ,

2015). Of note, we and others previously reported discovery of mGlu$_5$ neutral allosteric ligands

(NALs) that have no measurable cooperativity with glutamate in the in vitro assays used for

compound assessment (O'Brien et al., 2003; Rodriguez et al., 2005; Hammond et al., 2010; Sams

et al., 2011; Gregory and Conn, 2015; Gregory KJ, 2015). In addition, mGlu$_5$ "partial NAMs"

have also been identified that display weak cooperativity, only partially blocking maximal

mGlu$_5$-mediated responses at concentrations that fully occupy the allosteric site on mGlu$_5$

(Rodriguez et al., 2005; Felts et al., 2010; Bradley et al., 2011; Gregory et al., 2012). This

provides a potential breakthrough and raises the possibility that partial NAMs could be

developed that provide *in vivo* efficacy without completely inhibiting mGlu$_5$ function. However,

previous mGlu$_5$ partial NAMs had relatively low potencies relative to MPEP and other full

NAMs and were not highly optimized for use in *in vivo* studies (Rodriguez et al., 2005; Gregory

et al., 2012). We now report optimization of mGlu$_5$ NAMs belonging to a picolinamide biaryl acetylene series and discovery of VU0477573 as a novel mGlu$_5$ selective partial NAM that displays high affinity and induces a partial inhibition of the maximal response of mGlu$_5$ to glutamate. VU0477573 has an excellent *in vivo* pharmacokinetic profile, achieves high brain exposure, and systemic administration of VU0477573 provides full CNS occupancy of the receptor *in vivo* and corresponding efficacy in a model of anxiolytic activity known to be responsive to prototypical mGlu$_5$ NAMs. These results confirm the potential utility of partial GPCR NAMs as a novel approach to reducing receptor signaling and achieving *in vivo* efficacy without completely eliminating receptor signaling.

MATERIALS AND METHODS

Reagents. Ammonium formate was purchased from Fisher Scientific (Fair Lawn, NJ).

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and antibiotics were

obtained from Life Technologies (Carlsbad, CA). MPEP (Gasparini et al., 1999), MTEP

(Cosford et al., 2003b), and VU0477573 (Jaeschke, Oct. 25, 2012) were synthesized in-house.

[^{3}H]myo-inositol was obtained from Perkin-Elmer (Boston, MA). L-glutamic acid was obtained

from Tocris Bioscience (Ellisville, MO). [^{3}H]-methoxyPEPy (76.3Ci/mmol) was custom

synthesized by PerkinElmer Life and Analytical Sciences (Waltham, MA). All other reagents

were purchased from Sigma-Aldrich (St. Louis, MO) and were analytical grade.

Cell Culture. HEK293 cell lines were maintained in complete DMEM supplemented with 10%

FBS, 2 mM glutamine, 20 mM HEPES, 0.1 mM nonessential amino acids, 1mM sodium

pyruvate, and antibiotic/antimycotic (Life Technolgies ,Carlsbad, CA), and G418 (500ug/mL;

Mediatech, Manassas, VA) at 37 ℃ in 5% CO_2 in a humidified cell incubator. Cell lines utilized

include HEK293A cells expressing low levels of rat $mGlu_5$ (HEK293A-rat-$mGlu_5$-low cells).

The cell lines used for selectivity studies are all HEK293A cells. Those used in the calcium

release assays include tREX human $mGlu_1$ (induced overnight with 50ng/mL tetracycline) and

the HEK293A-rat-$mGlu_5$-low cells. Additional cell lines used in selectivity studies express G

protein-Inwardly Rectifying Potassium (GIRK) channels. These include rat $mGlu_2$ GIRK, rat

$mGlu_3$ GIRK, rat $mGlu_4$ GIRK, human $mGlu_6$ GIRK, rat $mGlu_7$ GIRK (L-AP4 was used as the

agonist in $mGlu_7$ studies; all other selectivity profiling used glutamate as the orthosteric agonist),

rat $mGlu_8$ GIRK (Niswender et al., 2008). The HA-tagged *N*-terminally truncated rat $mGlu_5$ cell line was utilized for [^{3}H]IP accumulation studies (Goudet et al., 2004; Noetzel et al., 2013).

Rat cortical astrocytes. Rat cortical astrocytes from Lonza (Basel, Switzerland) were received, stored in liquid nitrogen, and thawed according to manufacturer's instructions. The astrocytes were plated on BD Falcon Primaria dishes in assay growth media (AGM) from Lonza, which consists of the assay basal media plus AGM SingleQuot supplements from Lonza. Approximately 4 hr after plating, the cells were fed with AGM according to the protocol and every 3 days until the cells reached confluent growth. The cells were plated in 96-well poly-D-lysine coated plates in 100μL of the AGM. Each well contained approximately 5×10^4 cells. The following day, the media was supplemented with G5 at a dilution of 1:100 in AGM. On the subsequent day, the fluorescence-based calcium assay was performed using the compound dilutions and conditions described for the HEK293A-rat-$mGlu_5$-low cells.

Fluorescence-Based Calcium Assays. Mobilization of intracellular calcium in response to drug treatment was assessed in HEK293A-rat-$mGlu_5$-low cells. Approximately 24 hr prior to the assay, cells were plated at a density of 50,000 cells/well in poly-D-lysine-coated clear-bottom, black-walled 96 well plates in assay buffer (DMEM with 10% dialyzed fetal bovine serum, 20 mM HEPES, and 1 mM sodium pyruvate). Cells were grown at 37 ℃ and 5% CO_2 overnight. The day of the assay, Fluo4-AM dye (Invitrogen, Carlsbad, CA) was used as a cell-permeant calcium indicator dye to assess receptor-mediated intracellular calcium mobilization on the Flexstation II microplate reader (Molecular Devices, Sunnyvale, CA). Test compounds were diluted into DMSO, for a final DMSO concentration of 0.3% at 3X concentration. Compounds

were assayed against an EC_{80} concentration of glutamate (350-400 nM). Compound alone was

added at 20 sec followed by an EC_{80} concentration of glutamate added at 80 sec to detect NAM

activity with a total read time of 120 sec. A 5-point smoothing function was applied to the raw

calcium fluorescent traces. The basal fluorescence of each well was assessed during the first 20

sec of the assay. The peak increase in fluorescence over basal was calculated, and the values

normalized to the maximal peak response due to glutamate stimulation (Gregory et al., 2012;

Noetzel et al., 2013).

For fold-shift assays, calcium flux was measured using the Functional Drug Screening

System (FDSS 6000; Hamamatsu Corporation, Bridgewater, NJ.). Cells were plated at 15,000

cells/well in black-walled poly(D)lysine-coated, 384-well plates (Grenier Bio-One, Monroe, NC)

in assay medium. Calcium assay buffer was utilized in the experiment (HBSS, Invitrogen; HBSS

containing 2.5 mM probenecid and 20 mM HEPES). On the day of the assay, cells were washed

on a BioTek ELx 405 plate washer (Bioteck, Winooski, VT) and the cells were incubated with

1 µL Fluo-4 AM prepared as a 2.3 mM stock in DMSO and mixed 1:1 with 10% (w/v) Pluronic

F-127 and diluted in assay buffer for one hour at 37 ºC and 5% CO_2. Unincorporated dye was

removed, cells washed on the BioTek ELx washer, and 20 µL of assay buffer was added to each

well. Compounds were diluted to 2X in assay buffer. After 10 min, cells were pre-incubated with

test compounds for 5 min and stimulated with glutamate for 2 min. Data were collected at 0.25

Hz during the pre-incubation and at 1 Hz during the glutamate addition. Raw data were

normalized as follows: based on the initial readings in the well, both cell number and non-

uniform illumination/imaging were controlled; signal amplitude using the data point immediately

preceding the addition of agonist was subtracted from each point on the trace and data were

normalized to the maximal response for each experiment. Concentration response curves were created using the Prism 5.0 (GraphPad Software Inc., San Diego, CA).

Selectivity Profiling

mGlu$_1$. The HEK293A tREX human mGlu$_1$ cell line was used to assess the activity of compounds against mGlu$_1$. Fluorescence-based calcium assays were performed on the FDSS as described above for the fold-shift protocol. Cells were plated and induced overnight with 50ng/mL tetracycline. Screening was performed against a concentration response curve (CRC) of glutamate. Either vehicle or a 10 µM final concentration of compound was added and followed 140 sec later by a CRC of glutamate.

Group II and Group III mGlus. In order to assess the effect of test compounds at the group II and group II mGlu receptors, a thallium flux assay was utilized on the FDSS as previously described (Niswender et al., 2008). Briefly, cells were plated at 15,000 cells/well in black-walled poly(D)lysine-coated, 384-well plates (Grenier Bio-One, Monroe, NC) in assay medium. FluoZin-2 AM dye (Invitrogen, Carlsbad, CA) was used as a cell-permeant indicator dye to assess receptor-mediated thallium flux on the FDSS. Screening was performed against a CRC of glutamate or (L-AP4 for mGlu$_7$) diluted in thallium buffer (125 mM NaHCO$_3$, 1 mM MgSO$_4$, 1.8 mM CaSO$_4$, 5 mM glucose, 12 mM Tl$_2$SO$_4$, 10 mM HEPES). Either vehicle or a 10 µM final concentration of compound was added and followed 140 sec later by a CRC of agonist. Fluorescence was measured using an FDSS 6000 and data were analyzed as described previously (Niswender et al., 2008).

Radioligand Binding. Membranes were prepared from HEK293A-rat-mGlu$_5$-low cells as follows. Cells were placed on ice and washed twice with ice-cold phosphate-buffered saline and harvested by scraping into ice-cold homogenization buffer (50 mM Tris-HCl, 10 mM EDTA, 0.9% NaCl, pH 7.4). Cells were homogenized using a Tekmar TP-18/10S1 homogenizer (Teledyne, Tekmar, Cincinnati, OH) using three 10 sec bursts, interspersed with 30 sec incubations on ice. The cell fractions were separated by centrifugation at 1000 xg for 10 min at 4 ℃. The supernatant was subsequently centrifuged at 30,000 xg for one hour at 4 ℃. The pellet was resuspended in ice-cold binding buffer (50 mM Tris-HCl, 0.9% NaCl, pH 7.4). For competition binding, 50μg/well of membranes were incubated with shaking for one hour at room temperature with ~ 3nM of [^{3}H]-methoxyPEPy and a range of concentrations of test ligand in binding buffer. 10 μM MPEP was used to determine nonspecific binding. The assay was terminated by rapid filtration through GF/B Unifilter plates (Perkin Elmer Life and Analytical Sciences) using a Brandel 96-well plate harvester (Brandel, Inc., Gaithersburg, MD) with three washes using ice-cold binding buffer to separate bound from free radioligand. Plates were dried overnight at room temperature. Microscint20 scintillation fluid was added at 40 μL per well to the plate (PerkinElmer Life and Analytical Sciences) and the radioactivity was counted on a TopCount scintillation counter (PerkinElmer Life and Analytical Sciences) following a 2 hr incubation period at room temperature. Concentration-response curves were generated with a four-parameter logistical equation in GraphPad Prism5.

ERK1/2 Phosphorylation Assay. Receptor-mediated extracellular signal-regulated kinases 1 and 2 (ERK1/2) phosphorylation was assessed using the AlphaScreen SureFire phosphoERK1/2 assay kit (PerkinElmer, Waltham, MA). HEK293A-rat-mGlu$_5$-low cells were plated on poly-D-

lysine-coated, clear 96-well plates at a density of 40,000 cells/well, in assay media, 16 to 24 hr before assay. The next day, media was aspirated and cells washed once with serum/glutamate-free (SF) media by gentle flicking and adding 100 µL/well of SF media followed by gentle flicking again. 100 µl /well of SF media was added to the cells and starved for 5-6 hr at 37 ℃ prior to the assay. After the incubation period, 50 µL/well of fresh SF media was added to the cells and equilibrated at RT for 15 to 20 min followed by pre-treating the cells firstly with 40 µL/well of 2X NAMs (compounds) in SF media for 1 min and secondly with 10 µL10X agonist (glutamate) or FBS (positive control) in SF media for 7 min. Immediately after the agonist treatment, the assay was stopped by removal of media by flicking the plate and 40 µL/well of 1X lysis buffer (from kit) was added to the cells and kept on a shaker for 10 min. Cell lysates were stored for 1 to 4 days at -20 ℃ prior to carry out the detection assay using the AlphaScreen SureFire phosphoERK1/2 assay kit. After thawing with shaking for 10 min, 4µL of lysate was transferred to a white 384-well plate. (Costar; Corning Life Sciences, Tewksbury, MA). Under very dim light, 7 µL of the reaction buffer mix was added to each well. The mixture was incubated at 37 ℃ for 90 min in the dark. The AlphaScreen signal was detected using an H4 synergy reader (BioTek Instruments, Winooski, VT) with standard AlphaScreen settings. The data are expressed a fold increase over the basal level of detected phosphoERK1/2.

Inositol Phosphate Accumulation. We used anion-exchange chromatography to assess either [^{3}H]inositol phosphate (IP) accumulation in HEK293A-rat-mGlu$_5$-low cells or to assess the basal level of [^{3}H]inositol phosphate (IP) accumulation to determine inverse agonist activity in HEK293 cells stably expressing HA-tagged N-terminally truncated rat mGlu$_5$ (Goudet et al., 2004; Noetzel et al., 2013). Cells were seeded at a density of 100,000 cells/mL/well on poly-D-

lysine coated 24 well plates (Fisher Scientific Co LLC, Suwanee GA) in the presence of 1µCi of

[^{3}H]myo-inositol per well. Cell plates were incubated at 37 °C, 5% CO_2 for approximately 24 hr

for HEK293A-rat-mGlu$_5$-low cells and for approximately 48 h for HEK293A cells stably

expressing HA-tagged N-terminally truncated rat mGlu$_5$. On the day of the assay the media was

aspirated from the cells and 1 mL of 10 µM compound (1X) or dimethylsulfoxide (DMSO)

matched vehicle in PI assay buffer (10X HBSS, 20 mM HEPES, 4.16 mM NaHCO$_3$, 30 mM

LiCl) was added to the cells and incubated at 37 °C, 5% CO_2 for 1 hr. After compound treatment,

buffer was removed from cells and reaction stopped with 1 mL of 10 mM formic acid (prepared

fresh) per well. Cells were incubated in formic acid at room temperature (RT) for 40 min and

stored overnight at 4 °C. IP accumulation was analyzed by anion-exchange chromatography as

follows. Cell lysates were transferred to freshly prepared AG 1-X8 anion-exchange resin-packed

(100g resin/400 mL of H$_2$0) 10 mL poly-prep® chromatography columns (Bio-Rad Laboratories,

Hercules, CA). The columns were washed with 9 mL of water, 9 mL of 5 mM myo-inositol, and

9 mL of water. [^{3}H]IP was eluted with 8 mL of PI eluent (200 mM ammonium formate, 100 mM

formic acid) into 20 mL scintillation vials (RPI, Mt. Prospect, ILL). Scintillation fluid (14 mL of

Ecoscint™ Ultra, National Diagnostics, Atlanta, GA) was added to each tube and mixed by

vigorous shaking for 30 sec. The vials were counted on a Packard Instruments Tri-Carb 2900TR

Liquid Scintillation Analyzer. The portion of the protocol including column preparation, washes,

and elution averaged approximately 4.5 hr (for 100 columns). Columns were regenerated with PI

regeneration solution (1M ammonium formate, 0.1 M formic acid) and followed with two water

washes. Columns were stored at 4 °C and used up to four times.

Extracellular Field Potential Recordings from Brain Slices. All animal studies and

experiment procedures were approved by the Vanderbilt University Institutional Animal Care

and Use Committee in accordance with the National Institutes of Health's Guide for the Care and

Use of Laboratory Animals. Transverse hippocampal slices were prepared from adult (7-11

weeks old) male C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME). In brief, mice were

anesthetized with isoflurane, decapitated and the brains quickly removed from the skull and

submerged in oxygenated (95% O_2/5% CO_2), ice-cold cutting solution (in mM: 210 sucrose, 3

KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 10 D-glucose, 0.5 $CaCl_2$, 8 $MgCl_2$). Transverse brain slices

(400 µm) containing the hippocampus were cut using a Leica VT1200S microtome (Leica

Microsystems Inc.), incubated in oxygenated artificial cerebrospinal fluid (ACSF) for 30 min at

32°C, and then maintained at room temperature for at least 30 min until transferred to a

submersion recording chamber. The chamber was continuously perfused with oxygenated ACSF

at a flow rate of 2 ml/min. The ACSF contained (in mM): 126 NaCl, 2.5 KCl, 2.0 $CaCl_2$, 1.3

$MgSO_4$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, and 10 D-glucose. A concentric bipolar-stimulating

electrode was placed in the stratum radiatum near the CA3-CA1 border in order to stimulate the

Schaffer collaterals. Recording electrodes were fabricated from borosilicate glass (Sutter

Instruments, Novato, CA) using a Narishige micropipette puller (PP-830, Narishige, Japan),

filled with ACSF, and placed in the stratum radiatum of area CA1. Extracellular field excitatory

postsynaptic potentials (fEPSPs) were evoked every 20 sec, and acquired using a MultiClamp

700B amplifier (Molecular Devices, Sunnyvale, CA) and pClamp 10.4 software (Molecular

Devices). Input–output curves were generated to determine the stimulus intensity that produced

50-60% of the maximum fEPSP slope before each experiment. This submaximal fEPSP slope

was used as a baseline for the remainder of the individual experiment. Stock solutions of mGlu

compounds were made using DMSO and diluted to the appropriate concentrations in ACSF with

the final DMSO concentration of 0.1%. All drugs were bath applied. Three sequential fEPSP

slopes were averaged. The time courses were normalized to the average slope calculated during

the predrug period (percent of baseline). Data were analyzed offline using Clampfit 10.4

(Molecular Devices) and GraphPad Prism 5.0 (GraphPad Software), and presented as mean ±

SEM.

Animals. CD-1 mice (Harlan Laboratories, Indianapolis, IN) were used in pharmacokinetic, *in

vivo* occupancy, and behavioral studies. All animals were group housed (4 animals per cage), in

polycarbonate cages with corn cob bedding in a colony room maintained at 21 ± 2 °C with 45 ±

10% relative humidity on a 12-h light/12-h dark cycle (lights on at 0600 h CST). Standard

rodent chow (Purina Mills, Richmond, Indiana) and water were provided *ad libitum*. Animals

ranged in weight from 30-60 g. This study was carried out in accordance with the Institutional

Animal Care and Use Committee of Vanderbilt University and the guidelines of the Committee

on Care and Use of Laboratory Animal Resources, as adopted and promulgated by the U.S.

National Institutes of Health.

***In Vivo* Pharmacokinetic Study**. Compound was formulated in 10% tween 80 in water and CD-

1 male mice (30-40 g) were administered VU0477573 intraperitoneally (i.p.). For the dose-

exposure study, mice were dosed 1, 3, 10, and 30 mg/kg of VU0477573 (10 ml/kg, i.p.) and

samples obtained at 15 and 45 min. For the time-course study, mice were dosed with 10 mg/kg

of VU0477573 i.p. and samples obtained at 15, 45, 60, 180, and 360 min. At the indicated time

points, mice were deeply anesthetized with isoflurane, rapidly decapitated, followed by

collection of trunk blood and whole brain. The brains were washed thoroughly in cold PBS and frozen immediately on dry ice. Plasma was separated by centrifugation (4000 rcf, 4 °C) and stored at -80 °C until analysis. On the day of analysis, frozen whole-mouse brains were weighed and diluted with 1:3 (w/w) parts of 70:30 isopropanol:water. The mixture was then subjected to mechanical homogenation employing a Mini-Beadbeater™ and 1.0 mm Zirconia/Silica Beads (BioSpec Products) followed by centrifugation. The sample extraction of plasma (20 μL) or brain homogenate (20 μL) was performed by a method based on protein precipitation using three volumes of acetonitrile containing an internal standard (50 ng/mL carbamazepine). The samples were centrifuged (3000 rcf, 5 min) and supernatants transferred and diluted 1:1 (supernatant: water) into a new 96 well plate, which was then sealed in preparation for LC/MS/MS analysis.

LC/MS/MS Bioanalysis of Samples from *In Vivo* Studies.

The *in vivo* exposure study was performed as a satellite study to the marble burying behavioral assay. All *in vivo* samples were analyzed via electrospray ionization (ESI) on an AB Sciex API-5500 QTrap (Foster City, CA) triple-quadrupole instrument that was coupled with Shimadzu LC-20AD pumps (Columbia, MD) and a Leap Technologies CTC PAL auto-sampler (Carrboro, NC). Analytes were separated by gradient elution using a Fortis C18 3.0 x 50 mm, 3 µm column (Fortis Technologies Ltd, Cheshire, UK) thermostated at 40 °C. HPLC mobile phase A was 0.1% formic acid in water, mobile phase B was 0.1% formic acid in acetonitrile. The gradient started at 10% 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 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). The calibration curves were constructed, and linear response was obtained in the range of 5 to 10,000 ng/mL, by spiking known amounts of VU0477573 in blank plasma. All data were analyzed using AB Sciex Analyst software v1.5.1.

Marble Burying Assay. Harlan CD-1 male mice weighing between 30 to 35 g were administered drug or vehicle (10% Tween80) i.p. 15 min prior to the start of the assay. Test sessions were performed between 10:00 AM and 4:00 PM. All dose groups consisted of 5 to 8 mice. Eight small Plexiglass cages (32 x 17 x 14 cm) were arranged in two rows of four cages on top of a large round table. Mice were transported from the colony room to the testing room and were allowed to habituate for 30 min. Mice were pretreated with a dose of MTEP or VU0477573 at 15 min prior to the initiation of the assay. Mice were treated with increasing doses of VU0477573 as compared to vehicle alone and 15 mg/kg MTEP. The mice were placed individually into cages in which 12 black glass marbles that are 14 mm in diameter were evenly spaced: 6.4 cm vertically and 4.25 cm horizontally from each other and from the walls of the cage. The marbles were placed on top of 2.5cm on Diamond Soft Bedding (Harlan Teklad, Madison, WI). The compound and comparator were evaluated in a counterbalanced design, in which all doses of compounds were tested in each session. Mice receiving the same dose were placed in cages on opposite sides of the table to control for context and lighting variations. Perforated clear plastic lids were placed on top of each cage. The amount of marble burying was recorded over a 30 min interval. The mice were removed from the cages, and the number of marbles buried was counted. A marble was considered buried if greater than two thirds of the

marble was covered by bedding. The sessions were videotaped using a Sony camcorder equipped with a Sony wide-angle lens mounted on a 1.5m tripod (Sony, Tokyo, Japan). Dose-response studies were analyzed by a between-group analysis of variance. If there was a main effect of the dose, each dose group was compared with the vehicle control group using a Dunnett's comparison. The calculations were performed using JMP Pro 10 (SAS Institute, Cary, NC) statistical software and graphed using SigmaPlot 12.0 (Systat Software, San Jose, CA)

In Vivo **Occupancy Study.** The $mGlu_5$ partial NAM VU0477573 was evaluated for receptor occupancy utilizing the method outlined in detail by Anderson et al. (Anderson et al., 2003). Briefly, CD-1 mice (40-60 grams) were administered VU0477573 (30 min, 10% Tween 80, i.p., 10 mL/kg). Each treatment group included 6-12 animals and the compound pretreatment time was 30 min. One min prior to tissue collection, [^{3}H]-methoxy-PEPy was administered (30 µCi/kg; 1 mL/kg injection volume in isotonic saline) through a lateral tail vein. Animals were then euthanized and bilateral hippocampus and cerebellum were rapidly dissected on a cooled dissecting tray. The bilateral hippocampus and cerebellum were immediately weighed and homogenized in 10 volumes of ice-cold buffer (10 mM potassium phosphate, 100 mM KCl, pH 7.4) using a Polytron. Homogenates (400 µL) were then filtered over GF/B membrane filters (Whatman) and washed twice with 5 mL ice-cold homogenization buffer to separate membrane bound from free radioactivity. Filters were then counted for radioactivity using a Beckman counter. The hippocampus was utilized since it is a region with high density of $mGlu_5$ receptors, while cerebellum was used as a reference region because it has a low density of $mGlu_5$ receptors. Nonspecific *in vivo* binding of [^{3}H]-methoxy-PEPy was estimated by measuring radioactivity in washed filters following administration of a saturating dose of MTEP (50 mg/kg i.p.).

RESULTS

Discovery of the mGlu$_5$ Partial NAM VU0477573. Our group has previously reported

the discovery of mGlu$_5$ NAMs that show weak negative cooperativity, including both Br-MPEPy

and M-5MPEP (Rodriguez et al., 2005). In addition, compounds with a similar structure,

including 5-MPEP and ML353, act as a neutral allosteric ligands (NALs) at mGlu$_5$ (Rodriguez et

al., 2005; Gregory and Conn, 2015). We searched our available mGlu$_5$ allosteric antagonists to

identify novel compounds with weak negative cooperativity. In screening compounds within the

picolinamide acetylene class series (Stauffer, 2011; Bridges et al., 2013; Turlington et al., 2013;

Gregory KJ, 2015), we noted that the mGlu$_5$ NAM VU0477573 (C_{18},H_{17},FN_2O; molecular

weight = 296.34) showed limited negative cooperativity in inhibiting the glutamate EC_{80}-induced

intracellular calcium release in HEK293A cells stably expressing rat mGlu$_5$ (HEK293A-rat-

mGlu$_5$-low). The structure of VU0477573 is shown in comparison to the prototypical biaryl

acetylene scaffold of the mGlu$_5$ NAMs MPEP and MTEP (Figure 1A) (Gasparini et al., 1999;

Gasparini et al., 2001; Cosford et al., 2003a; Cosford et al., 2003b).

VU0477573 shows approximately 80% inhibition of the response to an EC_{80}

concentration of glutamate *in vitro* in inducing intracellular calcium mobilization, whereas

MPEP and MTEP completely inhibit the response to an EC_{80} glutamate concentration (Figure

1B, Table 1). Therefore, the percent glutamate maximal response is reduced to approximately

20% by VU0477573. The high potency of the compound (32 nM) is similar to that of both

MPEP (13 nM) and MTEP (52 nM). To test the mode of action of VU0477573, we examined the

effect of a fixed concentration of compound in HEK293A-rat-mGlu$_5$-low cells in the presence of

increasing concentrations of glutamate. In the presence of increasing concentrations of

VU0477573, the glutamate concentration response curve shifts to the right along with a decrease

in the maximal effect of glutamate that reaches a limit, consistent with a noncompetitive

allosteric antagonist (Figure 1C). Even at a concentration of 10 μM, a concentration

approximately 300 fold over the in vitro IC_{50}, VU0477573 did not result in full blockade of the

glutamate response, as compared to treatment with 10 μM MPEP.

The percent inhibition of a weakly cooperative NAM depends on the concentration of

orthosteric agonist used in the assay. In the fold shift assay (Figure 1C), 10 μM VU0477573

inhibits an approximate $EC_{90} – EC_{95}$ concentration of glutamate (1 μM) by approximately 50%

and completely inhibits the response to an EC_{50-60} concentration of glutamate (0.3 μM) (Figure

1C). In the CRC assay, which utilizes an EC_{80} value of glutamate, we see an 80% inhibition by

VU0477573 of the EC_{80} response (Figure 1B). Therefore, the percent glutamate maximal

response is reduced to approximately 20% by VU0477573. In the NAM fold shift assay, an EC_{80}

of glutamate would be between 0.3-1 μM. Therefore, there is a good correlation observed

between the NAM CRC (Figure 1B) and fold shift assays (Figure 1C) for the partial inhibition by

VU0477573 of glutamate-mediated intracellular calcium release. The failure of VU0477573 to

fully block the glutamate response is consistent with weak, saturable negative cooperativity, or

"partial NAM" activity, for $mGlu_5$ in this assay.

To be useful as an *in vivo* tool for $mGlu_5$ behavioral assays, compounds need to show

$mGlu_5$ subtype selectivity among the metabotropic glutamate receptors. We assessed the

selectivity profile of VU0477573 using stable cell lines individually expressing the other

metabotropic glutamate receptor subtypes. We examined the effect of 10 μM of VU0477573

against an agonist concentration-response curve. Individual cell lines were assessed for either

glutamate- (or, for $mGlu_7$, L-AP4)-mediated intracellular calcium release ($mGlu_1$) or thallium

flux (mGlu$_{2, 3, 4, 6, 7, 8}$), as indicated. We observed that VU0477573 shows no activity against the other mGlu receptor subtypes, indicating that it is functionally selective for mGlu$_5$ (Figure 1D).

VU0477573 Binds to the MPEP Site. The majority of mGlu$_5$ allosteric modulators discovered to date bind to the MPEP site of mGlu$_5$. There is growing evidence that allosteric modulators are able to bind to allosteric sites distinct from the MPEP site. A small subset of mGlu$_5$ PAMs, including CPPHA (O'Brien et al., 2004; Zhao et al., 2007; Chen et al., 2008), VU0357121 (Hammond et al., 2010), VU0028316 (Rodriguez et al., 2010), and NCFP (Zhao et al., 2007; Noetzel et al., 2013), interact noncompetitively with the MPEP site. In addition, the mGlu$_5$ partial NAM VU0029251 reduced maximal [^{3}H]-methoxyPEPy binding by only approximately 50% (Rodriguez et al., 2010). To determine whether VU0477573 binds to the prototypical MPEP allosteric binding site, we performed inhibition binding studies. Using the allosteric antagonist [^{3}H]-methoxyPEPy, known to bind to the MPEP site, we demonstrate that VU0477573 fully displaces the radioligand, consistent with a competitive interaction with the MPEP site (Figure 2A, Table 1).

VU0477573 shows Partial NAM activity at mGlu$_5$ in measures of IP accumulation. To assess the negative allosteric activity of VU0477573 in an endpoint assay, where the duration of incubation with agonist allows the agonist to come to equilibrium, we examined glutamate-induced IP accumulation in the HEK293A-rat-mGlu$_5$-low cells. Similar to intracellular calcium release, increasing concentrations of VU0477573 caused the glutamate CRC to shift to the right along with a decrease in the maximal effect of glutamate. We again observed that this effect reached a limit, consistent with VU0477573 acting as a noncompetitive allosteric antagonist

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(Figure 3). Even at a maximal concentration of 10 μM, VU0477573 did not result in full

blockade of the glutamate-induced IP accumulation, as compared to treatment with 10 μM

MPEP. This assay provides additional supportive data that VU0477573 does not fully block the

maximal glutamate response in multiple in vitro assays of receptor activity, consistent with weak

negative cooperativity, or "partial NAM" activity, for mGlu$_5$.

VU0477573 is an mGlu$_5$ Partial NAM in Rat Cortical Astrocytes. To verify that the partial

negative allosteric activity observed in HEK293A-rat-mGlu$_5$-low cells is also observed in a

native system, we evaluated the activity of VU0477573 in rat cortical astrocytes. Cortical

astrocytes endogenously express mGlu$_5$ without the expression of other mGlu subtypes and serve

as an excellent *in vitro* native experimental system (Peavy et al., 2001; Peavy et al., 2002). In

addition, the astrocyte level of mGlu$_5$ expression is similar to that of HEK293A-rat-mGlu$_5$-low

cells (Noetzel et al., 2012). We first examined the effect of a fixed concentration of VU0477573

in rat cortical astrocytes (Lonza, Basel, Switzerland) in the presence of increasing concentrations

of glutamate. The compound shows a rightward shift of the glutamate concentration response

curves and a decrease in the maximal effect of glutamate, consistent with a noncompetitive

allosteric antagonist (Figure 4A). As in HEK293A-rat-mGlu$_5$-low cells, the 10 μM maximal

concentration of VU0477573 did not fully block the glutamate response as compared to 10 μM

MPEP, consistent with weak negative cooperativity. The compound also shows concentration-

dependent, but saturable, inhibition of intracellular calcium release in the presence of an EC$_{80}$

concentration of glutamate. VU0477573 inhibits the intracellular calcium release by

approximately 70% of the glutamate maximal response in rat cortical astrocytes, as compared to

MPEP as a control (Figure 4B). Therefore, the percent glutamate maximal response is reduced to

approximately 30% by VU0477573. The pIC$_{50}$ for VU0477573 is 7.90 ± 0.18 (13nM) and for

MPEP is 8.26 ± 0.33 (5.5 nM) in astrocytes. The percent glutamate maximal response is 31.5 ±

4.7 for VU0477573 and 6.5 ± 2.9 for MPEP. Comparing the activity of VU0477573 in the NAM

CRC assay (Figure 4B), which utilizes an EC$_{80}$ value of glutamate, and the fold shift assay

(Figure 4A), The maximal effect of 10 μM VU0477573 in the fold shift assay (Figure 4A) at 50

μM glutamate is approximately 80% and at 1 μM glutamate is 100% inhibition (Figure 4A). The

EC$_{80}$ of glutamate for the fold shift assay is approximately 25 μM. (Figure 4A), and 10 μM

VU0477573 demonstrated approximately 80% inhibition (Figure 4A). This value correlates well

with the maximal inhibition observed in the NAM CRC assay (Figure 4B) in rat cortical

astrocytes. Again, consistent with the results observed in HEK293A-rat-mGlu$_5$-low cells,

VU0477573 also shows weak negative cooperativity, or partial NAM activity, for mGlu$_5$ in this

native *in vitro* system.

**VU0477573 shows partial blockade of DHPG-induced inhibition of excitatory transmission

at SC-CA1 synapses.** To determine if VU0477573 has partial NAM activity at mGlu$_5$ in a native

system, we examined DHPG-induced inhibition of synaptic transmission at the Schaffer

collateral (SC)–CA1 synapse in mouse hippocampal brain slice preparation. Since both mGlu$_1$

and mGlu$_5$ have been shown to play a role in modulation of glutamatergic transmission at SC-

CA1 synapses (Huber et al., 2001; Mannaioni et al., 2001; Faas et al., 2002), we performed the

experiments in the presence of the known mGlu$_1$ NAM VU0469650 (Lovell et al., 2013) to

block mGlu$_1$ activity. In the presence of VU0469650 pretreatment, 100μM DHPG inhibited the

extracellular field excitatory postsynaptic potential (fEPSP) slope (Figure 5 A, B). The addition

of 10 μM VU0477573 partially blocked this response, while the addition of 10 μM MPEP fully

blocked the response (Figure 5A, B). The effects of VU0477573 and MPEP on DHPG induced inhibition were statistically significant at the 20 minute time point, with VU0477573 showing partial activity, blocking the DHPG-mediated fEPSP slope by approximately 40% as compared to full blockade by MPEP (Figure 5C). Therefore, as in the assays using mGlu$_5$ expressing cell lines described above, VU0477573 shows partial activity as compared to full blockade induced by MPEP, of DHPG-mediated inhibition of glutamatergic transmission at the SC-CA1 synapse in mice.

VU0477573 fully inhibits glutamate-induced extracellular signal-related kinase 1/2 phosphorylation. Allosteric ligands have the capacity to show differential efficacy in downstream signaling pathways. This property has several labels including functional selectivity, biased agonism, or stimulus trafficking (Conn et al., 2009; Kenakin, 2011). mGlu$_5$ allosteric ligands are known to show functional bias, with varying levels of efficacy in different signaling pathways (Zhang et al., 2005). Numerous examples exist showing functional selectivity among allosteric modulators for other GPCRs (Maj et al., 2003; Sachpatzidis et al., 2003; Wei et al., 2003; Mathiesen et al., 2005; Sheffler and Conn, 2008; Kenakin and Miller, 2010; Niswender et al., 2010; Digby et al., 2012; Noetzel et al., 2013). We examined the effect of VU0477573 on glutamate-stimulated extracellular signal-related kinase 1 and 2 phosphorylation (phosphoERK1/2) in HEK293A-rat-mGlu$_5$-low cells using the ERK Surefire Assay Kit. VU0477573 induces full inhibition of glutamate-mediated phosphoERK1/2 phosphorylation, similar to MPEP (Figure 6A), returning the levels of phosphoERK1/2 to basal levels comparable to vehicle-treated cells. The pIC$_{50}$ is 8.13 ± 0.70 (7.41 nM) for VU0477573 as compared to 8.59 ± 0.31 (2.57 nM) for MPEP. In addition, VU0477573 induces a rightward shift of the glutamate

CRC and full inhibition of the maximal effect of glutamate, consistent with a full noncompetitive

allosteric antagonist (Figure 6B).

These data may suggest that VU0477573 has differential cooperativity with glutamate

depending on the measure of receptor function. However, as reported previously, mGlu$_5$ is

coupled more weakly to phosphoERK1/2 responses in HEK293A-rat-mGlu$_5$-low cells relative to

calcium mobilization, such that some NAMs with lower cooperativity in calcium assays fully

inhibit glutamate-mediated phosphoERK1/2 (Gregory et al., 2012). To compare these responses,

we utilized the operational model of allosterism (Leach et al., 2007) to estimate VU0477573

affinity (pK_B) and cooperativity (logβ) values (Table 1) from the shift in glutamate

concentration-response curves for intracellular calcium mobilization (Figure 1B). In this analysis

we assumed that affinity modulation was neutral and the pK_A was held constant as validated

previously (Gregory et al., 2012). We then used the values obtained from the operational

analysis of the Ca^{2+} mobilization data for VU0477573 to simulate the phosphoERK1/2 data

(Supplementary Figure 1). The logτ_A was constrained at -0.36, according to previously published

results reflecting the decreased coupling efficiency of glutamate for phosphoERK1/2 as

compared to calcium mobilization in this cell line (Gregory et al., 2012). If we assume that

VU0477573 has the same apparent affinity and cooperativity with glutamate between Ca^{2+}

assays and phosphoERK1/2, then the simulation predicts that the maximal glutamate response

for phosphoERK1/2 will be fully inhibited (Supplementary Figure 1). Importantly, in support of

this assumption, simulation of the VU0477573 EC$_{80}$ inhibition curves in both Ca^{2+} and

phosphoERK1/2 assays predicts that VU0477573 will be more potent in phosphoERK1/2 than

Ca^{2+}, as we observed. Therefore the full blockade of glutamate observed in phosphoERK1/2 is

most likely due to the low stimulus response coupling of the system rather than biased

cooperativity of VU0477573 between these two different endpoints. Fitting the fold shift data

(Figure 6B, expressed as fold over basal) to the operational model of allosterism, the apparent

pK_B is 6.88 ± 0.10, (with the constraints of $\log\tau_B = -100$, $\log K_A = -6.155$, $\log \alpha = 0$, $\log \beta = -100$,

$E_m = 9.1$ for FBS maximal response, and basal $= 1.0$)

VU0477573 Does Not Show Inverse Agonist Activity in a PI Hydrolysis Assay. Ligands that

bind to GPCRs have the potential to show a range of modes of action, including full agonism,

antagonism, full inverse agonism, and neutral interactions that do not show functional effects.

The mGlu$_5$ NAMs MPEP and MTEP act as inverse agonists, decreasing the basal level of

inositol phosphate accumulation (Gasparini et al., 1999; Roppe et al., 2004; Lea and Faden,

2006). Fenobam, the atypical anxiolytic and mGlu$_5$ NAM, also exhibits inverse agonist

properties (Porter et al., 2005). The inverse agonist activity seen with mGlu$_5$ allosteric ligands

such as MPEP has been proposed to correlate with the adverse side effects associated with

mGlu$_5$ NAMs (Gasparini et al., 1999; Roppe et al., 2004; Porter et al., 2005; Lea and Faden,

2006; Rodriguez et al., 2010). Examination of mGlu$_5$ NAM candidates for inverse agonist

activity may prove important as these agents are further developed and characterized. When

expressed in heterologous systems, ligand-independent signal transduction is correlated with an

increased level of heterologous GPCR expression (Tiberi and Caron, 1994). To assess inverse

agonist activity of VU0477573, we utilized an [³H]IP accumulation assay and HEK293A cells

stably expressing an HA-tagged N-terminally truncated rat mGlu$_5$ (Noetzel et al., 2013). This

receptor lacks the extracellular *N*-terminal orthosteric agonist binding domain, and is

unresponsive to glutamate stimulation. Therefore, the accumulated [³H]IP in this assay utilizing

this cell line represents constitutive mGlu$_5$ receptor activity and is not complicated by the

presence of glutamate. These studies reveal that VU0477573 (10 μM and 30 μM) does not show inverse agonist activity as compared to the prototypical mGlu$_5$ NAMs MPEP and MTEP, at 10 μM and 30 μM concentrations (Figure7).

VU0477573 Demonstrates Excellent Pharmacokinetic Properties. To determine if VU0477573 achieved the brain exposure necessary for *in vivo* behavioral assays, we first performed a dose-response exposure study in mice. VU0477573 was well-solubilized in the 10% Tween80/90% water vehicle used for these studies. The clogP value for the compound is 3.35, which was calculated using ChemBioDraw Ultra v. 14.0, Cambridge Software Corporation. We performed *in vivo* pharmacokinetic studies of VU0477573 in male CD-1 mice after i.p. administration of 1, 3, 10, and 30 mg/kg. We obtained blood and brain samples at both 15 and 45 min time points (Figure 6A and Table 2). VU0477573 showed excellent brain penetrance, with a good correlation between the unbound brain concentration (43.7 nM at a 3mg/kg dose at 15 min post-dosing) and efficacy in the intracellular calcium release assay (IC$_{50}$ = 32 nM). At the 15 min exposure time point, VU0477573 showed an approximately linear increase in total brain and plasma exposure with increasing dose in CD-1 mice (Figure 8A). The unbound brain-to-plasma concentration ratio (K$_{p\ uu}$) is 0.77 for the 3 mg/kg dose of compound at 15 minutes, demonstrating excellent brain exposure of VU0477573 in mice. We next performed a time-course study at the 10mg/kg dose. The T$_{max}$ at the 10 mg/kg dose in the time course is 15 min (Figure 8B). The compound shows sufficient brain exposure over the hour needed to run behavioral assays. The suitable brain exposure of the compound prompted us to progress VU0477573 into behavioral studies *in vivo*.

VU0477573 Achieves Full Occupancy of mGlu$_5$ *in vivo*. Based on its excellent brain exposure,

we would expect VU0477573 to achieve high occupancy of mGlu$_5$ in the CNS after systemic

dosing. Prior to using this compound for in vivo behavioral studies, we performed studies to

definitively confirm determine if the compound achieved full occupancy of the receptor *in vivo*.

Using CD-1 mice, we assessed the occupancy of the compound at mGlu$_5$ using displacement of

the radioligand [^{3}H]-methoxyPEPy (Figure 9A). Increasing doses of VU0477573 resulted in full

occupancy of the receptor with an RO$_{50}$ of 2.6 ± 1.9 mg/kg, corresponding to 50% receptor

occupancy. For this assay, we utilized the cerebellum as a reference region with low mGlu$_5$

expression levels to confirm low radioactive counts, similar to the method detailed in Anderson

et al. (Anderson et al., 2003) (Supplemental Figure 2).

VU0477573 Displays Dose-dependent Efficacy in a Marble Burying Behavioral Model of

Anxiety. To determine the potential efficacy of VU0477573, we performed a marble burying

assay in CD-1 mice, which is a standard behavioral model of novelty-induced anxiety (Spooren

et al., 2000). Mice are known to bury foreign objects in deep bedding (Deacon, 2006), and the

anxiolytic benzodiazepine inhibits burying behavior (Broekkamp et al., 1986; Njung'e and

Handley, 1991). The prototypical mGlu$_5$ NAM MPEP as well as fenobam, are also effective in

this behavioral assay (Spooren et al., 2000; Nicolas et al., 2006). VU0477573 dose-dependently

reduced the number of marbles buried as compared to vehicle (Figure 9B). The mGlu$_5$ NAM

MTEP at 15 mg/kg was included as a positive control. While 1mg/kg of VU0477573 did not

result in a significant decrease in the number of marbles buried, the 3, 10, and 30 mg/kg doses

did result in a statistically significant reduction in total marbles buried. As compared to the

potency value (IC$_{50}$) of 32 nM in the calcium release assay in HEK293A-rat-mGlu$_5$-low cells,

the efficacy in the marble burying assay observed at 3 mg/kg corresponds well to the unbound

brain concentration of 43.7 nM. The 1mg/kg dose provides unbound brain concentrations of

approximately 24 nM, which is lower than the in vitro IC_{50} of the compound and did not exhibit

efficacy in the marble burying assay. Therefore, there is good correlation between the unbound

brain concentration, the efficacy seen in the marble burying assay, and the observed potency of

the compound in the intracellular calcium release assay (Figure 9B). Also, these data are in close

agreement with the doses of RO_{50} value, corresponding to 50% receptor occupancy, determined

in the receptor occupancy studies (Figure 9A). This behavioral assay demonstrates that

VU0477573 shows efficacy in a standard rodent model of anxiety. Therefore, compounds with

weak negative cooperativity, or partial NAMs, of $mGlu_5$ may show potential as therapeutics in

anxiety disorders.

DISCUSSION

The discovery of allosteric modulators of metabotropic glutamate receptors has led to an abundance of research to identify therapeutic candidates for CNS disorders. CNS indications for mGlu$_5$ NAMs include Fragile X syndrome, L-dopa induced dyskinesia in Parkinson's disease, Alzheimer's disease, addiction and anxiety. Unfortunately, mGlu$_5$ NAMs have not been successful thus far as clinical therapeutics, due in part to the induction of side-effects, including psychotomimetic activity (Pecknold et al., 1982; Porter et al., 2005; Rodriguez et al., 2005; Abou Farha et al., 2014) or lack of efficacy in the treatment of L-DOPA induced dyskinesia (Petrov et al., 2014) and fragile X syndrome (FRAXA mavoglurant posting (Novartis), FRAXA basimglurant posting (Roche)) and the doses tested. Recent strategies aimed at avoiding the side effect liabilities of mGlu$_5$ NAMs include the use of new chemical scaffolds different from MPEP, identifying compounds with partial NAM activity, and assessing inverse agonist activity as a potential predictor of side effect liability. We present here the discovery and characterization of VU0477573 a high affinity mGlu$_5$ selective partial NAM (for glutamate-mediated calcium mobilization) with no inverse activity and pharmacokinetic properties ideal for *in vivo* studies.

mGlu$_5$ NAMs have been associated with adverse side effects in both preclinical species and humans. One adverse event observed in patients is the induction of psychomimetic-like behaviors. Previous human studies have demonstrated that noncompetitive, use-dependent *N*-methyl-D-aspartate subtype of glutamate receptors (NMDAR) antagonists, such as phencyclidine (PCP) and ketamine, exacerbate all three symptom clusters observed in individuals with schizophrenia and are psychotomimetic in healthy individuals (Krystal et al., 1994; Adler et al., 1998; Domino and Luby, 2012). mGlu$_5$ is a closely associated signaling partner of NMDAR. Therefore, complete inhibition of mGlu$_5$ may mimic the effects of NMDAR blockade *in vivo*.

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Early clinical trials using fenobam reported effects that dissociative side effects (Pecknold et al.,

1982) and serious psychotomimetic side effects were recently reported for a newer class of

mGlu$_5$ NAM (Swedberg and Raboisson, 2014). Unfortunately, these adverse effects were not

predicted in preclinical studies. However, partial NAMs may be useful in treatment regimens

where one desires to maintain some measure of receptor activity while inhibiting the ability of

the native orthosteric agonist to induce excessive receptor activation. The discovery of

VU0029251 as a potent systemically active partial NAM that achieves high CNS occupancy

after systemic dosing provides an exciting new tool for fully evaluating the range of effects of

mGlu$_5$ partial NAMs in animal models. Also, the finding that VU0477573 has robust efficacy in

at least on rodent model of anxiolytic activity suggests that complete blockade of mGlu$_5$ and

mGlu$_5$-mediated inverse agonist activity are not required for efficacy in this in vivo measure.

Importantly, VU0477573 has ~10-fold higher affinity for mGlu$_5$ than the previously reported

partial NAMs, M-5MPEP and Br-MPEPy (Rodriguez et al., 2005) and greater than 30-fold

higher affinity than VU0029251 (Rodriguez et al., 2010). Moreover, VU0477573 has acceptable

pharmacokinetic properties necessary to evaluate the effect of partial NAMs *in vivo*.

For the most part, discovery programs for mGlu$_5$ allosteric modulators continue to rely on

a single measure of receptor activation, i.e. glutamate-mediated intracellular calcium

mobilization, to classify allosteric modulator pharmacology. It has become increasingly apparent

that allosteric ligands can exhibit biased modulation of the orthosteric agonist (Zhang et al.,

2005; Gregory et al., 2012; Noetzel et al., 2013; Rook et al., 2015). M-5MPEP has been

suggested to have differential negative cooperativity, partially inhibiting agonist-stimulated

[^{3}H]IP accumulation and completely blocking agonist-stimulated Ca^{2+} oscillations (Bradley et

al., 2011). VU0477573 only partially inhibited agonist-mediated intracellular calcium release

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and fully inhibited ERK1/2 phosphorylation. These phenotypic differences in the pharmacology of VU0477573 on the ERK1/2 phosphorylation versus intracellular calcium release pathways raise the possibility of identifying partial NAMs with functional bias. This feature may be leveraged in future development efforts to achieve activity in the desired downstream pathway from mGlu$_5$. If specific signaling pathways are linked to clinical applications, ligands that show functional bias may allow for the development of tailored therapies. However, as we demonstrated, these phenotypic differences can also arise from differential system bias. In this case, VU0477573 most likely causes complete blockade of glutamate-induced ERK1/2 phosphorylation due to the fact that mGlu$_5$ is weakly coupled to phosphoERK1/2 relative to calcium mobilization. Similar conclusions were drawn regarding the phenotypic differences for the partial NAMs VU0366248 and M-5MPEP (between glutamate stimulated Ca^{2+} mobilization compared with phosphoERK1/2) (Gregory et al., 2012). Nevertheless, VU0366249, which has lower cooperativity with glutamate in the Ca^{2+} assay than VU0366248, M-5MPEP and VU0477573, retains its partial NAM phenotype in the phosphoERK1/2 assay (Gregory et al., 2012). These data highlight the importance of considering not only ligand pharmacology but also the signal transduction pathways mediating receptor effects when classifying ligands.

The inverse agonist activity seen with mGlu$_5$ allosteric ligands such as MPEP (Gasparini et al., 1999; Roppe et al., 2004; Lea and Faden, 2006) has been proposed to correlate with the adverse side effects observed for the mGlu$_5$ NAMs (Rodriguez et al., 2010). It remains to be seen whether or not the lack of inverse agonism for VU0477573 is associated with decreased adverse effect liability. Additional complementary screening assays predictive of adverse side effects are needed. Comparison of the available mGlu$_5$ full and partial NAMs in assays indicative of psychotomimetic-like activity, including disruption of prepulse inhibition of the acoustic startle

response and potentiation of PCP-induced hyperlocomotion may elucidate the predictive value of

inverse agonist activity on potential psychotomimetic side effects (Spooren et al., 2000).

Importantly, VU0477573 has anxiolytic efficacy at doses that correlate well with receptor

occupancy, suggesting that inverse agonist activity, as detected using PI hydrolysis experiments,

is not required for anxiolytic efficacy. As more mGlu$_5$ partial NAMs are identified, further

characterization will shed light on their potential utility as therapeutic agents and the relative

contribution of affinity, cooperativity and inverse agonist activity. The ideal scenario to assess

the potential utility of mGlu$_5$ partial NAMs would be the identification of a set of compounds

that show similar affinities and pharmacokinetic properties but with varying degrees of

cooperativity with glutamate at mGlu$_5$ from a small (i.e. 25%) to high (i.e. 80% inhibition)

extent. With this putative group of compounds showing a range of mGlu$_5$ partial NAM activity,

it would then be possible to assess the degree of cooperativity needed to show efficacy while not

eliciting side effects. One could examine the desired balance between the activity needed for

efficacy and that associated with adverse effects in preclinical models.

In summary, the discovery of the mGlu$_5$ partial NAM VU0477573, which shows efficacy

in the anxiolytic behavioral model of marble burying and achieves 100% *in vivo* receptor

occupancy, provides a promising step in the development of mGlu$_5$ allosteric agents. The

identification of VU0477573 is proof-of-concept that mGlu$_5$ NAMs can have limited

cooperativity and can achieve full receptor occupancy *in vivo* and demonstrate efficacy in a

preclinical model of anxiety. Ongoing studies will determine the efficacy of mGlu$_5$ partial NAMs

in additional behavioral models of psychosis, addiction and depression.

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FOOTNOTES

FIGURE LEGENDS

Figure 1: Discovery of the partial mGlu$_5$ NAM VU0477573. (A) Chemical structures of mGlu$_5$ negative allosteric modulators: MPEP, MTEP, 5-MPEP, Br-MPEPy, and VU0477573. (B) VU0477573 (black inverted triangles) shows dose-dependent partial NAM activity in glutamate-stimulated intracellular calcium release in HEK293A-rat-mGlu$_5$-low cells in comparison with MPEP (black squares) and MTEP (open circles). (C) Increasing concentrations of VU0477573 (10 nM inverted black triangles, 30 nM black circles, 1 μM black triangles, 10 μM black diamonds) induce a rightward shift in the potency and decreased maximal response to glutamate-stimulated intracellular calcium release in HEK293A-rat-mGlu$_5$-low cells as compared to MPEP (10 μM black squares) and Vehicle (open triangles). Data are plotted as a percentage of the maximal response to glutamate and error shown as S.E.M. with n=3 experiments, points performed with 2-4 replicates. (D) VU0477573 is selective for the mGlu$_5$ receptor subtype. HEK293 cells stably expressing each mGlu subtypes were assessed for either intracellular calcium or thallium flux and the leftward or rightward fold shift of the agonist concentration-response curve was calculated. Data represent the mean ± SD of two individual experiments with 4-6 replicates each.

Figure 2: VU0477573 binds to the MPEP site VU0477573 fully competes with [^{3}H]-methoxyPEPy binding to the MPEP site. Membranes from HEK293A-rat-mGlu$_5$-low cells were incubated with increasing concentrations of VU0477573 (black inverted triangles), MPEP (black squares) or MTEP (open circles) in the presence of 3 nM of [^{3}H]methoxyPEPy. Data represent the mean ± SEM of three individual experiments performed in duplicate. Data are plotted as the percentage of the total [^{3}H]methoxyPEPy binding.

Figure 3: VU0477573 shows partial mGlu$_5$ NAM activity in [^{3}H]IP accumulation. Increasing concentrations of VU0477473 (10 nM black inverted triangles, 1 μM black triangles, 10 μM black diamonds) show a dose-dependent rightward shift in the potency and decreased maximal response to glutamate in HEK293A-rat-mGlu$_5$-low cells as related to MPEP (10 μM black squares) and Vehicle (clear triangles). Data are plotted as a percentage of the maximal response to glutamate and error shown as S.D. with n=3 experiments, with data points performed in duplicate.

Figure 4: VU0477573 shows partial mGlu$_5$ NAM activity in rat cortical astrocytes. (A) Increasing concentrations of VU0477473 (10 nM inverted black triangles, 30 nM black circles, 10 μM black diamonds) show a dose-dependent rightward shift in the potency and decreased maximal response to glutamate in rat cortical astrocytes as related to MPEP (10 μM black squares) and Vehicle (clear triangles). Data are plotted as a percentage of the maximal response to glutamate and error shown as S.E.M. with n=3-4 experiments, with 2-4 replicates per experiment. (B) VU0477573 (triangles) shows dose-dependent partial NAM activity in agonist-stimulated intracellular calcium release in rat cortical astrocytes as compared to MPEP (squares). Data are plotted as a percentage of the maximal response to glutamate and error shown as S.E.M. with n=6 experiments, with 2-4 replicates per experiment.

Figure 5. Effects of VU0477573 and MPEP on DHPG-induced inhibition of glutamatergic transmission at SC-CA1 synapses. **A.** Representative traces of fEPSPs in control (thin black traces), and after applications of mGlu$_1$ NAM VU0469650 (10 μM, grey traces) followed by

combination of VU0469650 and 100 µM DHPG, VU0469650 and 10 µM VU0477573, and

VU0469650 and 10 µM MPEP (thick black traces in A1, A2 and A3, respectively). **B.** Average

time courses of effects of VU0469650 followed by combination of VU0469650 with DHPG (n =

6), VU0477573 (n = 9) or MPEP (n =8) on the slope of fEPSPs at SC-CA1 synapses. The grey

vertical bar indicates the time point at which the data were taken to make statistical comparisons.

C. Summary bar graph for the data taken from the time point indicated by the grey vertical bar in

B, and presented as the percentage of maximal DHPG effect, demonstrating that VU0477573

partially blocked the DHPG effect to $38.1 \pm 14.2\%$ of maximum DHPG response whereas MPEP

completely blocked the DHPG effect to $-2.3 \pm 3.8\%$ of maximum DHPG response. One-way

ANOVA analysis revealed significant differences between groups $[F(2,20) = 19.39, p <$

$0.0001]$. The post-hoc Bonferroni's test showed significant differences between

DHPG+VU0469650 and DHPG+VU0469650+VU0477573 groups (** $p < 0.01$) and between

DHPG+VU0469650+VU0477573 and DHPG+VU0469650+MPEP groups (* $p < 0.05$).

Figure 6. VU0477573 Fully Inhibits Glutamate-mediated Extracellular signal-related kinase 1/2

Phosphorylation. **(A)** VU0477573 (inverted triangles) shows full dose-dependent inhibition of

glutamate-mediated ERK1/2 phosphorylation to basal levels comparable to vehicle (left black

square) and MPEP (squares) in HEK293A-rat-mGlu$_5$-low cells. The concentration response

curve represents the mean ± S.E.M from 4 independent experiments with 4 replicates per

condition. (B) Increasing concentrations of VU0477573 (10 nM black inverted triangles, 100 nM

black circles, 1 µM black triangles, 10 µM black diamonds) induce a rightward shift in the

potency and decreased maximal response to glutamate-stimulated intracellular calcium release in

HEK293A-rat-mGlu$_5$-low cells in glutamate-mediated ERK1/2 phosphorylation as compared to

MPEP (black squares) and vehicle (clear triangles). Data are plotted as a percentage of the maximal response to glutamate and error shown as S.E.M. with n=5 experiments, with 3-6 replicates per experiment.

Figure 7. VU0477573 does not show inverse agonist activity in [^{3}H] inositol phosphate accumulation. The basal level of [^{3}H]IP accumulation was determined as a measure of inverse agonist activity in HEK293A cells stably expressing HA-tagged *N*-terminally truncated mGlu$_5$. The bar graph represents the mean ± S.E.M from three to five independent experiments performed with 3-6 replicates per treatment, expressed as a percentage of the basal [^{3}H]IP accumulation. * denotes $p < 0.0001$ vs. vehicle (one-way ANOVA followed by Bonferroni's multiple comparison test).

Figure 8. VU0477573 has an excellent pharmacokinetic profile in CD-1 mice. Pharmacokinetic studies in CD-1 male mice show (A) VU0477573 is brain penetrant at 15 minutes post dosing with the total brain level increasing in an approximately linear fashion with increasing dose; (B) Time exposure data at 10mg/kg demonstrates excellent brain exposure over time with a T_{max} of 0.25 hours. Data are mean ± S.E.M. with three to four animals per group.

Figure 9. VU0577573 displays efficacy in marble burying and achieves full receptor *in vivo* occupancy. (A) Systemic administration of VU0477573 (1 – 30 mg/kg i.p.) dose-dependently displaces hippocampal [^{3}H]-methoxyPEPy binding in CD-1 mice. Data are mean ± S.D. of 6 – 12 animals per group. (B) Systemic administration of VU0477573 (1 – 30 mg/kg i.p.) dose-dependently inhibits marble burying in CD-1 mice, with a control MTEP dose of 15 mg/kg. Data

are mean ± S.E.M. of 5-8 animals per group. * $p < 0.05$ vs. vehicle, (one-way ANOVA followed

by Dunnett's test). Inhibition of marble burying correlates with increased brain exposure. Bars

denote marbles buried. Open circles correspond to unbound brain exposure.

Table 1: VU0477573 *in vitro* activity on rat mGlu$_5$ in functional assays and radioligand binding

Compound	Ca assay % Glut Max (±SEM)	Ca assay pIC$_{50}$ (±SEM)	Ca assay IC$_{50}$ (nM)	Binding pK$_i$ (±SEM)	Binding K$_i$ (nM)
*VU0477573	16.43 ± 6.20	7.49 ± 0.16	32	7.85 ± 0.06	14
MPEP	-1.56 ± 0.89	7.87 ± 0.23	13	8.19 ± 0.03	6
MTEP	-3.11 ⊥ 1.26	7.28 ⊥ 0.15	52	7.65 ± 0.03	22

* The operational model of allosterism parameters for negative allosteric modulation of glutamate-mediated intracellular calcium mobilization in HEK293A-rat-mGlu$_5$-low cells by VU0477573 are: $\log\tau_A = 0.57 \pm 0.13$, $pK_B = 7.73 \pm 0.10$, $\log\beta = -0.44 \pm 0.10$.

Table 2: Mouse PK Results for VU0477573

Dose (mg/kg)	Time (hr)	Unbound Plasma (nM)	Unbound Brain (nM)
1	0.25	36.4	27.7
	0.75	16.4	20.9
3	0.25	69.7	53.7
	0.75	82.0	43.7
10	0.25	302	239
	0.75	335	164
30	0.25	1009	670
	0.75	783	569

Figure 1

A

MPEP

MTEP

M-5MPEP

Br-5MPEPy

VU0477573

B

- ▼ VU0477573
- ■ MPEP
- ○ MTEP

Percent Glutamate Max

Log [NAM] M

C

- △ Vehicle
- ▼ 10 nM VU0477573
- ● 30 nM VU0477573
- ▲ 1 μM VU0477573
- ◆ 10 μM VU0477573
- ■ 10 μM MPEP

Peak Response (Percent Glutamate Max)

Log [Glutamate] M

D

Agonist fold shift

mGlu$_1$ mGlu$_2$ mGlu$_3$ mGlu$_4$ mGlu$_6$ mGlu$_7$ mGlu$_8$
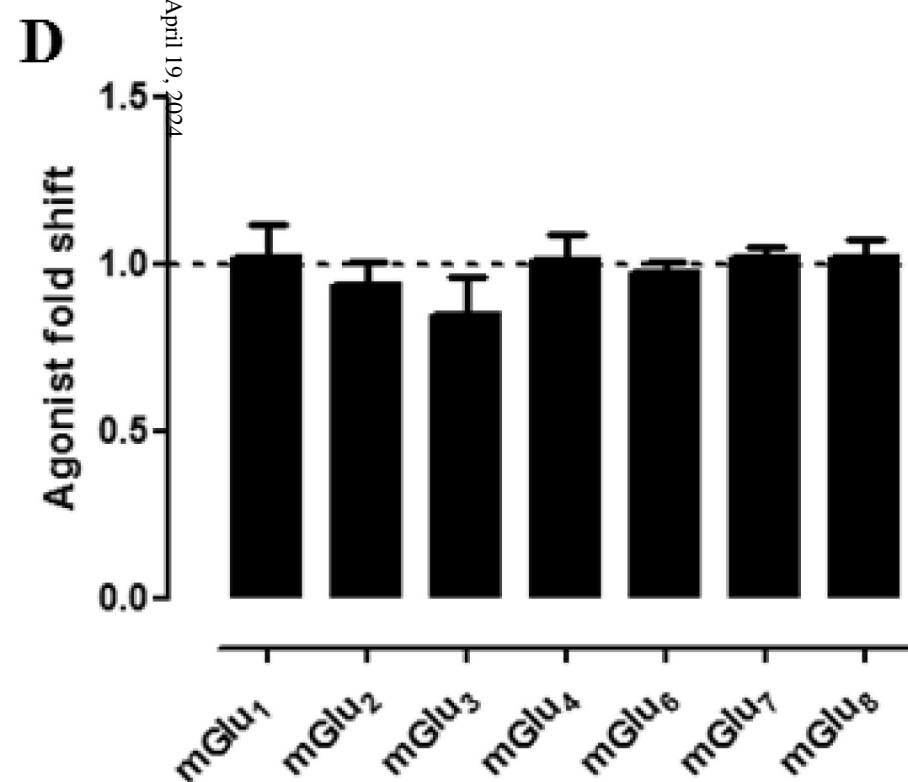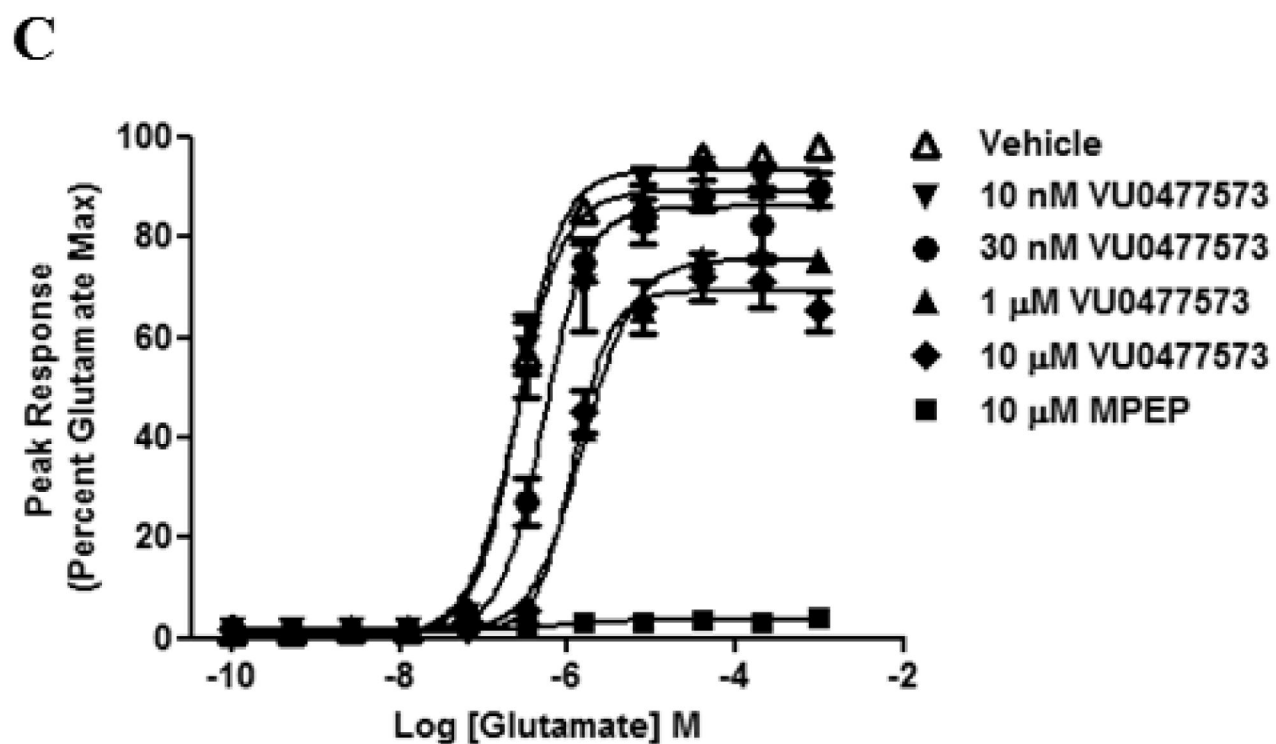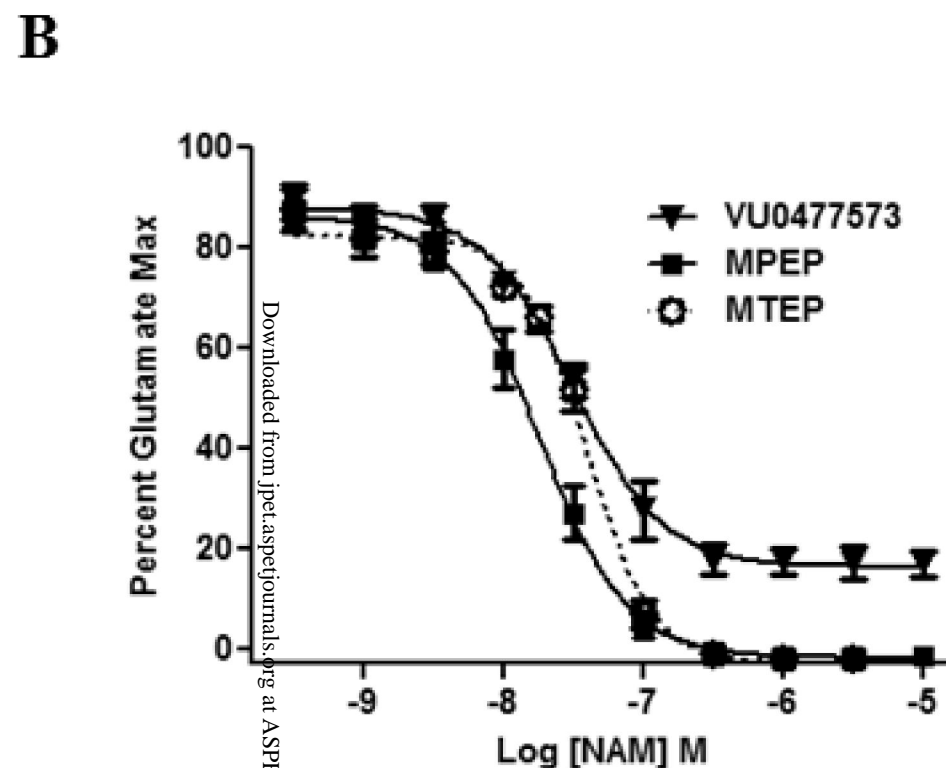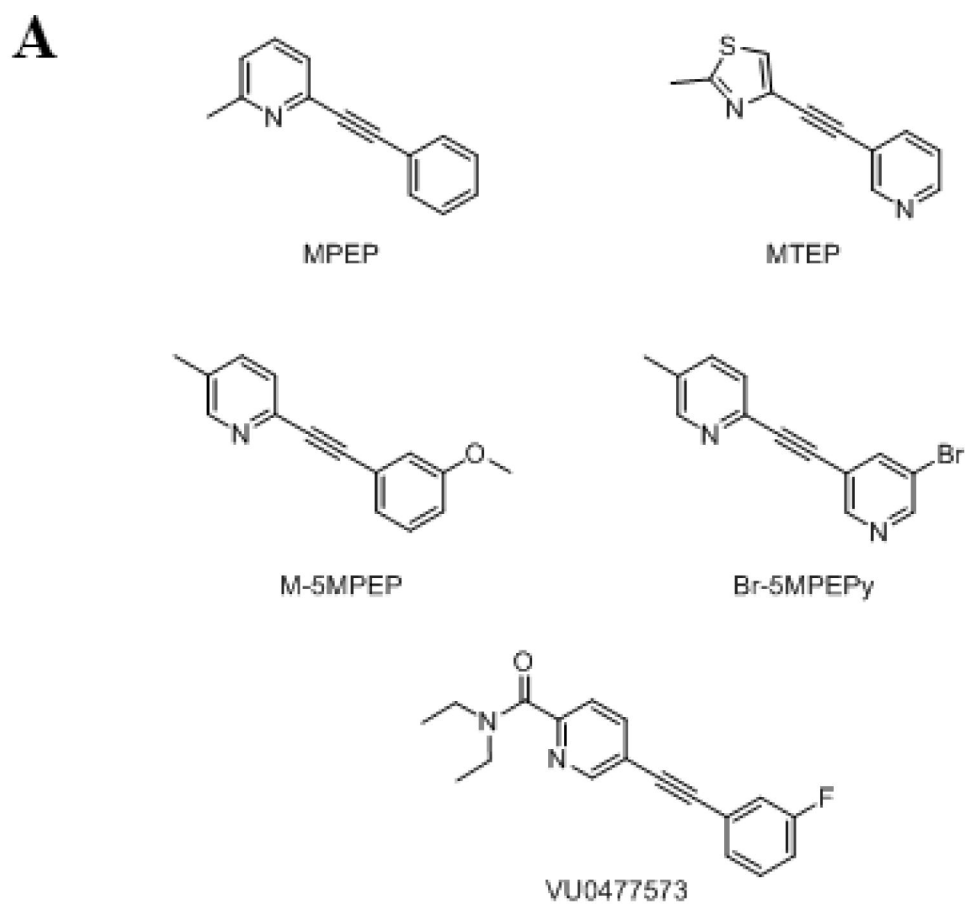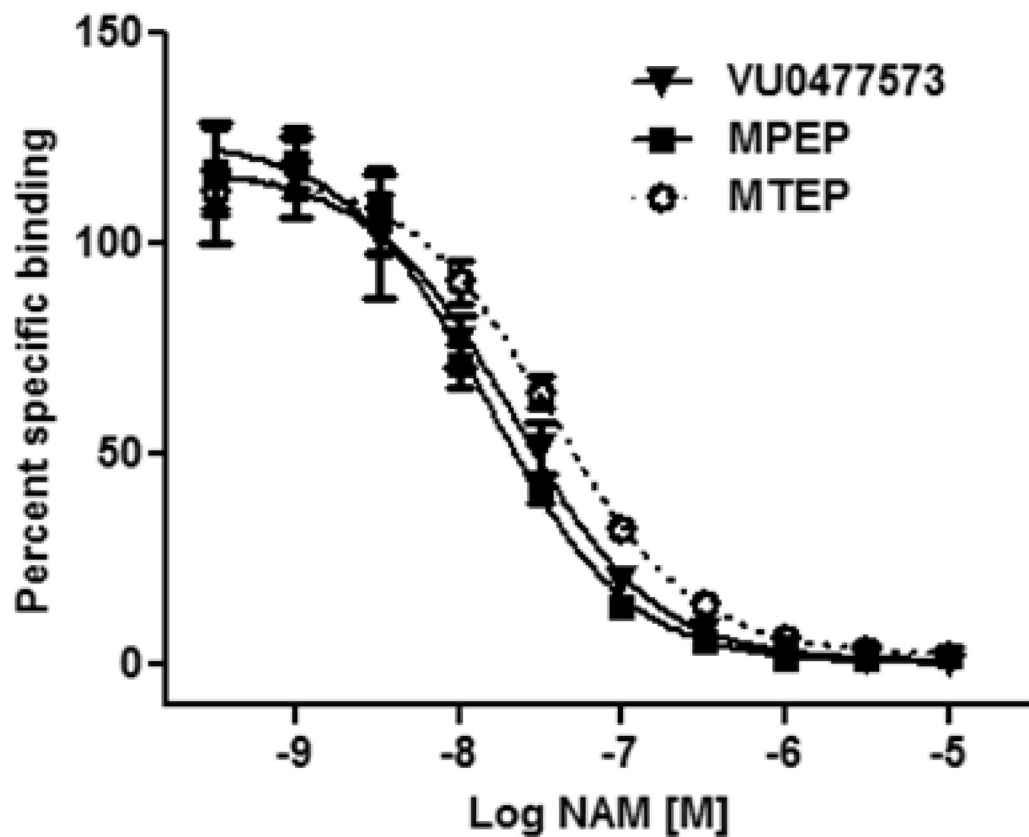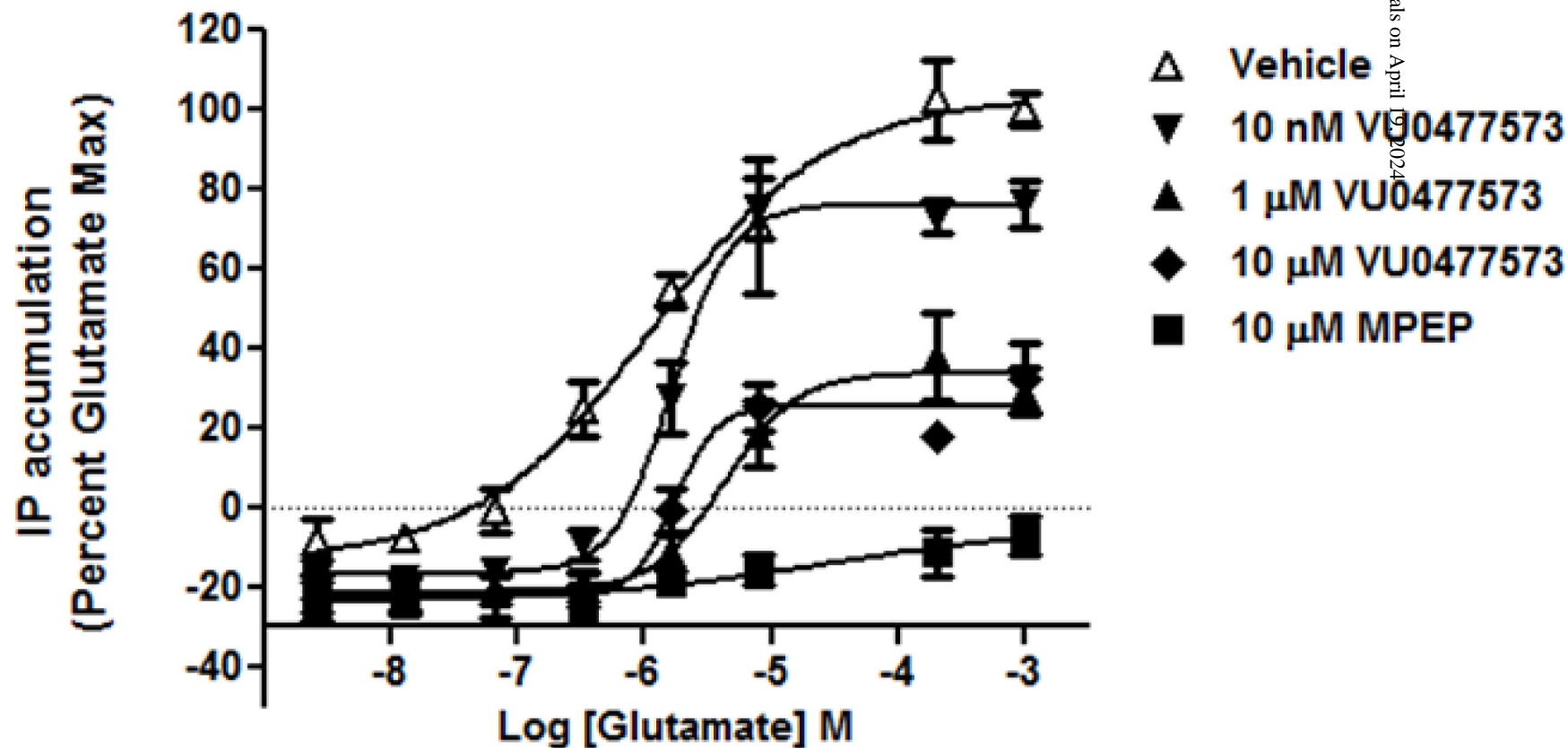
Figure 2

Figure 3

Figure 4

A
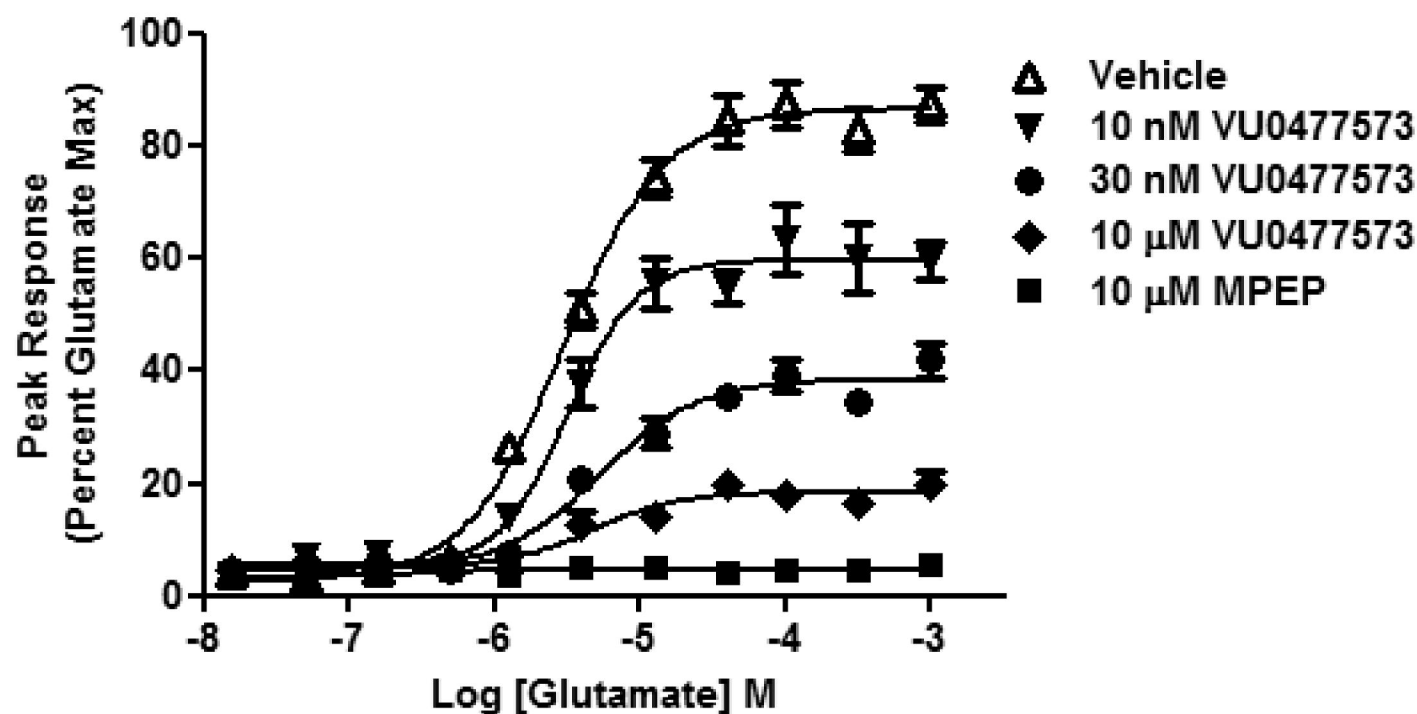
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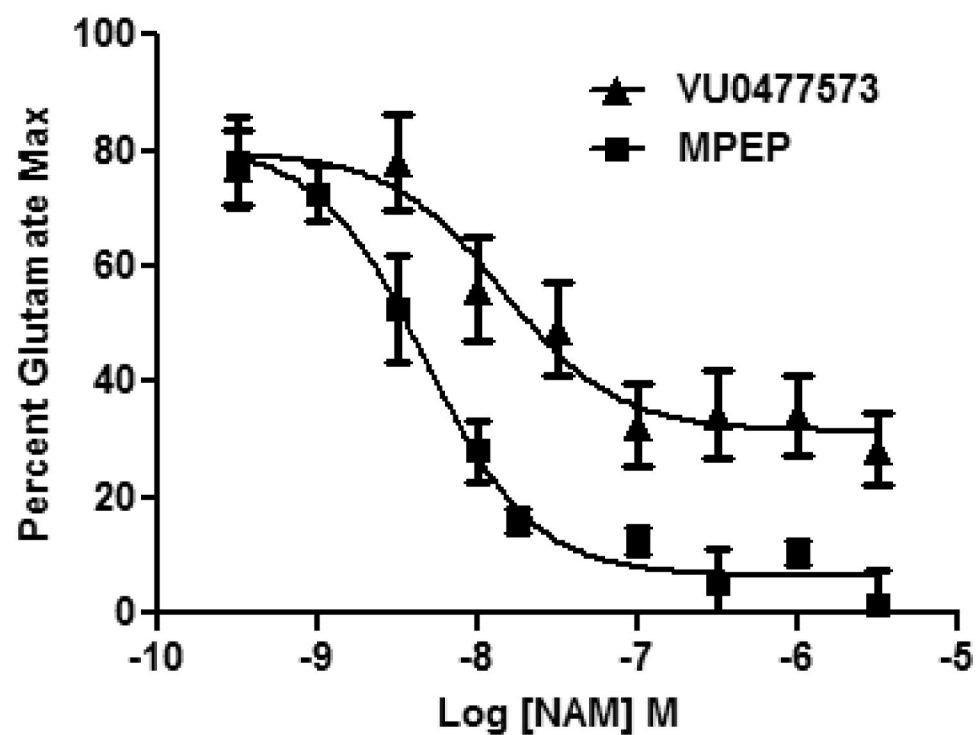
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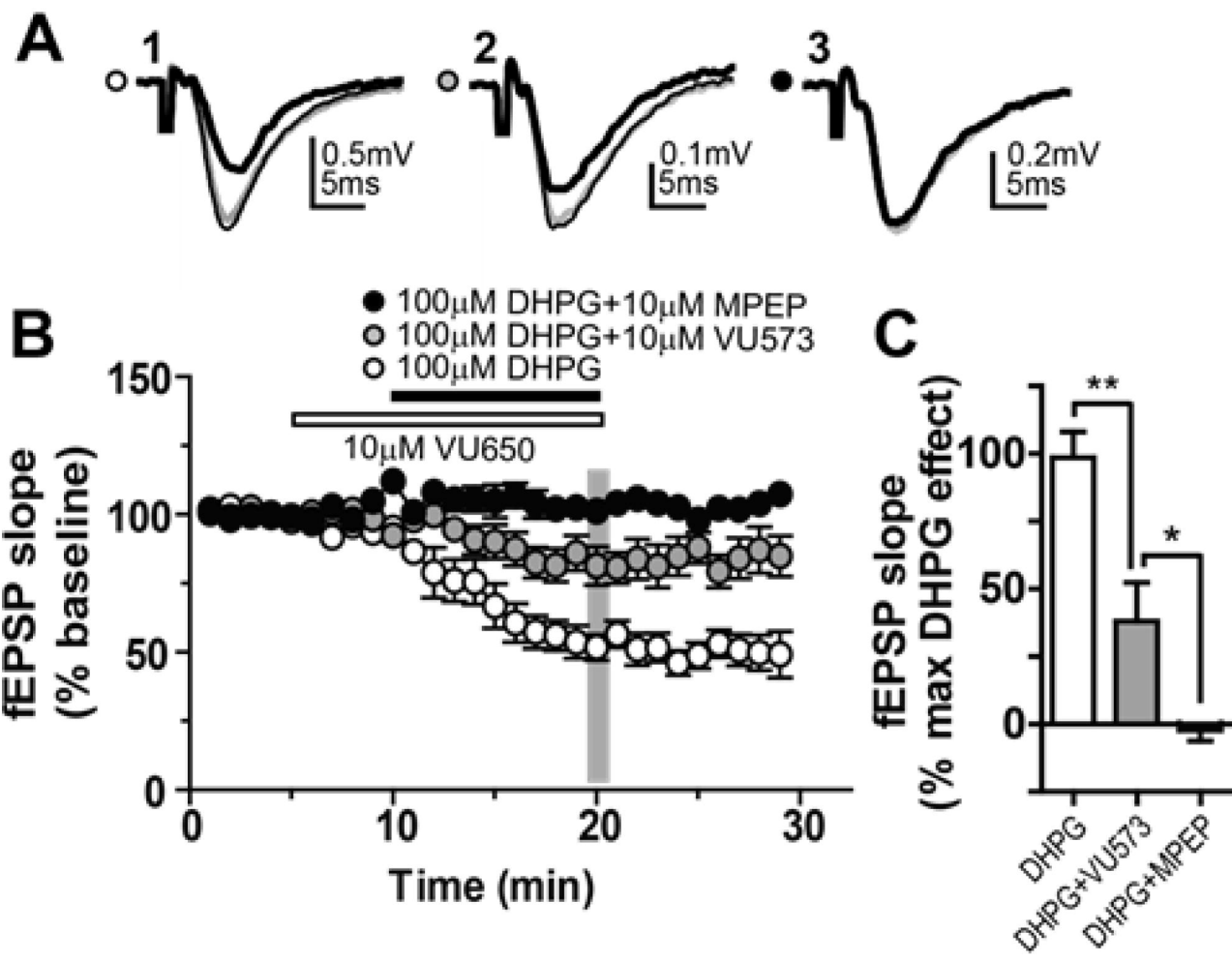
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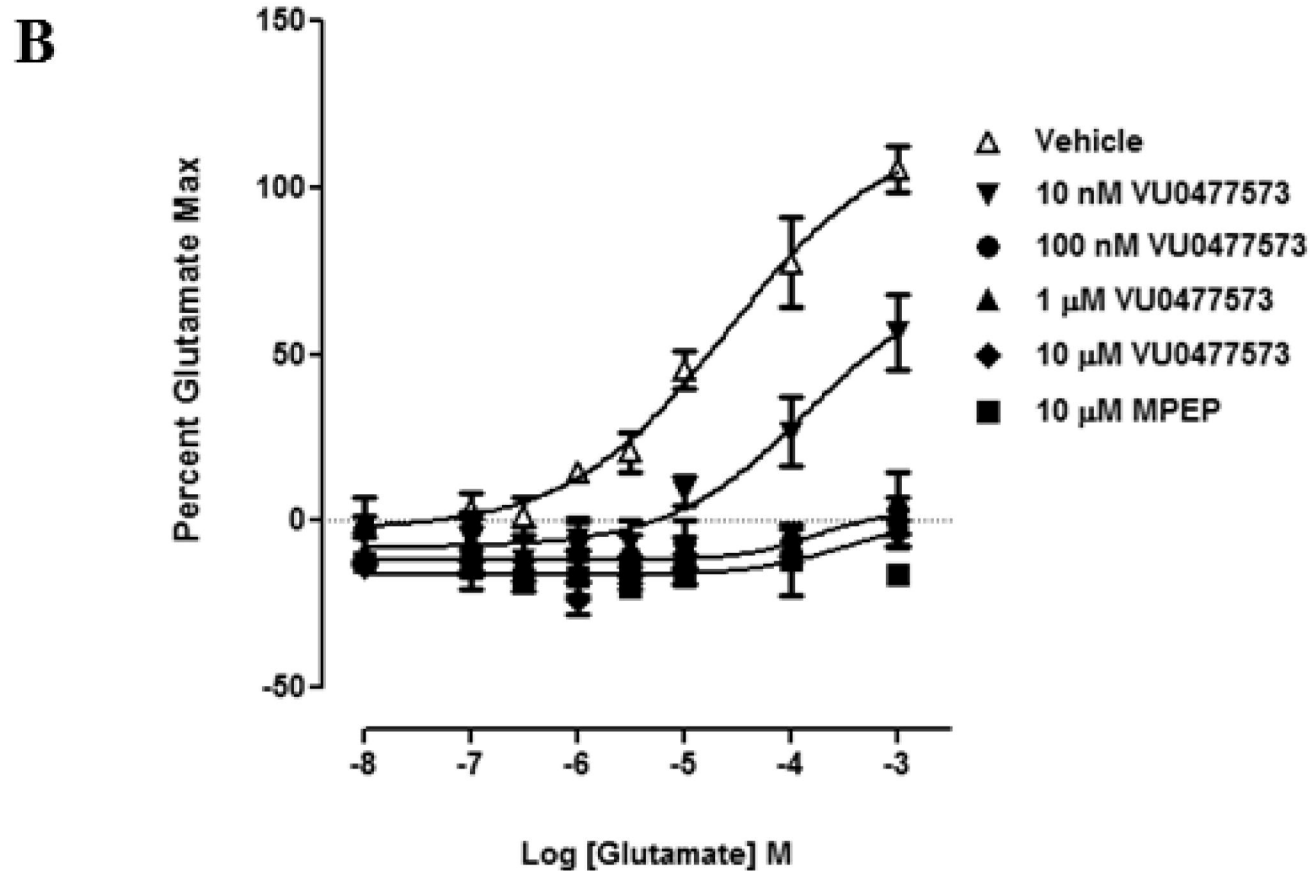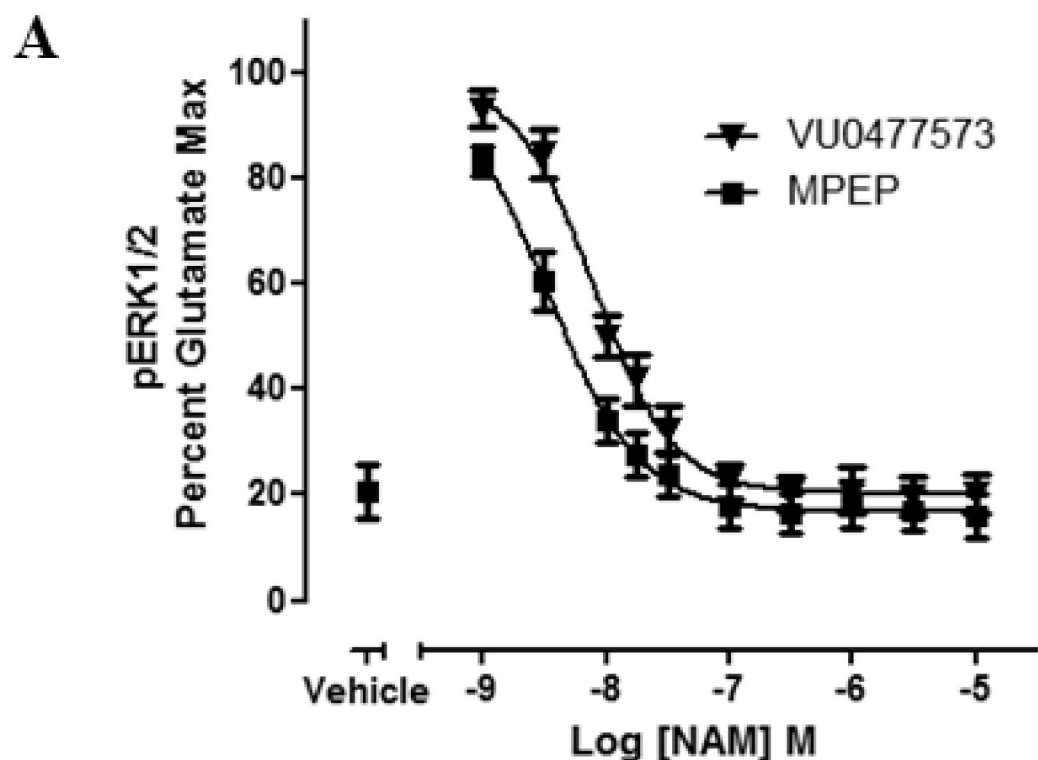
Figure 7

Figure 8

Figure 9