Discovery of a Novel Chemical Class of mGlu₅ Allosteric Ligands with Distinct Modes of Pharmacology

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Abstract

We previously discovered a positive allosteric modulator (PAM) of the metabotropic glutamate receptor subtype 5 (mGlu₅) termed 4-N-[4-chloro-2-[(1,3-dioxo-1,3-dihydro-2H-isooindol-2-yl)methyl]phenyl]-2-hydroxybenzamide (CPPHA) that elicits receptor activation through a novel allosteric site on mGlu₅, distinct from the classical mGlu₅ negative allosteric modulator (NAM) MPEP allosteric site. However, a shallow structure–activity relationship (SAR), poor physicochemical properties, and weak PAM activity at rat mGlu₅ limited the utility of CPPHA to explore allosteric activation of mGlu₅ at a non-MPEP site. Thus, we performed a functional high-throughput screen (HTS) and identified a novel mGlu₅ PAM benzamide scaffold, exemplified by VU0001850 (EC₅₀ = 1.3 μM, 106% Glu max) and VU0040237 (EC₅₀ = 350 nM, 84% Glu Max). An iterative parallel synthesis approach delivered 22 analogues, optimized mGlu₅ PAM activity to afford VU0357121 (EC₅₀ = 33 nM, 92% Glu Max). An iterative parallel synthesis approach delivered 22 analogues, optimized mGlu₅ PAM activity to afford VU0357121 (EC₅₀ = 33 nM, 92% Glu Max). An iterative parallel synthesis approach delivered 22 analogues, optimized mGlu₅ PAM activity to afford VU0357121 (EC₅₀ = 33 nM, 92% Glu Max). An iterative parallel synthesis approach delivered 22 analogues, optimized mGlu₅ PAM activity to afford VU0357121 (EC₅₀ = 33 nM, 92% Glu Max).

Keywords: mGlu₅, metabotropic, glutamate, allosteric, potentiator

Glutamate is the major excitatory neurotransmitter in the mammalian central nervous system (CNS). It plays important roles in virtually all major CNS circuits by acting through ionotropic glutamate receptors (iGluRs), as well as metabotropic glutamate (mGlu) receptors. The eight mGluR subtypes are family C G-protein coupled receptors (GPCRs) that are divided into three groups based on sequence homology and coupling to signal transduction cascades. Group I includes mGlu1 and mGlu5; these receptors couple to Gᵣ/q and induce increases in intracellular calcium. Group II (mGlu2 and mGlu3) and group III (mGlu4, mGlu6, mGlu7, and mGlu8) receptors couple to Gᵣ/i/o and inhibit cAMP formation (I). The family C GPCRs are characterized by a large bilobed (termed venus fly trap) extracellular ligand binding domain where the endogenous ligand glutamate and other orthosteric ligands bind (Figure 1A). Because of the highly conserved nature of the orthosteric binding site, ligands that modulate receptor function at this site have poor subtype selectivity among the mGlu receptors. An alternative approach to targeting the highly conserved orthosteric glutamate site is to develop compounds that act at allosteric sites on mGlu receptors that are removed from the orthosteric site in the heptahelical transmembrane domain and may be less highly conserved. This approach is proving highly successful for multiple GPCRs. Negative allosteric modulators (NAMs) were the first allosteric ligands reported for the mGlu receptors and have been described for mGlu1 and mGlu5 (2, 3); moreover, positive allosteric modulators (PAMs), molecules that bind at an allosteric site, do not activate the receptor directly but potentiate activation of the receptor by the orthosteric agonist glutamate, have been reported for mGlu3, mGlu4, and mGlu5 (4).

Recent anatomical, cellular, molecular, and behavioral findings suggest that selective activators of the metabotropic glutamate receptor subtype 5 (mGlu₅) may provide a novel strategy for the treatment of the psychotic symptoms and cognitive impairments observed.

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in individuals with neuropsychiatric disorders (5–7). However, it has been historically difficult to produce agonists of mGlu5 that are selective for this subtype as compared to other mGlu receptors. Recently, our laboratory and others have made major breakthroughs in this area by developing selective positive allosteric modulators (PAMs) of this receptor. MPEP is the prototypical mGlu5 NAM with a well characterized allosteric binding site (Figure 1B; see references 2, 8, and 9). Several distinct chemotypes have been identified that function as mGlu5 PAMs and are exemplified by DFB, MPPA, CDPPB/VU-29, and the ADX series (ADX47273, (S)-FTOPM, and (S)-FPOPM) (9–12). All of these PAMs potentiate mGlu5-mediated responses by binding to the well-characterized, negative allosteric modulator MPEP binding site. Interestingly, CPPHA is the only known mGlu5 PAM that does not bind to the MPEP site and acts via a unique site on the receptor to potentiate mGlu5 responses (13, 14). These data suggest that multiple allosteric sites exist on mGlu5. Also, CDPPB, ADX47273, and (S)-FTOPM display intrinsic agonist activity at moderate to high concentrations in Chinese hamster ovary (CHO) or human embryonic kidney (HEK293) cells expressing rmGlu5, whereas MPPA, DFB, (S)-FPOPM, and CPPHA have no effect on mGlu5 alone, and only potentiate responses of the receptor to glutamate (i.e., pure PAM) (10, 11, 13, 15). In addition, we have shown that CPPHA and DFB display differential modulatory function on mGlu5-mediated phosphorylation of extracellular signal-regulated kinases 1 and 2 (ERK1/2) (16). Together, these findings indicate that mGlu5 PAMs can have differential effects on downstream receptor trafficking by virtue of the allosteric site the ligand occupies to exert conformational changes leading to potentiation. Thus, it will prove critically important to identify additional novel classes of mGlu5 PAMs and allosteric binding sites to further our understanding of differential mGlu5 potentiation and receptor trafficking.

CPPHA, due to its unique allosteric binding site and pharmacology, has the potential to serve as an important pharmacological tool to further investigate functional selectivity among classes of mGlu5 PAMs. Unfortunately, due to diminished potency on rodent mGlu5, shallow SAR, and poor physicochemical properties, CPPHA is of limited utility to translate into in vivo studies (17). Here, we describe the identification, SAR and in vitro pharmacological characterization of a novel chemical class of mGlu5 PAMs that are reminiscent of, yet distinct from, CPPHA that activate mGlu5 at a non-MPEP and non-CPPHA allosteric site. Moreover, this new series of mGlu5 PAMs, represented by VU0357121 (EC50 = 33 nM, 92% Glu max), possess potent activity on the rat receptor. This effort also identified VU0365396, the first non-MPEP neutral allosteric ligand.

**Results and Discussion**

A number of significant issues have hampered the basic science efforts driving mGluR research across all receptor subtypes: (1) a lack of truly selective tool compounds to clearly elucidate the physiological role of given subtypes, (2) receptor trafficking phenomenon
by different allosteric ligands and by receptor activation at different allosteric binding sites, and (3) subtle molecular switches that elicit alternate modes of pharmacology. In order to develop an mGlu5 PAM that could be developed as a potential therapeutic agent, we must first understand the functional relationship between distinct allosteric binding sites and downstream receptor trafficking. In order to accomplish this, it will be important to understand the similarities and differences between novel mGlu5 PAMs that act at different allosteric sites.

**Discovery of a Novel Benzamide Class of mGlu5 PAMs by HTS**

Our laboratory performed a functional high-throughput screen (HTS) (18–20) that led to the identification of about 20 structural classes of mGlu5 PAMs. The present study focuses on a novel benzamide chemical scaffold that was identified in that screen. Sixty-five compounds were identified that belong to this structural class, and 10 were found to have high potencies with EC50 values ≤1 μM from HTS stock solutions. This class is of particular interest because these compounds share a benzamide backbone that is similar to that of CPPHA yet are distinct. These compounds display little intrinsic mGlu5 agonist activity in the calcium mobilization assay and induce a robust enhancement of the response to a suboptimal concentration (EC20) of glutamate (Figure 2). To test their PAM activity, HEK293 cells expressing rat mGlu5 were preloaded with calcium-sensitive fluorescent dye and received vehicle or compound (10 μM) followed by an EC20 concentration of glutamate 5 min later. Receptor-induced calcium mobilization responses were monitored using Functional Drug Screening System (FDSS, Hamamatsu, Japan).

**Design, Synthesis, and SAR of Benzamide Analogues**

As previously stated, the lead optimization program that led to the discovery of CPPHA faced several challenges, making it difficult to develop compounds related to CPPHA that retained activity on rodent mGlu5 (17). The three most potent compounds within the benzamide chemical class (VU0001850, VU0003712, and VU0040237) are shown in Figure 3. With the identification of our three main hits from the screen, we had the opportunity to determine whether slight modifications to their chemical structures would lead to tractable SAR for rat mGlu5 or afford modulation in the mode of mGlu5 pharmacology. For chemical lead optimization, we elected to employ a parallel synthesis approach in which we resynthesized the three initial hits along with a library of 22 analogues to obtain an initial understanding of the SAR in a 25-member library of analogues 10 (Figure 3A).

The first set of analogues varied both the aryl/heteroaryl amide (blue) as well as different ethers (green). The chemistry involved treatment of a p-alkoxy benzoic chloride 11 and an aryl/heteroaryl amine 12 to provide analogues 10 or the corresponding benzoic acid 13 could be coupled to 12 under standard carbodiimide conditions to delivers analogues 10 (Figure 3B). All compounds were purified by mass-directed preparative HPLC to >98% purity.

These novel analogues were assayed in a single-point screen, along with the three original hits, to determine their ability to potentiate a suboptimal concentration of glutamate using the FlexStation fluorometric imaging plate reader from Molecular Systems. HEK293 cells expressing mGlu5 were incubated with a fixed concentration of test compound for 109 s and then stimulated with an EC20 concentration of glutamate. At 10 μM, all three of the original hits (VU0001850, VU0040237, and VU0040237), as well as three of the novel analogues (VU0357121, VU0365393, and VU0125936), potentiated the response to suboptimal glutamate concentrations above a 50% response, which was used as a cutoff reference (Figure 4A). Screening the compounds at a concentration of 1 μM revealed that two of the original hits (VU0001850 and VU0040237) as well as two of
the newly synthesized compounds (VU0357121 and VU0125936) potenti-ated the response to glutamate at submicromolar concentrations (Figure 4B), and these compounds were chosen for further analysis as mGlu5 PAMs. As we had previously seen with CPPHA, SAR was quite shallow for this new series, with few active PAMs.

To further evaluate the compounds that were identified as potent mGlu5 PAMs (VU0001850, VU0040237, VU0357121, and VU0125936), we determined their ability to potentiate mGlu5 responses to glutamate in two separate calcium mobilization assays. First, the test compound’s concentration–response relationships were examined by incubating mGlu5-expressing cells with increasing concentrations of compound in the presence of a fixed suboptimal concentration of glutamate (EC50).

Figure 5 shows the concentration–response relationships for these compounds, and compound potencies ranged from ~33 nM to 1 μM. A second measure of potentiation was performed to assess the ability of the compounds to increase the potency of a glutamate concentration–response curve (fold-shift assay). This is measured as a leftward shift of the glutamate concentration–response curve (CRC) and decrease in the EC50 of glutamate in the presence of test compound as compared to a glutamate concentration–response curve in the presence of vehicle only. HEK293 cells expressing mGlu5 were pretreated with vehicle or a fixed concentration (12.5 μM) of the mGlu5 PAM, followed by increasing concentrations of glutamate. The test compounds caused 2- to ~6-fold shifts in the glutamate concentration–response curve at mGlu5 (Figure 6), providing further evidence for their ability to potentiate mGlu5-mediated responses to glutamate.

Figure 7 highlights the SAR for the benzamide series after one round of library synthesis. The initial HTS hits VU0001850 and VU0040237 were found to possess EC50 values of 1.3 μM and 350 nM on rat mGlu5, respectively. Both VU0001850 and VU0040237 potentiated the response to the EC50 glutamate concentration to 106% and 84% of the response to a maximal concentration of glutamate, respectively (% Glumax), and induced leftward shifts (~5.2× and ~3.9×, respectively) of the glutamate CRC (Figure 7A). The two new analogues, VU0357121 and VU0125936, represented improvements over the HTS leads. Addition of a second fluorine into the 4-position of VU0040237 provided VU0357121 (EC50 = 33 nM, 92% Glumax, a second fluorine into the 4-position of VU0040237, and ~2.6-fold shift) and a 10-fold increase in potency over the HTS hit. Similarly, homologation of the n-propyl ether in VU0001850 to an n-butyl ether analogue VU0125936 provided an increase in rat mGlu5 potency (EC50 = 664 nM, 112% Glumax, and ~5.8-fold shift).

Again, further libraries resulted in no significant active mGlu5 PAMs, and the SAR was highly reminiscent of the CPPHA SAR. As depicted in Figure 7B, alternate chain lengths and/or cyclic constraints in the ether linkage abolished PAM activity. Minor modifications, such as N-alkylation of the secondary amide, led to inactive analogues. Finally, aryl substitution other than fluorine and alternative heterocycles for the 2-pyridyl moiety also resulted in no measurable PAM activity. These data, coupled with the structural similarities of these compounds to CPPHA, led us to speculate that this series of mGlu5 PAMs may bind at either the CPPHA site or an overlapping, shallow allosteric site, as MPEP-site PAMs generally afford more robust SAR.

VU0357121 and Analogues Are Selective for mGlu5 Relative to Other mGluR Subtypes

Before using these compounds to explore mGlu5 allosteric sites, it was necessary to ensure that they were in fact selective for mGlu5 relative to other mGluR subtypes. All four compounds were tested against mGlu receptors 1–4, 6, 7, and 8, and displayed similar selectivity profiles;
therefore, data for the most potent PAM, VU0357121, is highlighted as representative (Figure 8). First, we looked at the effects of the compounds on mGlu1 because this receptor has the closest homology to mGlu5 (1), which leads to a high degree of cross-activity of compounds. Fold-shift studies were performed as described above, except that HEK293 cells expressing rat Glu1 were used. These compounds did not cause any shift in the glutamate concentration—response curve when added at 30 μM and only induced a slight depression in the maximum glutamate response, demonstrating a lack of PAM activity at mGlu1 (Figure 8). Our laboratory recently developed an assay that detects the activity of compounds that interact with G1/o-coupled GPCRs, such as the group II and III mGlu receptors (27). This assay measures thallium flux mediated...
by G-protein-regulated inwardly rectifying potassium channels (GIRKs). By performing fold-shift assays using HEK/GIRK cells that stably express either mGlu2, 3, 4, 6, 7, or 8, we found that the test compounds did not modulate the response of any of these receptors to agonist, providing more evidence for their subtype selectivity (Figure 8). These studies confirmed that these compounds are highly selective as PAMs of mGlu5 and are in fact inactive or very weakly antagonizing at other mGlu receptor subtypes.

**VU0357121 and Analogues Do Not Bind at the MPEP Allosteric Site**

Because of the structural similarity between these novel mGlu5 PAMs and CPPHA and the shallow SAR, we hypothesized that they might interact with a site on the receptor that is distinct from the MPEP binding site. To test this hypothesis, we assessed the ability of these test compounds to compete for binding of the radiolabeled MPEP analogue [3H]methoxy-5-(pyridin-2-ylethynyl)pyridine ([3H]methoxy-PEPy) (22) to evaluate their interaction with the allosteric MPEP site on mGlu5. The radioligand was added to membranes prepared from HEK293 cells stably expressing mGlu5 with or without the test compound, and reactions were allowed to incubate for 1 h. It was found that, unlike MPEP ($K_i = 35 \pm 10.4 \mu M$), test compounds did not inhibit steady state [3H]methoxyPEPy binding at concentrations of up to 100 μM, 3 orders of magnitude higher than those required for their PAM activity, suggesting that the ability of these agents to enhance glutamate sensitivity of mGlu5 is likely due to the interaction at a site on the receptor distinct from the MPEP binding site (Figure 9). Slight inhibition of radioligand binding at higher concentrations may indicate some interaction between the MPEP site and the putative site of action of these PAMs.

A neutral allosteric ligand or silent allosteric modulator (SAM) binds to an allosteric site but does not have positive or negative allosteric modulator activity when measuring a defined response. However, a SAM competes for binding with PAMs and NAMs and thereby blocks the effects of positive and negative allosteric modulators without affecting the action of the orthosteric ligand through its binding to an allosteric site (10, 23). Our laboratory previously reported that the neutral ligand 5-MPEP binds to the same site as its analogue MPEP and is able to inhibit the function of the NAM MPEP as well as PAMs such as VU-29 and CPPHA (14, 23). Interestingly, 5-MPEP causes a competitive inhibition of VU-29 activity, whereas it induces a noncompetitive blockade of CPPHA function, providing further evidence for multiple modulatory sites on mGlu5, and distinct sites of action for MPEP-like compounds and CPPHA. To further test the hypothesis that the novel benzamide class of mGlu5 PAMs described here acts at a site that is distinct from the MPEP site, we utilized the SAM 5-MPEP as a tool to investigate its effects on VU0357121 activity in two separate assays. First, we examined 5-MPEP activity on the CRC of VU0357121-induced potentiation of glutamate-induced increases in calcium mobilization. The mGlu5-expressing HEK293 cells were treated with fixed concentrations (333 nM to 10 μM) of 5-MPEP or vehicle and increasing concentrations of VU0357121, followed by the addition of an EC20 concentration of glutamate. Interestingly, in this kinetic assay 5-MPEP induces progressive rightward shifts and reductions in the maximal response to VU0357121 potentiation of glutamate responses (Figure 10A), suggesting that 5-MPEP is likely to block the response to VU0357121 by a non-competitive mechanism of action. To further explore the interaction between these allosteric modulators in an end-point assay, we performed phosphoinositide (PI) hydrolysis studies to measure the effects of 5-MPEP on the VU0357121 agonist CRC. mGlu5-expressing HEK293 cells loaded with [3H]myo-inositol were treated with vehicle or a fixed concentration (10 μM) of 5-MPEP, followed by various concentrations of VU0357121. The resulting radiolabeled phosphoinositides were measured and normalized to the response elicited by 1 mM glutamate. Consistent with our findings in the calcium assay, this concentration of 5-MPEP
caused a rightward shift in the VU0357121 CRC, coupled with a decrease in maximal potentiation (Figure 10B). As a control, we also investigated the effects of the same concentration of 5-MPEP on the VU-29 agonist curve. 5-MPEP (10 μM) induced a 20-fold parallel rightward shift of the VU-29 CRC at a magnitude consistent with the binding affinity for 5-MPEP (K_i = 388 nM) and with the effect observed in the kinetic assay. These data are consistent with our previous studies and suggest that 5-MPEP acts in a competitive manner with VU-29 (data not shown). These findings are consistent with our hypothesis that the benzamide class of mGlu5PAMs acts at an allosteric site other than the MPEP site and provide further evidence that there is a functional interaction between these two sites.

VU0357121 and Analogues Do Not Possess mGlu5 NAM Activity

Studies with previous series of mGlu5 allosteric modulators reveal that slight structural changes within a series can lead to striking changes in the modes of mGlu5 pharmacology of these compounds (PAM to NAM and NAM to PAM) (Figure 11). In fact, close analogues of the first mGlu5 PAM described, DFB, displayed a wide-range of modes of pharmacology with only slight structural...
changes (70). Replacement of the 3,3'-diF with a 3,3'-diOMe, DOMEB 14, provided an equipotent mGlu5 NAM, while the 3,3'-diCl congener, DCB 15, was the first neutral mGlu5 ligand. Subsequently, a series of mGlu5 partial antagonists 16 and 17 have also led to neutral ligands such as 5-MPEP 18 (23). More recently, another mGlu5 partial antagonist 5-PPEP 20 was converted into a potent, full NAM by the addition of a methyl group in the 3-position 19; in contrast, addition of either a 4-methyl group 21 or an amino-methyl moiety 22 afforded potent mGlu5 PAMs (Figure 11). Similar switches in the mode of mGlu5 pharmacology have been noted in multiple mGlu5 NAM series and in the ADX mGlu5 PAM series (15, 24, 25). Notably, this has only been observed in MPEP site allosteric ligands and never observed in the non-MPEP site CPPHA series. To avoid potential metabolites in vivo that might possess switches in the mode of pharmacology, it is imperative to establish whether subtle structural changes in compounds in this series of non-MPEP site ligands have a similar propensity to display fundamentally different modes of pharmacology.

Therefore, these compounds were also screened in a manner to identify NAMs or noncompetitive antagonists of mGlu5. For the antagonist screen, we determined whether the test compounds could inhibit the effect of glutamate on mGlu5-expressing HEK293 cells. The calcium mobilization assay was performed as described above, with cells being treated with a submaximal concentration of glutamate (∼EC80). In contrast to the blockade induced by the mGlu5 NAM MPEP, VU0357121 and analogues did not inhibit the effect of glutamate on mGlu5; in fact, the majority of compounds induced a mild potentiation of the EC80 glutamate response (Figure 12).

Identification of a Non-MPEP Site Neutral Ligand

To date, the only known neutral allosteric ligands or SAMs for mGlu receptors are ligands at the MPEP site on mGlu5. To expand our investigation of putative non-MPEP allosteric sites on mGlu5, it would be helpful to have a neutral ligand that acts at a different site on the receptor and possibly at the same site as VU0357121. We utilized the benzamide analogues that were synthesized earlier to determine whether any neutral ligands were present among these compounds. To screen our compounds for this activity, we determined their ability to block the VU0357121-induced potentiation of mGlu5-mediated responses to glutamate. To accomplish this, we
again used a calcium assay, but cells were pretreated with the test compound (10 μM) and VU0357121 (50 nM) followed by stimulation with an EC20 concentration of glutamate. Several compounds significantly inhibited the PAM activity of VU0357121 (Figure 13), but VU0365396 caused the most robust inhibition and was therefore chosen for further characterization. Interestingly, VU0365396 (30 μM) induced a parallel rightward shift in the VU0357121 CRC (Figure 14A), suggesting that this compound is a neutral ligand that acts in a competitive manner at the same allosteric site as VU0357121. In contrast, VU0365396 (30 μM) had no effect on CPPHA-induced potentiation of mGlu5 responses (Figure 14B). The lack of effect on CPPHA PAM activity was somewhat surprising but provides clear evidence that these mGlu5 PAMs do not act at the same site and raises the possibility that these sites may not interact functionally. However, it is possible that limited affinity of the neutral ligand at its own site or limited negative cooperativity led to a lack of effect on CPPHA responses. Unfortunately, limitations with solubility precluded studies with higher concentrations of VU0365396.

**Mutations Differentially Alter Responses to VU0357121 and CPPHA**

We previously reported the effects of single point mutations in mGlu5, which can inhibit the responses of mGlu5 to specific PAMs and NAMs (8, 14, 20). These mutants were tested for their ability to alter responses to VU0357121 (1 μM) in transiently transfected HEK293 cells using the calcium mobilization assay. Mutation of the alanine at the 809 position of rat mGlu5 to a valine (A809V/rmGlu5) reduces the binding and function of MPEP and inhibits functional responses to PAMs that act at the MPEP site, such as VU-29 (8, 14, 20) but has no effect on responses to CPPHA (14). In contrast, the F585I/rmGlu5 mutation inhibits the PAM activity of CPPHA but has no effect on responses to VU-29 activity (14). We replicated these previous findings and found that the response to VU-29 but not CPPHA is lost in the A809V/rmGlu5 mutant, whereas the response to CPPHA but not VU29 is lost in the F585I/rmGlu5 mutant (data not shown). Interestingly, we found that the A809V/rmGlu5 mutation inhibited the ability of VU0357121 to shift the glutamate concentration–response curve (Figure 15A), whereas the response to VU0357121 was not altered by the F585I/rmGlu5 mutation (Figure 15B). The finding that the F585I mutation was without effect is consistent with studies above suggesting that CPPHA and VU0357121 do not act at identical sites. The finding that the response was inhibited by the A809V mutation is somewhat surprising in light of the results from the molecular pharmacology studies and further illustrates the complexity of the relationships between putative allosteric sites on mGlu5. In addition, these data highlight the fact that mutagenesis studies must be interpreted with caution. While previous studies and the current data provide strong evidence that multiple mGlu5 modulators do not bind to identical binding sites, it is possible that they interact with overlapping sites that share critical residues. Also, in the absence of direct structural information about the class C GPCRs, models of class C GPCR structure and functional effects of specific mutations do not provide
definitive information about the nature of the binding of these ligands. However, the results of these mutagenesis studies combined with the current and previous molecular pharmacology studies provide further evidence that there is some interaction between the putative binding sites of CPPHA, VU0357121, and the MPEP binding site that are important for the action of these structurally and functionally diverse allosteric modulators.

In summary, using a functional HTS screen and subsequent library synthesis approach, we have discovered a novel series of highly selective mGlu5 PAMs and a neutral ligand that have properties that suggest that they do not act as traditional MPEP site ligands. Also, our results suggest that these compounds do not interact with the receptor in a manner that is identical to that of CPPHA. Our present and previous studies suggest the presence of distinct allosteric sites or modes of action for VU0357121, CPPHA, and MPEP-site ligands such as VU-29. If this is the case, it is not yet clear whether these sites physically overlap or whether they are physically distinct but functionally interact through allosteric mechanisms. While the weight of evidence suggests that these allosteric modulators interact with different binding sites, it is important to consider that until the binding pockets for each of these compounds is fully elucidated, it is impossible to definitively determine whether these compounds bind to the same or distinct binding sites. For instance, if the interaction of each of the allosteric modulators at mGlu5 has not reached equilibrium, this could influence the nature of the shifts in PAM CRCs that are observed with neutral ligands (26). Also, the lack of apparent binding to the MPEP site could be explained by very high cooperativity such that occupancy of less than 0.1% of the receptor leads to full potentiation of the response to glutamate. Finally, allosteric ligands could bind to a single monomer in a functional homodimer, and this could impact the pharmacological profile. While unlikely, it is important to consider these possibilities until we have definitive

Figure 14. VU0365396 competitively inhibits VU0357121 function but has no effect on CPPHA activity. (A) VU0365396 induces a parallel rightward shift in the VU0357121 concentration–response curve, indicating a competitive interaction between the two compounds. Cells expressing mGlu5 were treated with increasing concentrations of VU0357121 and either vehicle or VU0365396 (30 μM, Δ) followed by EC20 glutamate. (B) The calcium mobilization response produced when mGlu5-expressing HEK293 cells were treated with increasing concentrations of CPPHA and EC20 glutamate do not differ in the absence or presence of VU0365396 (30 μM). Data were obtained from three separate experiments, each performed in duplicate, and are expressed as the mean ± SEM. Data were normalized to the average maximum response obtained from each experiment as determined by 100 μM glutamate. Concentration–response curves were generated by nonlinear curve fitting.

Figure 15. Mutations that inhibit the function of MPEP/VU-29 also inhibit VU0357121 function, but mutations that inhibit CPPHA activity do not. The ability of VU0357121 (1 μM) to function when specific mGlu5 mutants were transiently expressed in HEK cells was assessed. The calcium mobilization assay was used to determine whether the mGlu5 PAM was able to enhance the glutamate concentration–response relationship at either the A809V/mGlu5 mutant or the F585I/mGlu5 mutant. The A809V mutation led to an inhibition of VU0357121 activity (A), whereas the F585I mutation had no effect on its function (B). Concentration–response relationships were generated by adding either vehicle or a fixed concentration (1 μM) of VU0357121 to transiently transfected HEK cells, followed by increasing concentrations of glutamate. Data were obtained from three separate experiments, each performed in duplicate, and are expressed as the mean ± SEM. Data were normalized to the average maximum response obtained from each experiment as determined by 100 μM glutamate.
structural information on mGlu5 and the allosteric binding sites.

**Methods**

**Compounds**

The Vanderbilt High Throughput Screening Center compound collection was obtained from ChemBridge Corporation (San Diego, CA) and ChemDiv, Inc. (San Diego, CA). L-Glutamate and MPEP were obtained from Tocris Bioscience (Ellisville, MO). [3H]methoxyPEP was obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO). CPPHA (17) and 5-MPEP (23) were synthesized as previously described.

**Chemical Synthesis**

**General.** All NMR spectra were recorded on a Bruker 400 MHz instrument. 1H chemical shifts are reported in δ values in ppm relative to CDCl3 at 7.27 ppm. Data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, br = broad, and m = multiplet), coupling constant (Hz), and integration. Low resolution mass spectra were obtained on an agilent 1200 series. Analytical thin layer chromatography was performed on Analtech silica gel GF 250 μm plates. Analytical HPLC was performed on an agilent 1200 series. Analytical HPLC was performed on an agilent 1200 series. Preparative purification was performed on a combi-flash companion. Solvents for extraction, washing, and chromatography were of HPLC grade. All reagents were purchased from Aldrich Chemical Co. and were used without purification.

**VU001850**

To a solution of 2-amino-5-chloropyridine (129 mg, 1.0 mmol) in dichloromethane (5 mL) was added N,N-diisopropylamidine (174 μL, 1.0 mmol), and chlorodipyrrolidinocarbenium hexafluorophosphate (PyClU) (365 mg, 1.0 mmol) under an argon atmosphere. The reaction was stirred for 1 h at ambient temperature. Saturated sodium bicarbonate (1 mL) was added and the reaction mixture filtered through an ISP phase separator. The organics were concentrated and purified by Gilson HPLC to afford the benzamide as a white crystalline solid (260 mg, 86%) upon concentration. 1H NMR (400 MHz, CDCl3) δ 8.02 (s, 1H), 7.21 (d, J = 8.8, 2H), 7.75 (d, J = 8.8, 2H), 6.85 (d, J = 8.8, 2H), 6.46 (d, J = 8.8, 1H), 4.50 (bs, 1H), 3.98 (t, J = 6.4, 6.4, 2H), 1.80–1.73 (m, 2H), 1.51–1.46 (m, 2H), 0.98 (t, J = 7.6, 7.4, 3H); LC-MS (214 nM) 2.0 min, m/z 306.2.

**VU0365396**

To a solution of 2,6-difluoroaniline (101 μL, 1.0 mmol) in dichloromethane (5 mL) was added N,N-diisopropylamidine (174 μL, 1.0 mmol) and 4-butoxybenzoyl chloride (189 μL, 1.0 mmol), and the reaction was stirred for 45 min at ambient temperature. Saturated sodium bicarbonate (1 mL) was added and the reaction mixture filtered through an ISP phase separator. The organics were concentrated and purified by Gilson HPLC to afford the benzamide as a white crystalline solid (285 mg, 93%) upon concentration. 1H NMR (400 MHz, CDCl3) δ 7.85 (d, J = 8.8, 2H), 6.99–6.88 (m, 3H), 4.04 (t, J = 6.4, 6.4, 2H), 1.84–1.77 (m, 2H), 1.56–1.47 (m, 2H), 1.00 (t, J = 7.6, 7.2, 3H); LC-MS (214 nM) 2.0 min, m/z 288.2.

**VU0357121**

To a solution of 2,4-difluoroaniline (101 μL, 1.0 mmol) in dichloromethane (5 mL) was added N,N-diisopropylamine (174 μL, 1.0 mmol) and 4-butoxybenzoyl chloride (189 μL, 1.0 mmol), and the reaction was stirred for 45 min at ambient temperature. Saturated sodium bicarbonate (1 mL) was added and the reaction mixture filtered through an ISP phase separator. The organics were concentrated and purified by Gilson HPLC to afford the benzamide as a white crystalline solid (290 mg, 95%) upon concentration. 1H NMR (400 MHz, CDCl3) δ 8.10 (d, J = 8.8, 2H), 7.01–6.95 (m, 5H), 4.06 (t, J = 6.4, 6.4, 2H), 1.85–1.75 (m, 2H), 1.57–1.46 (m, 2H), 1.00 (t, J = 7.6, 7.2, 3H); LC-MS (214 nM) 2.0 min, m/z 306.2.

**VU0125936**

To a solution of 2-amino-5-chloro-pyridine (129 mg, 1.0 mmol) in dichloromethane (5 mL) was added N,N-diisopropylamine (174 μL, 1.0 mmol) and 4-butoxybenzoyl chloride (189 μL, 1.0 mmol), and the reaction was stirred for 45 min at ambient temperature. Saturated sodium bicarbonate (1 mL) was added and the reaction mixture filtered through an ISP phase separator. The organics were concentrated and purified by Gilson HPLC to afford the benzamide as a white crystalline solid (200 mg, 86%) upon concentration. 1H NMR (400 MHz, CDCl3) δ 8.02 (s, 1H), 7.21 (d, J = 8.8, 2H), 7.75 (d, J = 8.8, 2H), 6.85 (d, J = 8.8, 2H), 6.46 (d, J = 8.8, 1H), 4.50 (bs, 1H), 3.98 (t, J = 6.4, 6.4, 2H), 1.80–1.73 (m, 2H), 1.51–1.46 (m, 2H), 0.98 (t, J = 7.6, 7.4, 3H); LC-MS (214 nM) 2.0 min, m/z 305.2.

**Cell Culture**

HEK293 cells stably expressing rat mGlu5 were grown in standard Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM l-glutamine, 20 mM HEPES, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 1× antibiotic-antimycotic (all from Invitrogen, Carlsbad, CA), and 500 μg/mL L-G418 sulfate from Mediatech, Inc. (Manassas, VA), at 37 °C in the presence 5% CO2.

**Fluorescence-Based Calcium Flux Assay**

Screening assays were completed within Vanderbilt University’s HTS laboratory and were performed as previously described (18–20). Hits from the primary screen including VU0001850, VU0003712, and VU0040237 were confirmed by testing for concentration-dependent activity on mGlu5 over a range of 4 log units including 10 μM, as depicted in Figure 2. Compounds were serially diluted 1:3 into 10-point
concentration–response curves (30 μM–1 nM final), transferred to daughter plates using the Echo acoustic plate reformat (Labcyte, Sunnyvale, CA), and tested as described in the primary screen except that test compounds were applied for 300 s followed by addition of EC20 concentrations of glutamate. All other mGlu5 calcium assays were performed in the following manner. Human embryonic kidney (HEK293) cells stably expressing mGlu5 were plated in black-walled, clear-bottomed, poly-D-lysine coated, 96-well plates (BD Bio-
coat, Bedford, MA) in 100 μL glutamate/glutamine-free 
medium (glutamine-free DMEM plus 10% dialyzed fetal bovine serum; Invitrogen) at 30,000 cells per well at least 24 h 
before the assay. Cells were incubated overnight at 37 °C in the presence of 5% CO2. After 19 h, followed by 20 μL of vehicle or 
test compound diluted into assay buffer to a 4× solution followed by EC20 glutamate. To determine the concentration–response 
relationships of the mGlu5 PAMs, cells received increasing concentrations of the test compound (10 μM or vehicle, followed 
by a concentration of glutamate that elicited a response that was 80% of the maximum glutamate response (EC80). To 
determine the concentration–response relationships of the mGlu5 PAMs, cells received increasing concentrations of the 
test compound, followed by stimulation with EC20 glutamate. To determine the effect of the mGlu5 PAMs on the glutamate 
dose–response relationship, cells received the test compound (12.5 μM or vehicle) followed by increasing concentrations of 
glutamate (with a top concentration of 100 μM). To screen for 
neutral allosteric site ligand activity, cells received increasing concentrations of 5-MPEP 
(333 nM to 10 μM), a fixed concentration of VU0365396 (30 μM), or vehicle prepared in the same 4× solution as 
inwardly rectifying potassium (GIRK) channels, a method 
that has been described in detail (21). These cell lines were 
grown in growth media containing 45% DMEM, 45% F-12, 
10% FBS, 20 mM HEPES, and 25 mM magnesium sulfate, 1.8 mM calcium sulfate, 5 mM glucose, 
12 mM thallium sulfate, and 10 mM HEPES) at 5× the final 
concentration to be assayed. Data were analyzed as pre-
viously described (21).

Radioligand Binding Assay

The allosteric antagonist MPEP analogue [3H]metoxo-
PEPy was used to evaluate the ability of test compounds to interact with the MPEP site on mGlu5 (22). Membranes were 
prepared from rat mGlu5 HEK293 cells (23). Compounds 
were diluted in assay buffer (50 mM Tris/0.9% NaCl, pH 7.4) 
to a 5× stock, and 100 μL of the test compound was added to 
each well of a 96 deep-well assay plate. Three hundred 
microliter aliquots of membranes diluted in assay buffer 
(20 μg/well) were added to each well. One hundred microliter of [3H]methoxyPEPy (2 nM final concentration) was added, 
and the reaction was incubated at room temperature for 1 h 
with shaking. After the incubation period, the membrane-
bound ligand was separated from free ligand by filtration 
through glass-fiber 96-well filter plates (Unifilter-96, GF/B, PerkinElmer Life and Analytical Sciences, Boston, MA). The 
contents of each well were transferred simultaneously to the 
filter plate and washed 3–4 times with assay buffer using a cell 
harvester (Brandel Cell Harvester, Brandel Inc., Gaithersburg, MD). Forty microliter of scintillation fluid was added to each 
well and the membrane-bound radioactivity determined by 
scintillation counting (TopCount, PerkinElmer Life and Analytical Sciences). Nonspecific binding was estimated using 
5 μM MPEP. Concentration–response curves were generated using a four parameter logistical equation in GraphPad Prism.

Phosphoinositide (PI) Hydrolysis Assay

HEK cells expressing mGlu5 were plated at 150,000 cells 
per well in the glutamate/glutamine-free assay medium de-
tailed above in 24-well plates 24 h prior to assay. Sixteen hours
prior to experiments, the cells received new assay media containing 1 μCi/mL [3H]myo-inositol (Perkin-Elmer LAS). On the day of the assay, cells were treated with vehicle or a fixed concentration of 5-MPEP (10 μM) in HBSS (containing 110 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, 25 mM glucose, and 58.4 mM sucrose at pH 7.4) supplemented with 20 mM HEPES and 30 mM LiCl, replacing the [3H]myo-inositol-containing media. Following vehicle or neutral ligand addition, a range of concentrations of VU0357121 were added, and cells were incubated for 1 h at 37 °C and 5% CO2. The accumulation of phosphoinositides was terminated by aspiration of ligand-containing buffer, followed by the addition of 10 mM formic acid (1 mL/well) and incubation for 1 h at room temperature to ensure the extraction of phosphoinositides. Columns packed with a 1 mL bed of AG 1-X8 Resin 100–200 mesh anion-exchange resin (formate form) (Bio-Rad; Hercules, CA) were washed twice with 10 mL of water. Following the water washes, the entire 1 mL sample volumes were added to the columns, avoiding the transfer of any cells. Columns were washed with 10 mL of water, followed by 10 mL of 5 mM myo-inositol. Total phosphoinositides (PIs) were eluted with 10 mL of 0.1 M formic acid/0.2 M ammonium formate into vials containing 3a70B liquid scintillation cocktail (Research Products International; Elk Grove Village, IL), and the radioactivity was measured by liquid scintillation counting. Following use, columns were regenerated with 10 mL of 0.1 M formic acid/1 M ammonium formate and washed with 30 mL of water. Data were corrected for baseline levels, normalized to the maximum response to 1 mM glutamate, and analyzed with GraphPad Prism to determine EC50 values.

**Transient Transfections**

HEK293 cells were grown in standard Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 20 mM HEPES, and 1× antibiotic–antimycotic. Cells were collected and plated in 100 mm tissue culture dishes (Costar; Corning Life Sciences, Acton, MA) in normal growth medium overnight before transfection. The next day, cells were transiently transfected with mutant forms of mGlu5 cDNA using FuGENE 6 (Roche Applied Science, Indianapolis, IN) according to the manufacturer’s instructions (2 μg of DNA, 24 μL of FuGENE 6, and 776 μL of Opti-MEM (Invitrogen, Carlsbad, CA)). The next day, cells were plated in black-walled, clear-bottomed, 96-well plates (Costar; Corning Life Sciences, Acton, MA) but pretreated with poly-D-lysine (Sigma, St. Louis, MO). Calcium assays were performed on FlexStation II, as described above, on the following day to study the effect of VU0357121 (1 μM) on the glutamate concentration–response activity of each mutant.

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**Author Contributions**

P.J.C. and C.W.L. oversaw and designed the molecular pharmacology and chemistry, respectively. A.R. oversaw the high-throughput screening and compound confirmation, as well as the molecular pharmacology. C.N. provided the selectivity data for mGluRs 2, 3, 7 and 8. A.H. performed all of the other in vitro pharmacology assays and the initial medicinal chemistry synthesis. K.G. performed PI hydrolysis studies. S.T. performed additional medicinal chemistry and characterized the compounds.

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**Abbreviations**

PAM, positive allosteric modulator; mGlu5, metabotropic glutamate receptor subtype 5; CPPHA, N-[4-[3-chloro-2-[(1,3-dioxo-1,3-dihydro-2H-isoindol-2-yl)methylphenyl]-2-hydroxybenzamide; NAM, negative allosteric modulator; MPEP, 2-methyl-6-(phenethyl)pyridine; SAR, structure–activity relationship; HTS, high-throughput screen; VU-0001850, N-(5-chloropyridin-2-yl)-4-propoxybenzamide; VU0040237, 4-butoxy-N-(2-fluorophenyl)benzamide; VU-357121, 4-butoxy-N-(2,4-difluorophenyl)benzamide; VU036-5396, 4-butoxy-N-(2,6-difluorophenyl)benzamide; CNS, central nervous system; iGluR, ionotropic glutamate receptor; mGluR, metabotropic glutamate receptor; GPCR, guanine nucleotide binding protein coupled receptor; cAMP, cyclic adenosine monophosphate; DFB, 3',3'-difluorobenzaldazine; MPPA, N-methyl-5-(phenethyl)pyrimidin-2-amine; CDPPB, 3-cyano-N-(1,3-diphenyl-1H-pyrazol-5-yl)benzamide; VU-29, 4-nitro-N-(1,3-diphenyl-1H-pyrazol-5-yl)benzamide; ADX27723, S-(4-fluoro-phenyl)-[3-[3-(4-fluoro-phenyl)-1,2,4]oxadiazol-5-yl]-piperidin-1-yl)-methanone; (S)-FTOPM, (S)-4-fluoro-phenyl)-[3-(3-thiophen-2-yl)-1,2,4-oxadiazol-5-yl]-piperidin-1-yl)methanone; (S)-FP0PM, (S)-(4-fluoro-phenyl)-[3-(3-pyridin-2-yl)-1,2,4-oxadiazol-5-yl]-piperidin-1-yl)methanone; CHO, Chinese hamster ovary; HEK293, human embryonic kidney; ERK1/2, extracellular signal-regulated kinases 1 and 2; FDSS, functional drug screening system; VU0003712, N-(5-chloropyridin-2-yl)-4-(2,2,2-trifluoroethoxy)methyl)benzamide; VU0365393, 4-butoxy-N-(2,5-difluorophenyl)benzamide;
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