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Chemical Modulation of Mutant $mGlu_1$ Receptors Derived from Deleterious *GRM1* Mutations Found in Schizophrenics

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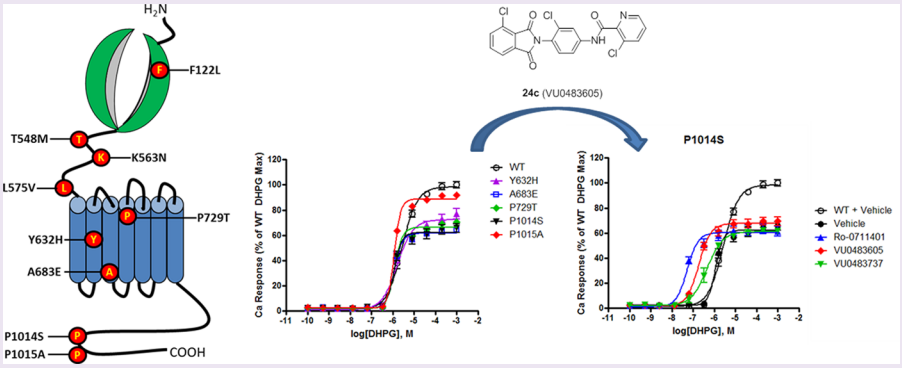
Supporting Information



ABSTRACT: Schizophrenia is a complex and highly heterogeneous psychiatric disorder whose precise etiology remains elusive. While genome-wide association studies (GWAS) have identified risk genes, they have failed to determine if rare coding single nucleotide polymorphisms (nsSNPs) contribute in schizophrenia. Recently, two independent studies identified 12 rare, deleterious nsSNPS in the *GRM1* gene, which encodes the metabotropic glutamate receptor subtype 1 ($mGlu_1$), in schizophrenic patients. Here, we generated stable cell lines expressing the $mGlu_1$ mutant receptors and assessed their pharmacology. Using both the endogenous agonist glutamate and the synthetic agonist DHPG, we found that several of the mutant $mGlu_1$ receptors displayed a loss of function that was not due to a loss in plasma membrane expression. Due to a lack of $mGlu_1$ positive allosteric modulators (PAM) tool compounds active at human $mGlu_1$, we optimized a known $mGlu_4$ PAM/$mGlu_1$ NAM chemotype into a series of potent and selective $mGlu_1$ PAMs by virtue of a double "molecular switch". Employing $mGlu_1$ PAMs from multiple chemotypes, we demonstrate that the mutant receptors can be potentiated by small molecules and in some cases efficacy restored to that comparable to wild type $mGlu_1$ receptors, suggesting deficits in patients with schizophrenia due to these mutations may be amenable to intervention with an $mGlu_1$ PAM. However, in wild type animals, $mGlu_1$ negative allosteric modulators (NAMs) are efficacious in classic models predictive of antipsychotic activity, whereas we show that $mGlu_1$ PAMs have no effect to slight potentiation in these models. These data further highlight the heterogeneity of schizophrenia and the critical role of patient selection strategies in psychiatric clinical trials to match genotype with therapeutic mechanism.

Schizophrenia, schizo-affective disorder, and bipolar disorder are heterogeneous, heritable psychiatric diseases with significant overlap in terms of genetic origins and clinical presentation. The exact etiology of the disease is mired in complex receptor and circuitry dysfunction arising from multiple genetic and environmental factors.[1−6] Two dominant hypotheses have guided therapeutic development: the "dopamine hypothesis" and the "NMDA receptor hypofunction hypothesis";[1−11] however, these receptors are also functionally linked through sophisticated signaling complexes by scaffolding proteins, thus connecting the two, seemingly distinct, approaches.[12,13] Moreover, genome-wide association studies (GWAS) of schizophrenia have identified a number of risk genes (e.g., *DISC1*, *COMT*),[14,15] single-nucleotide polymorphisms (SNPs), rare *de novo* copy number variations (CNVs), and increased deletions and/or duplications at coding regions.[16−20] Recently, Frank and co-workers[21] examined rare coding single nucleotide polymorphisms (nsSNPs), not detected by CNV scans and SNP arrays, as possible contributors to psychiatric disease. DNA sequencing of 10 hub genes, from both schizophrenics and controls, identified a cluster of disease only nsSNPs, with the most significant being

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GRM1, the gene encoding the metabotropic glutamate receptor subtype 1 ($mGlu_1$); importantly, this is the first study genetically linking $mGlu_1$ with schizophrenia.[21] A subsequent study by Ayoub et al.[22] confirmed the presence of deleterious *GRM1* mutations in schizophrenia but also found that families with these mutations were also affected by multiple neuropsychiatric conditions, such as depression, anxiety, drug abuse, and epilepsy. The nsSNP mutations identified (Figure 1) were

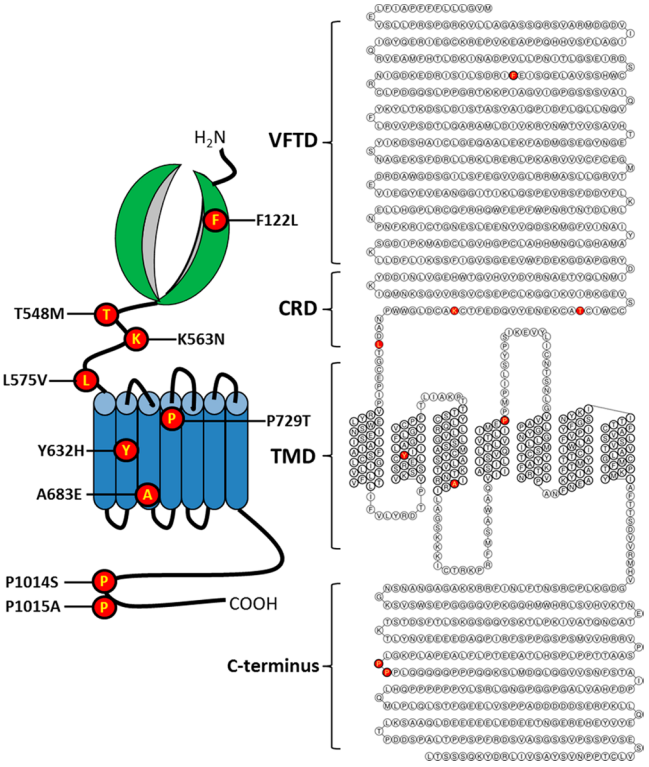

Figure 1. Representative (9 of 12) non-synonymous single nucleotide polymorphisms (nsSNPs) in $mGlu_1$ found in schizophrenia patients that are evaluated in this study. Schematic structure and snake plot of $mGlu_1$ are presented. nsSNPs are shown in red circles at their approximate location. VFTD: venus flytrap domain. CRD: cysteine-rich domain. TMD: transmembrane domain.

predicted to have deleterious effects on receptor function, and preliminary data with a functional quisqualate phosphoinositide hydrolysis (IP_1) assay suggested that, in transiently transfected COS-1 cells, receptors encoding these mutations displayed loss of function with no to minimal loss in plasma membrane expression using ELISA with an anti-$mGlu_1$ antibody.[21,22] Prior to these studies, $mGlu_1$ was not regarded as a major schizophrenia target, as opposed to its associated family member $mGlu_5$;[23] however, observations from post-mortem schizophrenic brains (increased expression of $mGlu_1$ due to NMDA hypofunction), sensorimotor gating deficits (PPI) in *GRM1* knockout mice, and the known role in both potentiating NMDA receptor function as well as synaptic plasticity have raised the possibility that $mGlu_1$ may also play a role.[24−26]

Based on our longstanding interest in schizophrenia, we pursued a more detailed pharmacological characterization of nine of the 12 mutant $mGlu_1$ receptors derived from these deleterious *GRM1* mutations found in schizophrenics, and demonstrated that the mutations are indeed loss of function mutations that can be rescued with novel, highly selective $mGlu_1$ positive allosteric modulators (PAMs), derived from a "double molecular switch"[27] of an $mGlu_4$ PAM chemotype.[28] The ligands developed show improvements over the one existing $mGlu_1$ PAM tool compound and enabled *in vivo* studies in a preclinical antipsychotic model. Results from these studies further emphasize the importance of patient selection for highly heterogeneous diseases such as schizophrenia.

RESULTS AND DISCUSSION

Pharmacological Characterization of the Schizophrenic $mGlu_1$ Mutant Receptors. Earlier work had employed transiently transfected cell lines[21,22] and assessed functional activity based on quisqualate-induced PI hydrolysis, a pathway known to be subject to ligand bias.[29] In order to fully characterize the pharmacology of the schizophrenic (Sz) $mGlu_1$ mutant receptors, we prepared stable cell lines carrying tetracycline-inducible human $mGlu_1$ wild-type (WT) and each of the nine mutations identified in schizophrenics (T548M, K653N, L575V, F122L, Y632H, A683E, P729T, P1014S, and P1015A) (Figure 1) in T-REx HEK-293 cells. As alluded to above, ligand bias and probe-dependence of allosteric modulator effects at $mGlu_1$ suggest different modes of receptor-agonist interactions;[29,30] therefore, to accurately assess the pharmacology of the nine schizophrenia mutants, we employed both the endogenous agonist glutamate, as well as the synthetic orthosteric agonist (*S*)-3,5-dihydroxyphenylglycine (DHPG). Figure 2 highlights the calcium response of the WT and nine mutant human $mGlu_1$ receptors upon stimulation with increasing concentrations of either glutamate or DHPG. When glutamate was used as the agonist (Figure 2A and B), all nine of the mutant $mGlu_1$ receptors displayed a loss of function, as judged by diminished calcium response, relative to $mGlu_1$ WT (mutants typically induced 45−65% of the WT response with the exception of K563N and P1015A, at ∼90% of WT response). With the synthetic group I mGlu agonist DHPG (Figure 2C and D), once again, the majority of mutant $mGlu_1$ receptors display a loss of function relative to $mGlu_1$ WT (typically 55−75% of WT response); however, K563N displays a slight gain of function (∼110% of WT), and P1015A only exhibits a slight diminution in activity (∼90% of WT response), suggesting some role for ligand bias across the mutants. Combined with the quisqalate-induced PI hydrolysis data, it is clear that the mutant $mGlu_1$ receptors generally display a loss of function across multiple agonists, and signaling pathways. It is possible that this loss of functional response was due to reduced receptor expression at the cell membrane. As mentioned earlier, data in transiently transfected mutant COS-7 cell lines using an ELISA-based read-out with $mGlu_1$ antibodies revealed that the majority of mutants retained plasma membrane expression comparable to WT $mGlu_1$.[21,22] We employed an independent assessment for plasma membrane expression of the nine mutants using our stable cell lines and a complementary method. Here, we biotinylated the cell surface of the nine mutant receptor-expressing and WT $mGlu_1$ cell lines, followed by capture with streptavidin resin and standard Western blotting with an $mGlu_1$-specific antibody. As shown in Figure 3, none of the mutants displayed reduced cell surface expression relative to WT $mGlu_1$, and, in fact, several displayed increased expression, despite a loss of calcium signaling. Again, the combined data strongly indicate that the nine mutant human $mGlu_1$ receptors are loss-of-function mutations with no loss of plasma membrane/cell surface expression.

Development of Novel $mGlu_1$ PAMs. Chemical modulation of $mGlu_1$, much like $mGlu_5$, cannot be effectively

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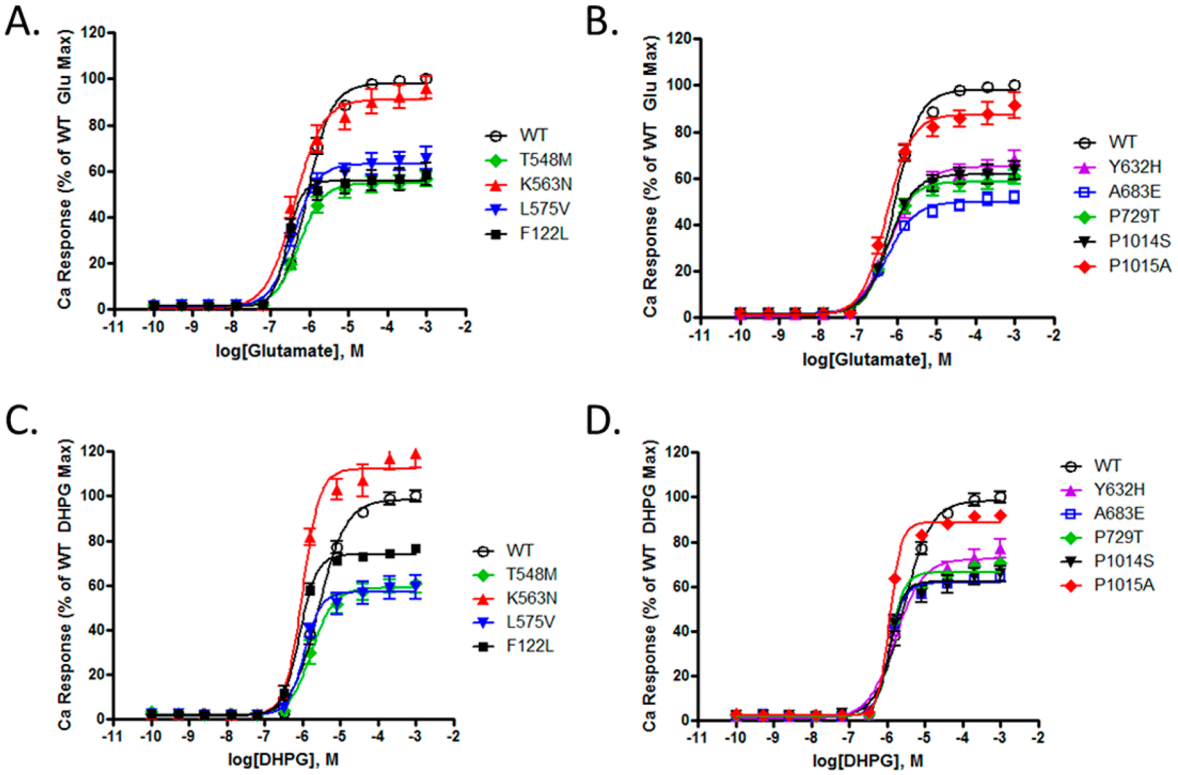

Figure 2. Calcium signaling induced by $mGlu_1$ receptors encoding mutations associated with schizophrenia is reduced compared to WT. The functional activity of the $mGlu_1$ schizophrenia (Sz) mutant receptors was examined in Ca mobilization assay. Stable cell lines carrying tetracycline-inducible $mGlu_1$ WT or Sz mutants in T-REx-293 cells were incubated with 20 ng/mL or 1 μg/mL TET for WT and all mutants, respectively, overnight to induce $mGlu_1$ expression. Cells were stimulated with the increasing amounts of pan mGlu receptor agonist, glutamate (A and B), or the group I mGlu-selective agonist, DHPG (C and D). The concentration−response curves were generated by normalizing the calcium responses to the % of the WT maximal response of each agonist. Data represent at least three independent experiments performed in duplicates.

mediated by orthosteric, "glutamate-like" agonists *in vivo*, due to the liability of excitotoxicity and seizures as a side effect.[23,31−33] In the case of $mGlu_5$, these concerns have been shown to be mitigated by mimicking physiological conditions and potentiating the receptor with a positive allosteric modulator (PAM). A multitude of $mGlu_5$ PAMs have been reported across literally dozens of chemotypes targeting multiple, distinct allosteric binding sites with robust *in vivo* efficacy and safety margins.[23] However, $mGlu_1$ is far less developed, and only five $mGlu_1$ PAMs have been reported (Figure 4).[31−33] Knoflach and co-workers from Roche pioneered the field in the early 2000s,[34] developing the first four $mGlu_1$ PAMs, **1** (Ro 01-6128), **2** (Ro 67-4853), **3** (Ro 67-7476), and **5** (Ro 07-11401)[35,36] with EC_{50s} in the 56 to 200 nM range; however, only **2** and **5** were active at both human and rat $mGlu_1$, highlighting a serious concern with species differences that was only recently understood in the studies characterizing the $mGlu_1$ NAM tool compounds.[30−36] In addition, the drug metabolism and pharmacokinetic (DMPK) profile of **5** was not optimal for *in vivo* studies (*vide infra*). Conn and co-workers later identified (VU-71) **4**, derived from the prototypical $mGlu_5$ PAM chemotype, as a weak rat $mGlu_1$ PAM (EC_{50} = 2.4 μM) with no activity at the human receptor.[37] In contrast, a wealth of $mGlu_1$ NAMs have been reported (**6**−**10**, Figure 4), with a range of species/ligand bias that can be used to explore the ability of NAMs to noncompetitively inhibit the mutant receptors.[31,32] Again, to fully evaluate the ability of small molecules to modulate the mutant $mGlu_1$ receptors, we needed to develop $mGlu_1$ PAMs in alternative chemotypes, beyond **5**, to ensure caveats with species differences and ligand bias did not preclude accurate interpretations and mask physiological responses.

In lieu of an $mGlu_1$ PAM high throughput screen (HTS), we pursued several avenues to identify novel $mGlu_1$ PAMs. A multidimensional, iterative parallel synthesis effort was employed to synthesize and screen several hundred analogs of **4**, resulting in little tractable SAR and no improvement in rat $mGlu_1$ PAM potency, and with no gain of human $mGlu_1$ activity; thus, this approach was discontinued. In parallel, we considered the phenomenon of "molecular switches" within allosteric ligands,[27] wherein subtle structural modifications can modulate modes of ligand pharmacology and/or subtype selectivity. Historically, there was evidence for cross-talk between the allosteric sites on the group III mGlu receptor, $mGlu_4$ and the group I mGlu receptor, $mGlu_1$ (Figure 5).[38] The first $mGlu_4$ PAM, (−)-PHCCC (**11**, EC_{50} = 1.4 μM) was highly selective against all the mGlu subtypes except $mGlu_1$, where it was an equipotent $mGlu_1$ NAM (IC_{50} = 2.1 μM). Incorporation of a "molecular switch" in the form of aza-congener **12** (VU0359516) not only enhanced PAM activity at $mGlu_4$ (EC_{50} = 380 nM) but also abolished activity at $mGlu_1$ (IC_{50} > 30 μM).[38] This observation led us to mine mGlu selectivity data from our $mGlu_4$ PAM program, where we had prepared hundreds of analogs around **13** (VU0400195, ML182), a potent and highly selective $mGlu_4$ PAM (EC_{50} = 291 nM).[28] This exercise led to the development of a highly selective $mGlu_1$ NAM **14** (VU0465334) within this chemotype ($mGlu_1$ IC_{50} = 220 nM, >10 μM versus $mGlu_4$) via a

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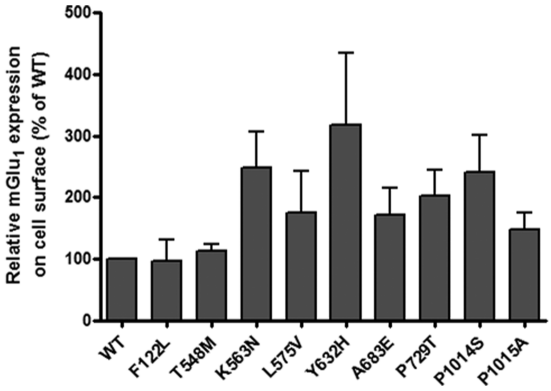

Figure 3. Decreased calcium signaling found in the $mGlu_1$ mutants associated with schizophrenia is not due to a loss of plasma membrane receptor expression. Biotinylation of cell surface membrane receptors was performed to compare the expression levels of the mutant receptors with the WT receptor. The day before biotinylation, cells expressing $mGlu_1$ WT and Sz mutants were treated in the same manner as shown in the calcium mobilization assay. Biotin labeling of the cell surface receptors was performed at 4 °C, and the biotin-labeled proteins were captured by streptoavidin resins. The streptoavidin-bound receptors were solubilized in a SDS sample buffer containing 150 mM dithiothreitol, and then, the proteins were resolved by 8% SDS-PAGE. The $mGlu_1$ expression was detected by Western blotting using an $mGlu_1$-specific antibody (clone 20, BD transduction Laboratories). Note that none of the Sz mutant receptors expressed at lower levels than the WT regardless of the calcium response shown in Figure 2. Data are represented as % of WT cell surface expression from four independent experiments (Mean ± SEM), two of which were concomitantly performed with the calcium assay.

"molecular switch" modification to the imide moiety.[30] Further mining identified **15** (VU0405623), possessing a substituted phthalimide moiety with potent, dual PAM activity at both $mGlu_4$ (EC_{50} = 61 nM) and $mGlu_1$ (EC_{50} = 75 nM), in essence a double "molecular switch", and a lead compound from which to develop a new, selective $mGlu_1$ PAM chemotype, distinct from Roche's **5**,[34−36] if $mGlu_4$ PAM activity could be eliminated.

Analogs of **15** were readily prepared in three synthetic steps from commercial **16a** (Scheme 1) following a divergent plan. A diversity library was developed by first treating **16** with phthalic anhydride to generate phthalimide **17** in 88% yield. Reduction of the nitro group and acylation under standard HATU conditions provided amide analogs **19** (structure−activity relationship (SAR) presented in Table 1), exploring diverse moieties, in good overall yields. Once the optimal amide moieties were identified in **19**, further analogs **22** were accessed by acylation of **16b** with picolinic acids under HATU conditions to deliver **20**. Subsequent nitro reduction and condensation with different phthalic anhydrides afforded functionalized phthalimide congeners **22** (SAR presented in Table 2), in good overall yields for the three step sequence.

As shown in Table 1, new analogs of **19** were screened against human and rat $mGlu_1$ as well as $hmGlu_4$, generating significant, tractable SAR with ideal compounds showing equipotent activity at both human and rat $mGlu_1$ to support future translational studies. The pyridyl isomers **19a−19c** demonstrated that the 2-position/picolinamides (**19a**) was essential for activity, with the 3- (**19b**) and 4-position (**19c**) congeners inactive at both $mGlu_1$ and $mGlu_4$. Interestingly, pyridazine congeners **19e** and **19f** were inactive, whereas the pyrazine derivative **19d** retained activity across human and rat $mGlu_1$ and $mGlu_4$. Functionalized picolinamides **19g−19o** were uniformly active at both $mGlu_1$ species, yet abolished activity at $mGlu_4$ (EC_{50}s >10 μM). Subsequent libraries held the 3-methyl picolinamide (**19k**) or 3-chloropicolinamide (**19l**) constant and surveyed alternative phthalimide moieties, producing analogs **23** and **24**, respectively, as human $mGlu_1$ PAMs (Table 2). Of these analogs, incorporation of a chlorophthalimide proved optimal, providing **23c** (VU0483737) and **24c** (VU0483605) (Figure 6). **23c** (VU0483737) is a potent $mGlu_1$ PAM at both human (EC_{50} = 370 nM) and rat (EC_{50} = 367 nM, pEC_{50} = 6.43 ± 0.13, 118 ± 7% Glu Max) and a very low efficacy $mGlu_4$ PAM (EC_{50} = 199 nM, 55% Glu Max). **24c** (VU0483605) proved superior with excellent $mGlu_1$ PAM activity at both human (EC_{50} = 390 nM) and rat (EC_{50} = 356 nM, pEC_{50} = 6.45 ± 0.11, 113 ± 5% Glu Max) and no activity as an $mGlu_4$ PAM (EC_{50} >10 μM). Thus, data mining, coupled with identification of a double "molecular switch", enabled the development of two novel $mGlu_1$ PAMs, equipotent against both human and rat $mGlu_1$, and selective against $mGlu_4$, despite originating from a potent and highly selective $mGlu_4$ chemotype. These two new compounds **23c** and **24c**, along with the Roche $mGlu_1$ PAM **5** and a cadre of $mGlu_1$ NAMs, afforded the requisite tools to explore chemical modulation of the schizophrenic $mGlu_1$ mutant receptors.

Small Molecule Modulation of the Schizophrenic $mGlu_1$ Mutant Receptors. As the mutant receptors displayed a loss of function in our standard calcium mobilization assay (Figure 2), we evaluated the ability of the $mGlu_1$ PAMs **5**, **23c**, and **24c** to potentiate the response of an increasing concentration of either glutamate (Figure 7) or the synthetic agonist DHPG (Figure 8) in the stable $mGlu_1$ WT and mutant cell lines. Figure 7A shows that all three PAMs potentiate WT $mGlu_1$, inducing a parallel left-ward shift of the glutamate concentration response curve (CRC), with **5** and **24c** showing 7.4- and 6.3-fold shifts respectively at 10 μM, while **23c** was only ~3-fold. Each of the nine mutants is profiled in Figure 7B-J, and all three PAMs are able to potentiate the decreased calcium response in the $mGlu_1$ mutants, and in the case of K563N and P1015A mutants, which show minimal decreases in calcium signaling, efficacy (%Glu Max) was also restored to the level of WT $mGlu_1$. As expected based on the data in Figure 2, the three PAMs exhibit a more robust potentiation of DHPG across both WT and mutant $mGlu_1$ receptors (Figure 8). Here, **5** displays a 16.6-fold shift at WT $mGlu_1$, whereas **24c** was 6.6-fold and **23c** was 3.4-fold. Once again, across all the mutant $mGlu_1$ receptors, the three PAMs potentiate the decreased calcium response. Hence, it is clear that the mutant $mGlu_1$ receptors can be chemically modulated with small molecule $mGlu_1$ PAMs to partially restore decreased calcium responses. These data suggest that chemical intervention with $mGlu_1$ PAMs in schizophrenic patients harboring these mutations could potentially be beneficial.

Finally, to complete the pharmacological characterization of the mutant $mGlu_1$ receptors, we examined the effect of an $mGlu_1$ NAM in high efficacy (K653N) and moderate efficacy (A683E) $mGlu_1$ mutant lines and compared it then to WT $mGlu_1$ (Supporting Information Figure 1). Cells were pre-treated with 1 μM of potent $mGlu_1$ NAM **7**, and immediately, cells were stimulated by glutamate or DHPG. In all cases, noncompetitive antagonism (decrease in % Glu Max) was noted, highlighting the fact that the mutant receptors behave as WT $mGlu_1$ in the presence of an $mGlu_1$ NAM. Thus, the

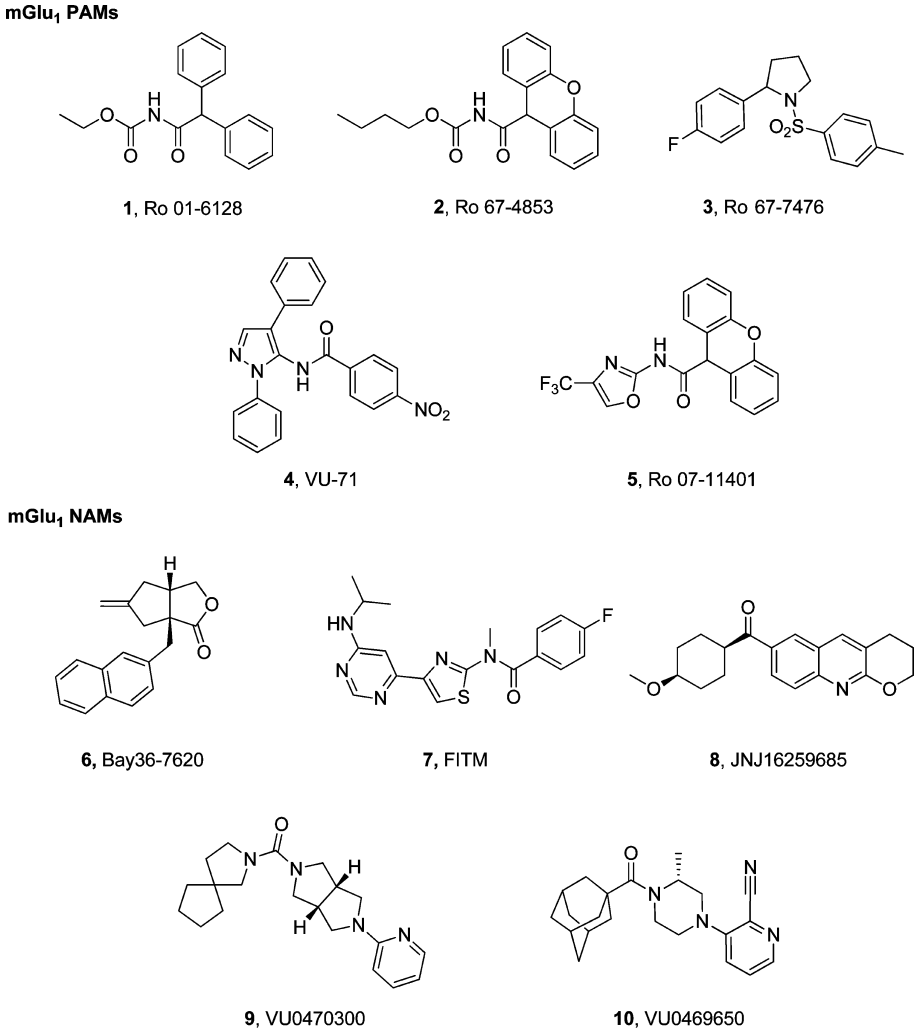

Figure 4. Structures of the only known $mGlu_1$ PAMs (**1**–**5**) and structures of representative $mGlu_1$ NAMs (**6**–**10**). Due to the existence of limited $mGlu_1$ PAMs with appropriate species selectivity, ancillary pharmacology, and disposition profiles, the therapeutic potential of $mGlu_1$ PAMs is vastly unexplored. **1**–**3** were the first $mGlu_1$ PAMs described, but only **2** and **5** are active at both human and rat $mGlu_1$. The rat/human species disconnect is a major issue within multiple $mGlu_1$ allosteric modulator chemotypes.

mutant receptors found in schizophrenic patients are capable of being modulated by both PAMs and NAMs, suggesting they may be targets for therapeutic intervention with small molecules.

Preparation for Translational Studies with Schizophrenic $mGlu_1$ Mutant Receptors. In preparation for future translational studies beyond cells and into animal models, we first needed to understand if the mutations in human $mGlu_1$ aligned with rodent $mGlu_1$. Fortunately, all of the human mutation sites in $mGlu_1$ are sequence aligned with rat $mGlu_1$ with a single exception; K563 is R563 in both rat and mouse $mGlu_1$ (Supporting Information Table 1). Thus, a future effort will focus on developing and characterizing mutant $mGlu_1$ mice. Based on these favorable data, we then assessed the DMPK profiles of the three $mGlu_1$ PAMs that were capable of modulating the mutant $mGlu_1$ receptors. In general (Supporting Information Table 2), both **23c** and **24c** displayed improved disposition profiles relative to **5** in terms of both intrinsic clearance and cytochrome P450 (CYP) inhibition profiles. For example, **24c** possessed moderate hepatic clearance in both rat (CL_{hep} 25.4 mL/min/kg) and human (CL_{hep} 9.46 mL/min/kg) and clean CYP profile (IC_{50} > 30 μM versus CYP1A2, 2C9, 2D6, and 3A4). In contrast, **5** possessed high hepatic clearance in both rat (CL_{hep} 63.3 mL/min/kg) and human (CL_{hep} 19.4 mL/min/kg) and a mixed CYP profile (IC_{50} > 30 μM versus CYP2C9 and 3A4, 1A2 IC_{50} = 13 μM, 2C9 IC_{50} = 0.6 μM). Moreover, in a single point plasma/brain level study (100 mg/kg i.p. at t = 1.25 h), **24c** was highly brain penetrant with a brain/plasma ratio (K_p) of 0.84, while **5** was modest with a brain/plasma ratio (K_p) of 0.19. Thus, these are acceptable first generation $mGlu_1$ PAM tool compounds for *in vivo* studies. These findings highlight the advantage of "molecular switches"[27] in effectively scaffold hopping within allosteric chemotypes to an advanced lead for another receptor subtype and opposing mode of pharmacology.

Historically, $mGlu_1$ NAMs have shown efficacy in multiple preclinical models that predict antipsychotic efficacy in WT mice and rats. We confirmed this in house, by evaluating the $mGlu_1$ NAM **7** in an amphetamine-induced hyperlocomotion (AHL) model (Supporting Information Figure 2). At doses of 10 and 30 mg/kg i.p., **7** completely reversed AHL. After a detailed perusal of the literature, it appeared that the activity of $mGlu_1$ PAMs had never been reported in this model. Therefore, we evaluated **24c** in WT rats at doses up to 100

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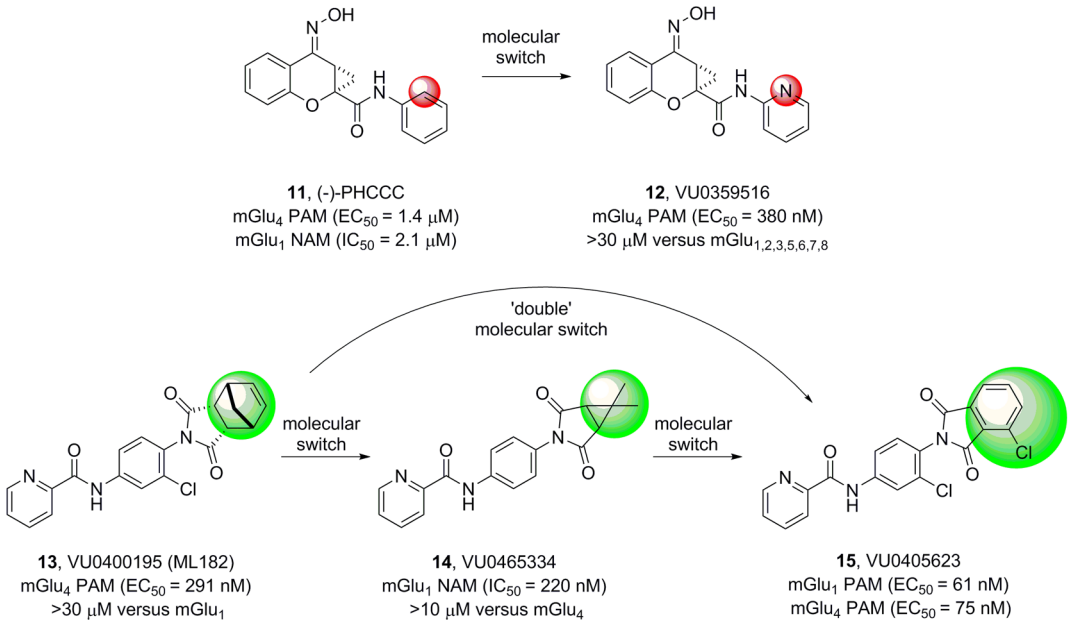
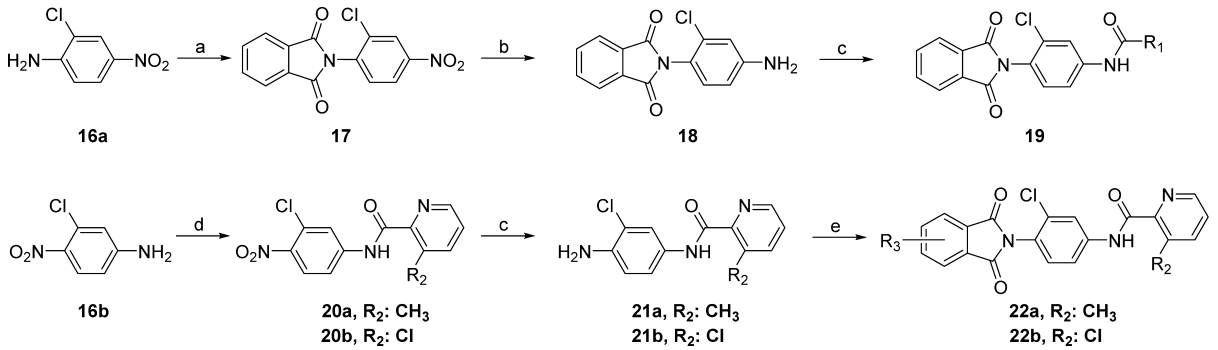


Figure 5. "Molecular switches" modulate mGlu subtype selectivity and mode of pharmacology. The first $mGlu_4$ PAM, (−)-PHCCC (**11**), possessed $mGlu_1$ NAM activity, suggesting cross-talk between the allosteric sites on $mGlu_1$ and $mGlu_4$. A subtle molecular switch in the form of an aza-analog, **12**, abolished activity at $mGlu_1$ and improved $mGlu_4$ PAM activity. An unrelated, highly selective $mGlu_4$ PAM chemotype, **13**, was subject to a "molecular switch" by virtue of modification of the succinimide moiety, yielding a selective $mGlu_1$ NAM **14**. Based on these observations, we screened our $mGlu_4$ PAM library in an effort to identify a new $mGlu_1$ PAM scaffold, and identified the phthalimide congener **15** as a dual $mGlu_1$/$mGlu_4$ PAM with comparable potency, via a "double" molecular switch. Extensive SAR within **13**−**14** demonstrated that it was possible to dial-out $mGlu_4$ activity; thus, **15** served as a new lead upon which to develop a novel and selective $mGlu_1$ PAM to profile against the Sz mutants.

Scheme 1. Synthesis of *N*-(3-chloro-4-(1,3-dioxoisoindolin-2-yl)phenyl)amides 19 and 22[a]



[a]Reagents and conditions. (a) phthalic anhydride, AcOH, reflux, 88%; (b) $SnCl_2$, HCl, dioxane, rt, 72−93%; (c) carboxylic acids, HATU, DIEA, DCM, rt, 45−90%; (d) picolinic acids, HATU, DIEA, μW 120 °C DCM, 75−78%; (e) phthalic anhydrides, μW 200 °C, *N*-methyl-2-pyrrolidone, 55−78%.

mg/kg i.p. and observed no effect up to the highest dose; in contrast, a slight potentiation of amphetamine was noted in the presence of good CNS exposure. Both **5** and **23c** behaved similarly. A key question that remains involved the potential for antipsychotic activity of an $mGlu_1$ PAM in genetic animals possessing the mutant $mGlu_1$ receptors? This further underscores the challenges in developing novel, single mechanism antipsychotic agents to be clinically tested for efficacy in a highly heterogeneous patient population. From these data, an $mGlu_1$ PAM would be expected to have no effect on patients with WT $mGlu_1$, but might give a signal in patients carrying the mutant $mGlu_1$ receptors.

In summary, we expressed each of the nine $mGlu_1$ mutant receptors, identified as *GRM1* mutations associated with schizophrenia, into stable cell lines and assessed their pharmacology. Employing both the endogenous agonist glutamate, and the synthetic agonist DHPG, we demonstrate that the mutant $mGlu_1$ receptors display reduced calcium signaling that does not result from a loss in plasma membrane expression. Based on a lack of $mGlu_1$ positive allosteric modulators (PAM) tool compounds, we optimized a known dual $mGlu_4$ PAM/$mGlu_1$ NAM chemotype into a series of potent and selective $mGlu_1$ PAMs by virtue of a double "molecular switch". Employing $mGlu_1$ PAMs from multiple chemotypes, we demonstrate that the mutant receptors can be potentiated by small molecules, and in some cases efficacy restored to that comparable to WT $mGlu_1$ receptors, suggesting schizophrenic patients harboring these mutations may be amenable to intervention with an $mGlu_1$ PAM. However, data generated in WT animals in AHL studies further highlight the heterogeneity of schizophrenia and the critical role of patient selection in psychiatric clinical trials to match genotype with

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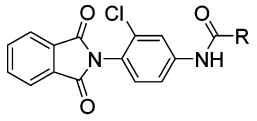
Table 1. Structures and Activities for Selected Picolinamide Analogs 19



19

Cmpd	R	hmGlu$_1$ EC_{50}[a] (μM)	hmGlu$_1$ pEC_{50} (±SEM)	hmGlu$_1$ % Glu Max (±SEM)	rmGlu$_1$ EC_{50}[a] (μM)	rmGlu$_1$ pEC_{50} (±SEM)	rmGlu$_1$ % Glu Max (±SEM)	hmGlu$_4$ EC_{50}[a] (μM)	hmGlu$_4$ % Glu Max
19a		0.125	6.90±0.11	66±6	0.140	6.82±0.15	62±8	0.0420	130
19b		>10.0	<5.00	-	>10.0	<5.00	-	>10.0	-
19c		>10.0	<5.00	-	>10.0	<5.00	-	>10.0	-
19d		0.728	6.14±0.16	105±2	1.09	5.96±0.03	85±6	0.0700	52
19e		>10.0	<5.00	-	>10.0	<5.00	-	>10.0	-
19f		>10.0	<5.00	-	>10.0	<5.00	-	>10.0	-
19g		1.02	5.95±0.58	92±4	0.378	6.42±0.08	64±13	>10.0	-
19h		0.637	6.21±0.35	105±2	1.09	5.96±0.03	85±6	>10.0	-
19i		1.05	5.98±0.27	93±12	0.49	6.31±0.13	95±5	>10.0	60
19j		2.42	5.62±0.21	83±12	>10.0	<5.00	-	>10.0	-
19k		0.760	6.12±0.09	91±7	1.41	5.85±0.26	109±13	>10.0	-
19l		1.29	5.90±0.12	102±3	0.566	6.25±0.03	77±11	>10.0	-
19m		>10.0	<5.00	-	>10.0	<5.00	-	>10.0	-
19n		>10.0	<5.00	-	>10.0	<5.00	-	>10.0	-
19o		0.568	6.25±0.34	106±8	0.0322	7.49±0.23	105±5	0.13	207

[a]Calcium mobilization mGlu$_1$ and mGlu$_4$ assays; values are average of n = 3 performed in triplicate.

therapeutic mechanism. Future efforts will examine mGlu$_1$ mutants harboring multiple mutations, and assessing the ability of mGlu$_1$ PAMs to modulate pharmacological responses.

METHODS

Molecular Pharmacology. Tetracycline-tested fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Lawrenceville, GA), and all other tissue culture reagents and Fluo-4-acetoxymethylester (Fluo-4-AM) were purchased from Life Technologies (Carlsbad, CA). Tetracycline hydrochloride (Sigma), L-glutamic acid (Tocris, Minneapolis, MN), and (*S*)-3,5-dihydroxyphenylglycine (DHPG) (Abcam, Cambridge, MA). EZ-Link Sulfo-NHS-SS-Biotin and NeutrAvidin agarose beads (Pierce Biotechnology, Rockford, IL).

Cell Culture and Mutagenesis. Tetracycline-inducible human mGlu$_1$ WT-T-REx-293 cells[39] were cultured at 37 °C in Dulbecco's Modified Eagle Medium (DMEM) growth medium containing 10% Tet-tested FBS, 2 mM L-glutamine, 20 mM HEPES, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, antibiotic/antimycotic, 100 μg/mL hygromycin, and 5 μg/mL blasticidin in the presence of 5% CO_2. To generate a collection of stable cell lines carrying tetracycline-inducible hmGlu$_1$ schizophrenic mutants,[21,22] site-directed mutagenesis of human mGlu$_1$ WT in pcDNA5/TO was performed using Quikchange II XL kit (Agilent Technologies, Santa Clara, CA), and all point-mutations were confirmed by sequencing. The mutant stable cell lines were generated in the same manner as the WT as previously described and cultured in the growth medium described above.

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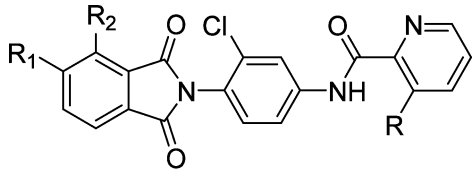
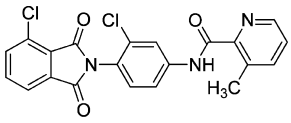
Table 2. Structures and Human $mGlu_1$ Activity for Selected Substituted Phthalimide Analogs 23 and 24



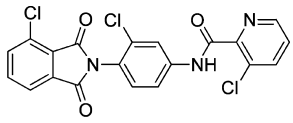
23, R = CH_3
24, R = Cl

compd.	R_1	R_2	EC_{50}[a] (μM)	pEC_{50} (±SEM)	% Glu max (±SEM)
23a	H	CH_3	0.176	6.75 ± 0.14	101 ± 7
24a			0.177	6.75 ± 0.19	110 ± 21
23b	CH_3	H	>10.0	<5.00	130 ± 26
24b			0.832	6.08 ± 0.07	82 ± 4
23c	H	CI	0.372	6.43 ± 0.01	126 ± 25
24c			0.391	6.41 ± 0.08	110 ± 15
23d	CI	H	2.02	5.69 ± 0.05	92 ± 11
24d			1.84	5.73 ± 0.15	86 ± 6
23e	H	F	0.418	6.38 ± 0.05	90 ± 15
24e			1.14	5.94 ± 0.09	117 ± 9
23f	F	H	>10.0	<5.00	
24f			3.88	5.41 ± 0.12	128 ± 5

[a]Calcium mobilization $mGlu_1$ assays; values are average of n = 3, performed in triplicate.



23c (VU0483737)
$hmGlu_1$ EC_{50} = 370 nM (126%)
$rmGlu_1$ EC_{50} = 367 nM (118%)
$hmGlu_4$ EC_{50} = 199 nM (55%)



24c (VU0483605)
$hmGlu_1$ EC_{50} = 390 nM (110%)
$rmGlu_1$ EC_{50} = 356 nM (118%)
$hmGlu_4$ EC_{50} >10,000 nM

Figure 6. Novel $mGlu_1$ PAMs derived from a double "molecular switch" of an $mGlu_4$ PAM/$mGlu_1$ NAM preferring chemotype.

Calcium Mobilization Assay. To examine the functional activity of all $hmGlu_1$ mutants compared to the WT, calcium flux was measured using the Functional Drug Screening System (FDSS7000, Hamamatsu, Japan), as previously described.[39] Briefly, the day before the assay, the $mGlu_1$-T-REx-293 cells were plated in black-walled, clear-bottomed, poly-D-lysine coated 384-well plates (BD Biosciences, San Jose, CA) at 20 000 cells/well in 20 μL of assay medium (DMEM supplemented with 10% dialyzed FBS, 20 mM HEPES, and 1 mM sodium pyruvate) that contains tetracycline (TET) to induce $mGlu_1$ expression; 20 ng/mL TET was used for WT and 1 μg/mL TET for all mutants. The next day, cells were washed with assay buffer (Hank's balanced salt solution, 20 mM HEPES, and 2.5 mM probenecid) using ELx microplate washer (BioTek Instruments, Winooski, VT) and immediately incubated with 20 μL of 1.15 μM Fluo-4 AM dye solution prepared in assay buffer for 45 min at 37 °C. The dye was removed and washed with assay buffer before measuring the florescent calcium traces in FDSS. Agonists, glutamate or DHPG, were 1:5 serially diluted into 11 point concentrations, added to cells, and incubated for 2 min. The agonist-mediated calcium response was calculated by subtracting the basal florescent peak before agonist addition from the maximal peak elicited by agonist. When testing the effect of the $mGlu_1$ PAMs, cells were first incubated with the PAMs (10 μM final concentration) or DMSO vehicle diluted in assay buffer for 2.2 min and then stimulated with glutamate or DHPG. Concentration response curves were generated using GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA). To determine the potency of $mGlu_1$ PAMs in calcium assays, all procedures are performed in a similar manner to the ones described above with the following modifications. Briefly, rat and human $mGlu_1$ WT-T-REx-293 cells were plated in poly-D-lysine coated 96-well plates at 80 000 cells/100 μL assay medium containing 10 ng/mL and 50 ng/mL TET, respectively. After incubating cells with the Fluo-4-AM dye solution, calcium flux was measured using Flexstation II (Molecular Devices, Sunnyvale, CA). Compounds serially diluted at half log concentrations in DMSO were further diluted in assay buffer. The compounds or DMSO vehicle were added to cells and incubated for 2.5 min, and an EC_{20} concentration of glutamate was added and incubated for 1 min. An EC_{max} concentration of glutamate was also added to cells that were incubated with DMSO vehicle to accurately calculate the EC_{20} calcium response. Data were normalized by subtracting the basal florescent peak before EC_{20} agonist addition from the maximal peak elicited by EC_{20} agonist and PAMs. Using GraphPad Prism 5.0, the concentration response curves were generated and the potencies of the $mGlu_1$ PAMs were determined.

Biotinylation of Cell Surface Expression of $mGlu_1$ Receptor. To determine the receptor expression level on the cell surface among the $mGlu_1$ WT and schizophrenia (Sz) mutant cell lines, cells were plated in a 10 cm dish at 8 × 10^6 cells/8 mL density in the assay medium containing either 20 ng/mL (WT) or 1 μg/mL (mutants) TET to induce the receptor expression. The next morning, dishes were placed on ice and gently washed with ice cold PBS. Immediately, cells were scraped in cold PBS into a 1.5 mL tube, and spun down at 500g for 4 min at 4 °C. All steps were carried at 4 °C unless otherwise noted. Cells were gently resuspended in 1 mL of cold PBS containing 2 mg of EZ-Link Sulfo-NHS-SS-Biotin and gently rocked for 30 min. Biotinylation was quenched by adding 500 uL of 150 mM Tris, pH 8.0, and cells were spun down and resuspended in 1 mL of 50 mM Tris, pH 8.0 to completely quench the reaction. Cells were spun down and resuspended in 1 mL of lysis buffer (25 mM Tris, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate containing protease inhibitors) and incubated for 30 min. The lysates were spun at 16 000g for 15 min, and the resulting supernatants were gently transferred to a new 1.5 mL tube to incubate with 60 μL of 50% slurry of NeutrAvidin beads by nutating at room temperature (RT) for 1 h. The beads were washed three times with lysis buffer and the biotinylated proteins bound to the beads were extracted by heating the beads at 65 °C for 5 min in SDS sample buffer containing 150 mM DTT. The proteins were resolved by 8% SDS-PAGE and the $mGlu_1$ expression level was determined by Western blotting using the $mGlu_1$–specific antibody (Clone 20, BD Transduction Lab). The density of the $mGlu_1$ protein bands was measured using Quantity One software (Bio-Rad, Hercules, CA).

DMPK Methods. *In Vitro.* The *in vitro* DMPK assays, including those assessing plasma protein binding (PPB), brain homogenate binding (BHB), hepatic microsomal intrinsic clearance (CL_{int}), and cytochrome P450 inhibition, were performed as described previously.[40] A potassium phosphate-buffered reaction mixture (0.1 M, pH 7.4) of test article (1 μM) and microsomes (0.5 mg mL^{-1}) was preincubated (5 min) at 37 °C prior to the addition of NADPH (1 mM). The incubations, performed in 96-well plates, were continued at 37 °C under ambient oxygenation and aliquots (80 μL) were removed at selected time intervals (0, 3, 7, 15, 25, and 45 min). Protein was precipitated by the addition of chilled acetonitrile (160 μL), containing glyburide as an internal standard (50 ng/mL), and centrifuged at 3000 rpm (4 °C) for 10 min. Resulting supernatants were transferred to new 96-well plates in preparation for LC/MS/MS analysis. The *in vitro* half-life ($t_{1/2}$, min, eq 1), intrinsic clearance (CL_{int}, mL/min·kg, eq 2) and subsequent predicted hepatic clearance (CL_{hep}, mL/min·kg, eq 3) was determined employing the following equations:

$$t_{1/2} = \mathrm{Ln}(2)/k \quad (1)$$

where k represents the slope from linear regression analysis (% test article remaining) vs time

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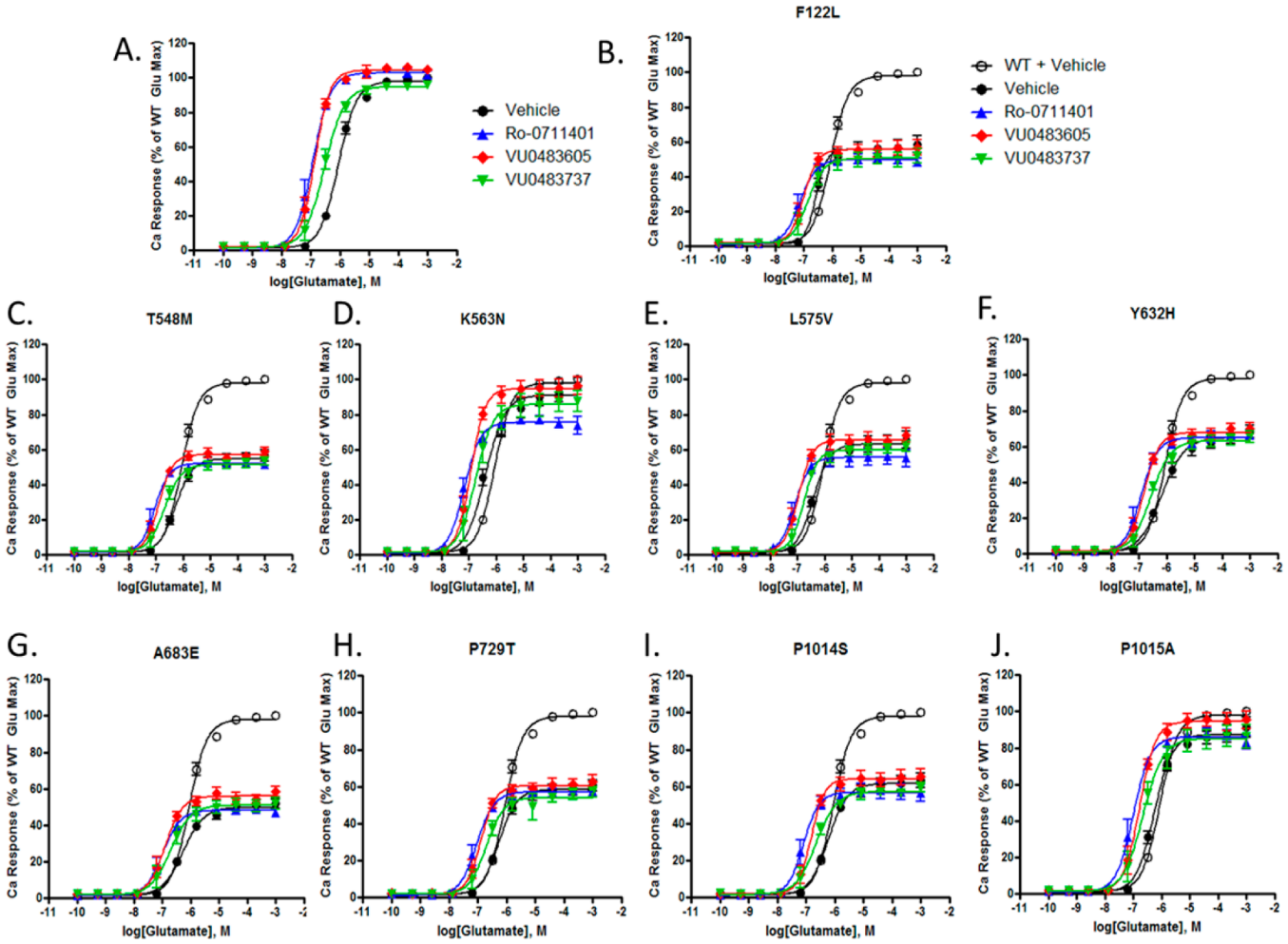

Figure 7. Treatment of $mGlu_1$ positive allosteric modulators partially restores the reduction in the glutamate-mediated calcium signaling in the Sz mutants. To examine if $mGlu_1$ PAM can potentiate the reduced calcium signaling induced by the Sz mutants, stable cells expressing the $mGlu_1$ WT and Sz mutants were incubated with 10 μM of Ro 07-11401 (**5**), VU0483605 (**24c**), VU0483737 (**23c**), or DMSO matched vehicle for 2.2 min, and immediately stimulated with the increasing concentration of glutamate. (A) $mGlu_1$ PAMs induce a leftward fold shift of the glutamate concentration−response curve in cells expressing the $mGlu_1$ WT receptor. Compared to an 870 nM EC_{50} for in the DMSO vehicle condition, both Ro 07-11401 (**5**) and VU0483605 (**24c**) shifted the glutamate EC_{50} to 118 nM and 139 nM (7.4- and 6.3-fold shift, respectively) and showed a slight increase in maximal glutamate response. VU0483737 (**23c**) displayed a lesser fold shift, shifting the EC_{50} to 294 nM. (B−J) All three $mGlu_1$ PAMs were able to potentiate the decreased glutamate-induced calcium response in the Sz mutants tested. Both Ro 07-11401 (**5**) and VU0483605 (**24c**) showed comparable fold shifts off all mutant responses; VU0483737 (**23c**) showed a lesser effect. Data shown was generated by normalizing the calcium responses to the % maximal glutamate-elicited response at the WT receptor in the presence of DMSO vehicle. Data represent four independent experiments performed in duplicate.

$$CL_{int} = (0.693/t_{1/2})\ (\text{rxn vol./mg microsomes}) \times (45\ \text{mg microsomes/g of liver})\ (20\ \text{g of liver/kg body weight}) \quad (2)$$

with scale-up factors of 20 (human) and 45 (rat)

$$CL_{hep} = \frac{Q \times CL_{int}}{Q + CL_{int}} \quad (3)$$

In Vivo. Male Sprague−Dawley rats (n = 2) weighing around 300 g were purchased from Harlon laboratories (Indianapolis, IN) and implanted with catheters in the carotid artery and jugular vein. The cannulated animals were acclimated to their surroundings for approximately 1 week before dosing and provided food and water *ad libitum*. Samples were collected into chilled, EDTA-fortified tubes, centrifuged for 10 min at 3000 rpm (4 °C), and resulting plasma aliquoted into 96-well plates for LC/MS/MS analysis. All pharmacokinetic analysis was performed employing noncompartmental analysis.

Plasma Protein Binding. Protein binding of the $mGlu_1$ PAMs were determined in rat plasma via equilibrium dialysis employing Single-Use RED Plates with inserts (ThermoFisher Scientific, Rochester, NY). Briefly, plasma (220 μL) was added to the 96 well plate containing test article (5 μL) and mixed thoroughly. Subsequently, 200 μL of the plasma-test article mixture was transferred to the *cis* chamber (red) of the RED plate, with an accompanying 350 μL of phosphate buffer (25 mM, pH 7.4) in the *trans* chamber. The RED plate was sealed and incubated 4 h at 37 °C with shaking. At completion, 50 μL aliquots from each chamber were diluted 1:1 (50 μL) with either plasma (*cis*) or buffer (*trans*) and transferred to a new 96 well plate, at which time ice-cold acetonitrile (2 volumes) was added to extract the matrices. The plate was centrifuged (3000 rpm, 10 min) and supernatants transferred to a new 96 well plate. The sealed plate was stored at −20 °C until LC/MS/MS analysis.

Liquid Chromatography/Mass Spectrometry Analysis. *In Vivo Experiments.* $mGlu_1$ PAMs were analyzed via electrospray ionization (ESI) on an AB Sciex API-4000 (Foster City, CA) triple-quadrupole instrument that was coupled with Shimadzu LC-10AD pumps (Columbia, MD) and a Leap Technologies CTC PAL autosampler (Carrboro, NC). Analytes were separated by gradient

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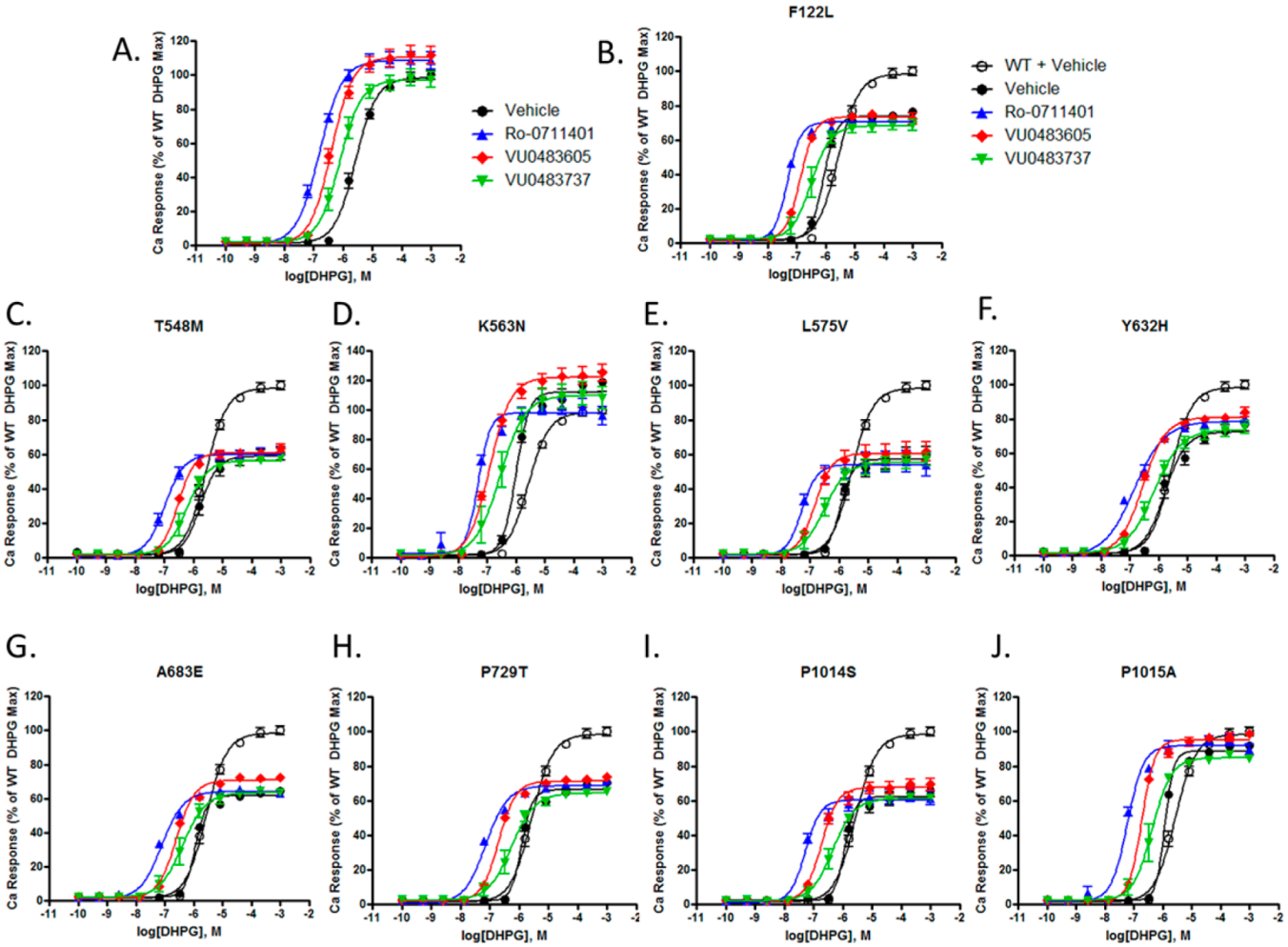

Figure 8. Potentiating effect of $mGlu_1$ positive allosteric modulators reveal a probe-dependency in the DHPG-mediated Ca signaling. Stable cells expressing the $mGlu_1$ WT and Sz mutants were incubated with 10 μM of Ro 07-11401 (**5**), VU0483605 (**24c**), VU0483737 (**23c**), or DMSO-matched vehicle for 2.2 min, and then stimulated with increasing concentrations of DHPG, group I mGlu-specific agonist. (A) $mGlu_1$ PAMs induce a leftward fold shift of the DHPG concentration−response curve at WT $mGlu_1$. Ro 07-11401 (5) exhibited a dramatic 16.6 fold shift, resulting in 159 nM of EC_{50} DHPG concentration compared to 2.5 μM when cells were treated with DMSO. Comparable to the fold shifts in glutamate response, both VU0483605 (**24c**) and VU0483737 (**23c**) shifted the DHPG EC_{50} value to 402 nM and 783 nM (6.6 and 3.4 fold shift, respectively). (B−J) All three $mGlu_1$ PAMs were able to potentiate the decreased DHPG-mediated calcium response in the Sz mutants tested. Both VU0483605 (**24c**) and VU0483737 (**23c**) showed comparable fold shifts of all mutant responses while Ro 07-11401 (**5**) showed a stronger effect. Data shown was generated by normalizing the calcium responses to the % maximal DHPG-elicited response at the WT receptor in the presence of DMSO vehicle. Data represent three independent experiments performed in duplicate.

elution using a Fortis C18 2.1 × 50 mm, 3.5 μm column (Fortis Technologies Ltd., Cheshire, U.K.) thermostated at 40 °C. HPLC mobile phase A was 0.1% NH_4OH (pH unadjusted), mobile phase B was acetonitrile. The gradient started at 30% B after a 0.2 min hold and was linearly increased to 90% B over 0.8 min, held at 90% B for 0.5 min, and returned to 30% B in 0.1 min, followed by a re-equilibration (0.9 min). The total run time was 2.5 min and the HPLC flow rate was 0.5 mL/min. The source temperature was set at 500 °C and mass spectral analyses were performed using multiple reaction monitoring (MRM) utilizing a Turbo-Ionspray source in positive ionization mode (5.0 kV spray voltage). All data were analyzed using AB Sciex Analyst 1.4.2 software.

In Vitro Experiments. The $mGlu_1$ PAMs were analyzed similarly to that described above (*In vivo*) with the following exceptions: LC/MS/MS analysis was performed employing a TSQ Quantum^{ULTRA} that was coupled to a ThermoSurveyor LC system (Thermoelectron Corp., San Jose, CA) and a Leap Technologies CTC PAL autosampler (Carrboro, NC). Chromatographic separation of analytes was achieved with an Acquity BEH C18 2.1 × 50 mm, 1.7 μm column (Waters, Taunton, MA).

Amphetamine-Induced Hyperlocomotion. *Animals.* Adult male Sprague−Dawley rats (Harlan, Indianapolis, IN) were used for the behavioral studies. They were group-housed under a 12-h light/dark cycle (lights on from 7 AM−7 PM) with food and water available *ad libidum*. All animal procedures were performed in accord with the guidelines set by the *Guide for the Care and Use of Laboratory Animals* and were approved by the Vanderbilt University Animal Care and Use Committee.

Drugs. All compounds were dissolved in 20% hydroxypropyl-β-cyclodextrin (BCD), and if necessary, the solutions were adjusted to pH 6−7 using 1 N NaOH; D-amphetamine hemisulfate (Sigma-Aldrich, St. Louis, MO) was dissolved in sterile water.

Reversal of Amphetamine-Induced Hyperlocomotion. Locomotor activity was assessed using SmartFrame open field activity chambers (40.5 cm × 40.5 cm × 38 cm; Kinder Scientific, Poway, CA) that were equipped with a 16 × 16 array of infrared photobeams.[41] Rats were habituated in the open field for 30 min and then pretreated with vehicle (20% BCD in water) or VU0483605 (100 mg/kg). Thirty minutes later, amphetamine (0.75 mg/kg free base) was administered IP and locomotor activity was recorded for an additional hour. The

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time course of locomotor activity (number of beam breaks per 5 min interval) over the whole 120 min testing session and total locomotor activity (total beam breaks from the time of amphetamine administration to the end of the study) are presented. Data are shown as means ± SEM and statistical comparisons were made by one- or two-way ANOVA using GraphPad Prism V5.04.

Chemistry Experimental. All commercial chemicals and solvents were reagent grade and were used without further purification unless otherwise specified. All reactions were carried out employing standard chemical techniques under inert atmosphere. Analytical thin layer chromatography was performed on 250 μm silica gel plates from Sorbent Technologies was employed routinely to follow the course of reactions. NMR spectra were recorded on a 400 MHz Bruker AV-400, 500 MHz Bruker DRX-500, and 600 MHz AV-II instruments. ^{1}H chemical shifts are reported as δ values in ppm relative to the residual solvent peak (DMSO-d_6 = 2.50, $CDCl_3$ = 7.26). Data are reported as follows: chemical shift, integration, multiplicity (s = singlet, d = doublet, dd = double of doublet, t = triplet, q = quartet, m = multiplet) and coupling constant (Hz). ^{13}C chemical shifts are reported as δ values in ppm relative to the residual solvent peak (DMSO-d_6 = 39.52, $CDCl_3$ = 77.16). Analytical HPLC was performed on an Agilent 1200 LCMS with UV detection at 214 and 254 nm along with ELSD detection. The purity of all tested compounds was greater than 98% based on analytical HPLC. Preparative purification of library compounds was performed on a Gilson 215 preparative LC system. Low resolution mass spectra were obtained on an Agilent 1200 LCMS with electrospray ionization. High resolution mass spectra were recorded on a Waters QToF-API-US plus Acquity system with electrospray ionization.

***N*-(2-chloro-4-nitrophenyl)-3-chloropicolinamide (20b).** In a microwave vial, 347 mg (2.20 mmol) of the 3-chloropicolinic acid were added and dissolved in 5 mL of DCE:DIEA (9:1), then 1254 mg (3.30 mmol) of HATU were added. The mixture was stirred for 5 min, and 456 mg (2.64 mmol) of 3-chloro-4-nitroaniline dissolved in 5 mL of DCE:DIEA (9:1) were added, followed by 3 drops of DMF. The reaction was heated in the microwave at 120 °C for 30 min. The reaction was cooled to RT and water was added, causing the precipitation of the product. The crude was filtrated *in vacuo* and carefully triturated with cold methanol to give a pale yellow powder, 517 mg (75%). ^{1}H NMR (400.1 MHz, DMSO-d_6) δ (ppm): 11.36 (1H, s, −NH), 8.69 (1H, dd, J = 4.6 Hz, J = 1.3 Hz), 8.23 (1H, d, J = 2.1 Hz), 8.17 (2H, m), 7.92 (1H, dd, J = 9.0 Hz, J = 2.1 Hz), 7.68 (1H, 1H, dd, J = 8.2 Hz, J = 4.6 Hz). ^{13}C NMR (100.6 MHz, DMSO-d_6) δ (ppm): 164.1, 149.7, 147.8, 143.7, 142.5, 139.6, 129.4, 128.0, 127.6, 127.1, 121.5, 118.9.

***N*-(4-amino-2-chlorophenyl)-3-chloropicolinamide (21b).** In a vial, 510 mg (1.635 mmol) of **20b** were resuspended in dioxane. The suspension was cold in an ice bath and purged with argon. A previously prepared solution of tin(II) chloride (1395 mg) in concentrated hydrochloric acid (5 M concentration of $SnCl_2$) was added dropwise to the suspension. After 2 h of stirring at RT, the reaction was neutralized carefully with aqueous potassium carbonate 20%, filtered and extracted with diethyl ether. The organic phase was dried with magnesium sulfate, filtered and the volatiles eliminated in vacuo to yield a pale yellow powder (428 mg, 93%). ^{1}H NMR (400.1 MHz, DMSO-d_6) δ (ppm): 10.42 (1H, s, −NH), 8.61 (1H, dd, J = 4.6 Hz, J = 1.2 Hz), 8.09 (1H, dd, J = 8.2 Hz, J = 1.2 Hz), 7.74 (1H, d, J = 2.1 Hz), 7.59 (1H, dd, J = 8.2 Hz, J = 4.7 Hz), 7.33 (1H, dd, J = 8.7 Hz, J = 2.2 Hz), 6.79 (1H, d, J = 8.7 Hz), 5.24 (2H, s, $-NH_2$). ^{13}C NMR (100.6 MHz, DMSO-d_6) δ (ppm): 162.9, 151.6, 147.6, 141.8, 139.0, 128.8, 126.7, 121.0, 120.4, 116.9, 115.7.

***N*-(3-chloro-4-(4-chloro-1,3-dioxoisoindolin-2-yl)phenyl)-3-chloropicolinamide (24c).** In a microwave vial, 20 mg (0.0709 mmol) of **21b** and 19 mg (0.106 mmol) of 3-chlorophthalic anhydride were added and dissolved in 1 mL of NMP. The vial was sealed and the mixture was heated at 200 °C for 20 min. After this time, the reaction was quenched with the addition of water and was worked up by extraction with DCM (2 mL, thrice). The organic phased was filtered through a phase separator, volatiles were evaporated; the crude was dissolved in DMSO and purified by preparative HPLC. Cream powder. ^{1}H NMR (400.1 MHz, $CDCl_3$) δ (ppm): 10.18 (1H, s), 8.55 (1H, dd, J = 4.5 Hz, J = 1.4 Hz), 8.19 (1H, d, J = 2.3 Hz), 7.91 (2H, m), 7.75 (3H, m), 7.48 (1H, dd, J = 8.2 Hz, J = 4.5 Hz), 7.33 (1H, d, J = 8.6 Hz). ^{13}CNMR (100.6 MHz, $CDCl_3$) δ (ppm): 165.2, 164.2, 160.7, 145.8, 144.5, 141.1, 139.7, 136.1, 135.3, 133.9, 133.7, 132.9, 132.0, 130.8, 127.6, 127.2, 124.7, 122.4, 120.8, 118.4. HRMS (TOF, ES+) $C_{20}H_{11}Cl_3N_3O_3$ [M + H]+ calc. mass 445.9866, found 445.9869.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental details of compound synthesis and characterization as well as supplemental figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

‖H.P.C. and P.M.G. contributed equally. C.W.L. and C.R.H. oversaw and designed the chemistry. P.M.G. and J.T.B. performed synthetic chemistry work. H.P.C. and C.M.N oversaw, designed, and interpreted the molecular pharmacology experiments. H.P.C., P.M.G., and J.T.B. performed the molecular pharmacology experiments. J.S.D. oversaw the drug metabolism assays, and R.D.M. performed the DMPK assays and bioanalytical work.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

mGlu, metabotropic glutamate receptor; CNS, central nervous system; PAM, positive allosteric modulator; NAM, negative allosteric modulator; HEK, human embryonic kidney; CRC, concentration−response curve; GPCR, G-protein-coupled receptor

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