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(3-Cyano-5-fluorophenyl)biaryl negative allosteric modulators of $mGlu_5$: Discovery of a new tool compound with activity in the OSS mouse model of addiction

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Abstract

Glutamate is the major excitatory transmitter in the mammalian CNS, exerting its effects through both ionotropic and metabotropic glutamate receptors. The metabotropic glutamate receptors (mGlus) belong to family C of the G-protein-coupled receptors (GPCRs). The eight mGlus identified to date are classified into three groups based on their structure, preferred signal transduction mechanisms, and pharmacology (Group I: $mGlu_1$ and $mGlu_5$; Group II: $mGlu_2$ and $mGlu_3$; Group III: $mGlu_4$, $mGlu_6$, $mGlu_7$, and $mGlu_8$). Non-competitive antagonists, also known as negative allosteric modulators (NAMs), of $mGlu_5$ offer potential therapeutic applications in diseases such as pain, anxiety, gastroesophageal reflux disease (GERD), Parkinson's disease (PD), fragile X syndrome, and addiction. The development of SAR in a (3-cyano-5-fluorophenyl)biaryl series using our functional cell-based assay is described in this communication. Further characterization of a selected compound, 3-fluoro-5-(2-methylbenzo[*d*]thiazol-5-yl)benzonitrile, in additional cell based assays as well as *in vitro* assays designed to measure its metabolic stability and protein binding indicated its potential utility as an *in vivo* tool. Subsequent evaluation of the same compound in a pharmacokinetic study using intraperitoneal dosing in mice showed good

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Dr. Rodriguez oversaw, designed, and performed the molecular pharmacology experiments.

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exposure in both plasma and brain samples. The compound was efficacious in a mouse marble burying model of anxiety, an assay known to be sensitive to $mGlu_5$ antagonists. A new operant model of addiction termed operant sensation seeking (OSS) was chosen as a second behavioral assay. The compound also proved efficacious in the OSS model and constitutes the first reported example of efficacy with a small molecule $mGlu_5$ NAM in this novel assay.

Keywords

$mGlu_5$; negative allosteric modulator; non-competitive antagonist; addiction

Introduction

Glutamate (L-glutamic acid) is the major excitatory transmitter in the mammalian central nervous system, acting through both ionotropic and metabotropic glutamate receptors. The metabotropic glutamate receptors (mGlus) belong to family C (also known as family 3) of the G-protein-coupled receptors (GPCRs). The mGlus are characterized by a seven transmembrane (7TM) α-helical domain that is connected via a cysteine-rich region to a large bi-lobed extracellular amino-terminal domain. The location of the orthosteric binding site is in the extracellular domain; however, all of the known allosteric binding sites are located in the transmembrane domain. The eight mGlus discovered to date have been classified according to their structure, preferred signal transduction mechanisms, and pharmacology. Group I receptors ($mGlu_1$ and $mGlu_5$) are coupled to Gαq, a process that results in an increase in intracellular calcium. Group II receptors ($mGlu_2$ and $mGlu_3$) and group III receptors ($mGlu_4$, $mGlu_6$, $mGlu_7$, and $mGlu_8$) are coupled to Gαi, which leads to decreases in cyclic adenosine monophosphate (cAMP) levels. Group I receptors are predominately located postsynaptically and typically enhance postsynaptic signaling. In contrast, the group II and III receptors are located presynaptically and typically have inhibitory effects on neurotransmitter release.[1,2]

A common issue with orthosteric ligands as potential therapeutics has been poor selectivity among the various mGlus due to a highly conserved binding site. A potential solution to such selectivity issues was discovered through the development of allosteric modulators.[3,4] An area of study within this field that has garnered significant attention has been the design of small molecule negative allosteric modulators (NAMs), also known as non-competitive antagonists, of $mGlu_5$.[5,6,7,8] Most of the early $mGlu_5$ NAM preclinical *in vivo* work was conducted with two structurally related tool compounds, 2-methyl-6-(phenylethynyl) pyridine (MPEP)[9] and 3-[(2-methyl-1,3-thiazol-4-yl)ethynyl]pyridine (MTEP)[10]. These compounds have demonstrated efficacy in numerous preclinical models of disease, including pain[11], anxiety[12,13,14,15,16], gastroesophageal reflux disease (GERD)[17,18], Parkinson's disease levodopa induced dyskinesia (PD-LID)[19], and fragile X syndrome[20,21]. Following several years of discovery and development work by many organizations, some positive clinical reports with $mGlu_5$ NAMs have emerged. Addex Pharmaceuticals has disclosed positive data from phase II clinical studies with the $mGlu_5$ NAM ADX10059 in GERD[22] and acute migraine[23]. FRAXA Research Foundation and Neuropharm have been exploring the potential of the agent fenobam for treating fragile X syndrome and early results in patients have been encouraging.[24] Finally, Novartis has recently reported positive results from a study designed to examine the efficacy, safety, and tolerability of AFQ056 in the management of PD-LID.[25] Such clinical validation with $mGlu_5$ antagonists has further increased interest in the target and encouraged continued research in the area.

In addition to the diseases outlined above, extensive work with MPEP and MTEP has established their utility in numerous animal models of drug addiction. An ability to attenuate

various cocaine seeking behaviors in mice[26,27], rats[28,29,30,31,32,33], and squirrel monkeys[34,35] with these compounds has been noted. Such a body of evidence provides a compelling case for an $mGlu_5$ NAM as a treatment for cocaine addiction. The intensely addictive properties of cocaine have been well established. Furthermore, the risk of relapse among addicts is high, even after long periods of abstinence. Potentially severe medical complications associated with cocaine abuse include cardiac arrest, seizures, stroke, and coma.[36] There are currently no FDA approved medications for the treatment of cocaine addiction, although there are some compounds under investigation in clinical trials.[37] In spite of the success observed with MTEP and MPEP in preclinical models of cocaine addiction, examples with other structurally distinct $mGlu_5$ NAMs in addiction models are lacking.

We have been interested in the identification of new chemotypes for the design of $mGlu_5$ non-competitive antagonists and have recently reported some of the results from this effort.[38,39,40] Our previously described work was based on the development of hits identified using a functional cell-based high-throughput screen of a collection of 160,000 compounds. We have also focused a portion of our $mGlu_5$ NAM effort on rational design approaches and have recently communicated the initial results from that effort.[41] One area of interest to us centered on the development of SAR in a (3-cyano-5-fluorophenyl)biaryl series and is the subject of this communication. An interesting new compound has emerged from this effort, 3-fluoro-5-(2-methylbenzo[*d*]thiazol-5-yl)benzonitrile. Herein we describe the profile of this compound in multiple cell based assays as well as *in vitro* assays designed to measure its metabolic stability and protein binding. Exposure of the compound in both plasma and brain samples following intraperitoneal dosing in mice indicated the suitability of the molecule for use as an *in vivo* tool compound. The compound was evaluated in a mouse marble burying assay since that model has been established as a useful tool for the assessment of $mGlu_5$ NAM activity. A new operant model of addiction termed operant sensation seeking (OSS) was chosen as a second behavioral assay. The compound also proved efficacious in the OSS model and constitutes the first reported example of efficacy with a small molecule $mGlu_5$ NAM in this novel assay.

Results and Discussion

An examination of some of the primary literature describing the SARs of various $mGlu_5$ NAM chemotypes revealed some common structural features.[42,43,44,45] One such feature was the presence of a 3-cyano-5-fluorophenyl ring in several of the most potent analogs across multiple chemical series (Figure 1). We developed a chemical plan in order to build around this common structural motif. Significant effort has been detailed by the referenced research groups around the phenyl portion of their respective templates. Our plan was to hold this phenyl ring constant with 3-cyano-5-fluoro substituents and to prepare new aryl and heteroaryl groups at position one. We had successfully employed a similar approach in the design of 3-Cyano-5-fluoro-*N*-arylbenzamides mGlu5 antagonists.[41] One of the advantages of such an approach was that analogs could be prepared in a single step, in this case through the Suzuki coupling of commercially available 3-cyano-5-fluorophenylboronic acid with readily available aryl and heteroaryl halides (Scheme 1). All syntheses were carried out using one of three different reaction conditions using microwave-assisted organic synthesis (MAOS)[46]. MAOS in combination with a high-throughput preparative LC/MS system allowed for the rapid purification of compounds and generation of timely SAR.[47]

One of the areas of obvious interest was 6,6-fused heterocycles, and we thus prepared ten examples within this class of compounds (Table 1). 2-Methyl-7-arylquinoline **3** was described as a potent $mGlu_5$ NAM in the literature[44] and was very potent in our functional assay as well. Our cell-based assay measures the ability of the compound to block the

mobilization of calcium by an EC_{80} concentration of glutamate in HEK293A cells expressing rat $mGlu_5$. The 2-methyl substituent in **3** proved a significant boost to activity as evidenced by the nineteen fold drop in potency seen with unsubstituted quinoline **5**. 1,8-Naphthyridine **6** was similar in potency to **5**. SAR around $mGlu_5$ antagonism for a series of 1,8-naphthyridines was previously described in the literature,[48] although **6** was not reported in that publication. While introduction of a nitrogen atom into chemotype **5** to afford **6** was well tolerated, a similar modification to provide quinoxaline **7** resulted in a compound inactive up to the highest concentration tested (30 μM). Quinoxaline **8** demonstrated only moderate potency; however, the compound was unique among this set of compounds in that its activity could be classified as a partial antagonist. Partial $mGlu_5$ antagonists have been reported and well characterized in the MPEP chemotype.[49,50] Such molecules only partially block the response to glutamate, even with increasing concentrations of the antagonist. Compound **8** appears to fall into the partial antagonist class of inhibitors, with a maximum antagonism of 80%. Introduction of a methyl substituent at the 2-position of **8** to afford analog **9**, a modification that likely has substantial effects on the conformation of the biaryl, reduced potency. Quinazolinone derivatives **10** and **11** were inactive and weak antagonists, respectively. Finally, in order to evaluate saturated analogs of quinoline **5**, we prepared tetrahydroquinoline **12** and benzoxazine **13**. Compound **12** was inactive up to 30 μM while compound **13** was only a weak antagonist.

The second area of interest was 5,6-fused heterocycles, and we prepared several new analogs within this class of compounds (Table 2). Benzoxazole **14** was inactive up to 30 μM; however, modification of the 2-substituent from methyl to ethyl afforded **15**, a partial antagonist with moderate potency. Further modification of this group to cyclopropyl gave weak antagonist **16**. Benzthiazole **17** lacked activity up to 30 μM; however, introduction of a methyl group at the 2-position afforded compound **18**, which was quite potent. The des-fluoro analog of **18** was prepared and tested previously by the NIDA research group;[51] however, it demonstrated only moderate affinity (K_i = 2.1 μM) in their radioligand binding assay. Benzoxadiazole **19** and benzthiadiazole **20** both were inactive up to the highest concentration tested. Indazole **21** was a weak antagonist that was subsequently methylated under standard conditions. The resultant regioisomers **22** and **23** were readily separable by flash chromatography. While compound **22** was devoid of activity, compound **23** demonstrated moderate potency.

As a supplement to the 5,6-fused heterocycles described previously, we also prepared several analogs around a benzimidazole scaffold (Table 3). While the unsubstituted analog **24** lacked activity at 30 μM, as we have seen before, installation of a methyl substituent at either the 1 or 2-position improved activity, affording weak antagonists **25** and **26**. Like compound **24**, imidazopyridine **27** was inactive up to 30 μM; however, in this case addition of a 2-methyl substituent in the form of analog **28** provided no potency improvement. On the other hand, addition of a 3-methyl substituent gave analog **29**, which possessed good potency. Such dramatic potency changes due to subtle or minor structural modifications are typical of allosteric modulators of $mGlu_5$ and other GPCR targets. Interestingly, dimethyl analog **30** was inactive up to 30 μM, indicating that the 2-methyl substituent is actually not tolerated in the case of these imidazopyridine analogs.

Having identified a new, potent non-competitive antagonist of $mGlu_5$ in the form of benzthiazole **18**, we decided to further profile this compound. A binding affinity determination measuring the ability of the compound to compete with the equilibrium of [^{3}H]3-methoxy-5-(pyridin-2-ylethynyl)pyridine[52], a close structural analog of MPEP, confirmed the interaction of **18** with the known $mGlu_5$ allosteric binding site (Figure 2A). The K_i value for binding was approximately eight fold less than the potency in the functional assay; however, at the highest concentration, the molecule is able to almost

completely block the binding of the radioligand. The results of the binding assay may indicate that although compound **18** clearly interacts with portions of the MPEP binding site, the binding sites for the two molecules may not be identical. Rat cortical astrocytes have been reported to predominantly express $mGlu_5$ and offer an attractive native system for the characterization of modulators of this receptor.[53] As such, we decided to examine the effect of a fixed concentration (10 μM) of **18** on the response to increasing concentrations of glutamate in these cultured cells. Not surprisingly, we observed a near complete blockade of the glutamate response under these conditions (Figure 2B). Compound **18** was also examined in cell based functional assays for its selectivity versus additional mGlus and was determined to be inactive against $mGlu_{1\text{-}4}$ and $mGlu_{7\text{-}8}$.

We were interested in evaluating the potential utility of compound **18** for use as an *in vivo* tool compound and therefore determined its stability in mouse and human liver microsomes (Table 4). The metabolic stability in both species was rather poor, indicating that a dosing route other than oral would likely be necessary in order to avoid a high first pass metabolism. The compound was also evaluated for the degree to which it is bound to relevant proteins. Binding to both mouse and human plasma proteins was similarly high. Additionally, the compound was highly bound to mouse brain homogenates. Being highly bound to protein can potentially limit the ability of the free drug available to interact with the receptor. Although this was obviously a concern with **18**, it was not viewed as prohibitive to its further progression as its exposure in the brain would ultimately determine its utility. We next evaluated **18** in a mouse pharmacokinetic study (10 mg/kg) using intraperitoneal dosing (Figure 3). Exposure in the systemic plasma ($AUC_{0\text{-}6H}$ = 801 ng·h/mL) was good, and exposure in the brain ($AUC_{0\text{-}6h}$ = 1530 ng·h/g) was nearly two fold higher than in plasma. While the maximum concentration in the brain was achieved at the initial time point (15 min.) and clearance was rapid, exposure remained above 500 ng/g until the one hour time point. Such a profile indicated that the molecule would most likely only be useful in an *in vivo* assay over that time frame.

Prior to evaluation of a new tool compound such as **18** in a novel behavioral assay, it was desirable to ensure its effectiveness in an assay known to be sensitive to other $mGlu_5$ antagonists. It is well known that mice will bury foreign objects such as glass marbles in deep bedding.[54] Low doses of anxiolytic benzodiazepines have been demonstrated to inhibit this behavior.[55,56] Moreover, the known $mGlu_5$ NAMs MPEP and fenobam are effective in this model.[15,16] These facts along with the relative convenience of this assay make it a useful *in vivo* screening tool. We examined both compound **18** as well as MTEP (positive control) in this assay using a 15 minute pretreatment with both compounds (Figure 4). The 15 mg/kg dose of MTEP produced a significant effect as expected. Significant inhibition of marble burying was also observed with **18** at 30 mg/kg. Evaluation of these results in the context of the prior pharmacokinetic study was considered potentially useful. In the pharmacokinetic study the average brain concentration of **18** at 30 minutes post dose was 3.75 μM. The marble burying assay was conducted between the 15 and 45 minute time points post dose so the 30 minute brain concentration is a relevant concentration to consider. If one assumed a dose linear increase in exposure, a 30 mg/kg dose should lead to brain exposures in excess of 10 μM. In considering the reasons that these relatively high brain concentrations of **18** are required for efficacy, one explanation may lie in the highly bound nature of the compound, which restricts the availability of free drug to engage the receptor.

The association between novelty and sensation seeking with elevated drug intake has been documented in humans[57,58] as well as rodents[59], which suggests overlap in the pathways mediating such behaviors. Novel stimuli and drugs of abuse both have been shown to increase dopamine levels in the nucleus accumbens shell but not in the core.[60] In contrast, natural reinforcers such as food only increase dopamine within the core.[61] A number of

operant models for the study of addiction have been described that measure the ability of various reinforcers to influence behavior.[62] A new model was recently described demonstrating that C57Bl/6J mice readily acquired operant responding to varied visual and auditory stimuli without prior training, a phenomenon termed operant sensation seeking (OSS).[63,64] In this assay mice will 'self-administer' visual cues in the form of flashing lights of random duration in combination with an auditory stimulus. Disruption of dopamine signaling with low doses of the dopamine antagonist *cis*-flupenthixol increased responding for OSS stimuli, similar to effects seen with cocaine self-administration.[65] Moreover, knockout mice lacking the D1 dopamine receptor failed to acquire operant responding to OSS stimuli,[63] while acquisition of operant behavior with food has been noted.[66] Mice lacking $mGlu_5$ also fail to acquire OSS despite having normal acquisition of food self-administration,[67] suggesting that OSS can be used as an *in vivo* screening tool for $mGlu_5$ antagonism. A previous report found that $mGlu_5$ knockout mice did not self-administer cocaine,[68] providing further evidence that the reinforcing effects of OSS may be more similar to psychostimulants than food. We were interested in examining the effects of the $mGlu_5$ NAM **18** in this model as a small molecule antagonist of this receptor had yet to be examined in this assay. Compound **18** was found to dose dependently reduce progressive ratio responding for OSS stimuli; however, there was no significant effects observed with food reinforcer (Figure 5). When the experiments were repeated using the known $mGlu_5$ NAM MTEP, we found the same effect, that OSS was dose-dependently reduced while food self-administration was not (Figure 6). Results with MTEP reinforce the conclusion that the observed effects are due to antagonism of $mGlu_5$. These results also indicate that OSS may be an additional useful model for screening small molecule antagonists of $mGlu_5$, particularly for their evaluation as a treatment for drug addiction.

In summary, we have discovered and characterized a new $mGlu_5$ NAM tool compound using a rational drug design approach based on common features of known antagonists. Compound **18** potently inhibited the mobilization of calcium by an EC_{80} concentration of glutamate in HEK293A cells expressing rat $mGlu_5$. A 10 μM concentration of **18** resulted in a near complete blockade of the glutamate response in rat cortical astrocytes. Its interaction with the known allosteric binding site was also confirmed with a radioligand binding assay. In spite of a relatively low stability in mouse liver microsomes and a high level of protein binding, exposure of **18** in mouse brains was supportive of further *in vivo* studies. Efficacy was observed in a marble burying model of anxiety as well as an operant model of addiction. While multiple $mGlu_5$ NAM compounds have previously been shown to inhibit marble burying, the experiments detailed herein with compound **18** and the well known tool MTEP constitute the first $mGlu_5$ NAMs reported to be efficacious in the OSS model. Compound **18** is an attractive tool compound as it can be readily prepared in a single step synthesis from commercially available starting materials. Further studies with **18** will be reported in the near future.

Methods

Synthesis and characterization of 3-fluoro-5-(2-methylbenzo[*d*]thiazol-5-yl)benzonitrile

To ten separate microwave vials were added 5-Bromo-2-methylbenzo[*d*]thiazole (0.250 g, 1.10 mmol), bis(tri-t-butylphosphine)palladium (0) (0.0560 g, 0.110 mmol) and 3-cyano-5-fluorophenylboronic acid (0.181 g, 1.10 mmo1) each. Aqueous cesium carbonate solution (1M, 7.5 mL, 7.5 mmol) and THF (7.5 mL) were added to each vial. Each reaction vial was microwaved for 10 minutes at 150 °C. The reaction mixtures were allowed to separate into two layers. Each organic layer (top layer) was removed, and all were combined. The combined organics were filtered through 0.20 μm nylon filters and washed with 5% methanol in dichloromethane. The filtrate was placed in a separatory funnel and washed with water. The organic layer was dried over $MgSO_4$, filtered, and concentrated *in vacuo*.

The residue was dissolved in 50% methanol in DCM and filtered through a plug of silica gel. The filtrate was diluted with DCM and filtered through filter paper to remove silica gel. The filtrate was concentrated *in vacuo*. The resultant solid was recrystallized from methanol to afford 1.50 g (51%) of the desired product. Prior to use *in vivo* the particle size of the material was reduced using a jet mill. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.14 (s, 1H), 7.96 (d, *J* = 8.3 Hz, 1H), 7.76 (s, 1H), 7.61 (d, *J* = 9.4 Hz, 1H), 7.55 (dd, *J* = 8.3, 1.4 Hz, 1H), 7.39 (d, *J* = 7.6 Hz, 1H), 2.94 (s, 3H). HRMS (ESI) *m/z* 269.0549 $[M+H]^+$ (269.0549 calculated for $C_{15}H_{10}N_2SF$).

Calcium mobilization assay

HEK 293A cells stably expressing $mGlu_5$ were plated in black-walled, clear-bottomed, poly-D-lysine coated 384-well plates (BD Biosciences, San Jose, CA) in 20 μL assay medium (DMEM containing 10% dialyzed FBS, 20 mM HEPES, and 1 mM sodium pyruvate) at a density of 20K cells/well. The cells were grown overnight at 37 °C in the presence of 6% CO_2. The next day, medium was removed and the cells incubated with 20 μL of 2 μM Fluo-4, AM (Invitrogen, Carlsbad, CA) prepared as a 2.3 mM stock in DMSO and mixed in a 1:1 ratio with 10% (w/v) pluronic acid F-127 and diluted in assay buffer (Hank's balanced salt solution, 20 mM HEPES and 2.5 mM Probenecid (Sigma-Aldrich, St. Louis, MO)) for 45 m at 37 °C. Dye was removed, 20 μL assay buffer was added and the plate was incubated for 10 m at room temperature. Ca^{2+} flux was measured using the Functional Drug Screening System (FDSS6000, Hamamatsu, Japan). Compounds were serially diluted 1:3 into 10 point concentration response curves (30 μM – 1 nM final) and transferred to daughter plates using the Echo acoustic plate reformatter (Labcyte, Sunnyvale, CA). Compounds were diluted into assay buffer to a 2x stock using a Thermo Fisher Combi (Thermo Fisher, Waltham, MA) which was applied to cells at t = 3s. Cells were incubated with the test compounds for 140 s and then stimulated with an EC_{20} concentration of glutamate; 74 s later an EC_{80} concentration of glutamate was added and readings taken for an additional 40 s. Data were collected at 1 Hz. Concentration response curves were generated using a four point logistical equation with XLfit curve fitting software for Excel (IDBS, Guildford, UK).

Radioligand binding assay

Membranes were prepared from rat $mGlu_5$ HEK293A cells. Compounds were diluted in assay buffer (50 mM Tris/0.9% NaCl, pH 7.4) to a 5x stock and 100 μL test compound was added to each well of a 96 deep-well assay plate. 300 μL aliquots of membranes diluted in assay buffer (40 μg/well) were added to each well. 100 μL [^{3}H]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). 40 μL scintillation fluid was added to each well and the membrane-bound radioactivity determined by scintillation counting (TopCount, PerkinElmer Life and Analytical Sciences). Non-specific binding was estimated using 5 μM MPEP. Concentration response curves were generated using a four parameter logistical equation in GraphPad Prism (GraphPad Software, Inc., La Jolla, CA).

Rat cortical astrocytes assay

Rat astrocytes (ScienCell, San Diego, CA, Cat# R1800) were thawed and plated into poly-L-lysine coated T-75 flasks (500K cells per flask) in astrocyte medium (ScienCell, San Diego, CA, Cat# 1801) and grown overnight at 37 °C in the presence of 6% CO_2. Astrocyte medium was changed after 16 hours and then every other day until cells reached confluence (approximately 1 week after thaw). Cells were plated in black-walled, clear-bottomed, poly-

L-lysine hand-coated 384-well tissue culture plates (Greiner Bio-One, Monroe, NC) in 20 μL assay medium (DMEM containing 10% dialyzed FBS, 20 mM HEPES, and 1 mM sodium pyruvate) at a density of 20K cells/well. Calcium assays were run as described above for HEK293A cells except that astrocytes were instead incubated in the FDSS with the test compounds for 140 s and then stimulated with a range of concentrations of glutamate and readings taken for an additional 40 s.

Stability in liver microsomes

The test compounds (1μM) were incubated for 15 min at 37 °C with shaking, in medium containing human/rodent liver microsomes, phosphate buffer, and the cofactor NADPH. Following incubation, the samples were extracted using ice-cold acetonitrile containing 0.1% formic acid and 50 ng/ml of an internal standard. The extracts were analyzed by means of HPLC/MS/MS using a ThermoFinnigan TSQ Quantum Ultra (Thermo Fisher Scientific, Waltham, MA) mass spectrometer in the positive ion mode, by selective reaction monitoring. The chromatographic separation was achieved on an Acquity UPLC BEH C18 column (1.7um; 2.1×50mm) at a flow rate of 0.8 ml/min. A gradient program was used with the mobile phase, combining solvent A (95: 5: 0.1% formic acid in water: acetonitrile) and solvent B (95: 5: acetonitrile: 0.1% formic acid in water) as follows: 20% B up to 0.5 min, ramped from 20–100% B by 1 min., and held at 100% B until 2 min. The composition was returned to 20% B by 2.2 min. The total run time was 5 minutes. The column temperature was maintained at 50 °C. The software Xcalibur version 2.2 was used to control the instrument and collect data. The electrospray ionization source was fitted with a stainless steel capillary (100 μm i.d.). Nitrogen was used as both the sheath gas and the auxiliary gas. The ion transfer tube temperature was maintained at 350 °C. The spray voltage, tube lens voltage, and pressure of sheath gas and auxiliary gas were optimized to achieve maximal response using the test compounds mixing with the mobile phase A (50%) and B (50%) at a flow rate of 0.8 ml/min. Collision-induced dissociation was performed on test compounds and internal standard under 1.5 mTorr of argon. The compounds were optimized individually for their optimal conditions analysis conditions using QuickQuan software (version 2.3). Percent test compound remaining following incubation was calculated based on the amount of compound in the incubated samples compared to similarly prepared un-incubated controls.

Plasma protein and brain homogenate binding assays

A 96-well rapid equilibrium dialysis (RED) apparatus (Thermo Scientific) was used to determine the free fraction in the blood and brain for compound. Mouse plasma and brain tissues were obtained fresh on the day of the experiment. 6-8 brain tissues were homogenized with PBS to a final composition of 1:3 (w/w) brain:PBS using a sonic dismembrator (Fisher Scientific) in an ice bath. Neat plasma and diluted brain homogenate were spiked with compound at 1000 ng/g concentrations, and 200 μl aliquots (n = 3 replicate determinations) were loaded into the sample chambers of RED plate. Dialysis versus PBS (350 μl) was carried out for 4 h in a temperature-controlled incubator at 37 °C using shaker at 130 revolutions/ min. At the end of the incubation period, 50 μl aliquots of blood, brain homogenate, or PBS were transferred to a 96 deep well plate, and the composition in each well was balanced with control fluid, such that the volume of PBS to blood or brain was the same. Sample extraction was performed by the addition of 300 μl of acetonitrile containing an internal standard. Samples were vortex mixed for 5 min and then centrifuged for 10 min and supernatants injected onto LC/MS/MS. The unbound fraction in plasma was determined as the ratio of the peak area in buffer to that in plasma. The unbound fraction in brain was determined as the ratio of the peak area in buffer to that in brain, with correction for dilution factor according to eq. 1,[69]

$$fu = \left(\frac{1}{D}\right) / \left(\frac{1}{fu}\,(apparent) + \frac{1}{D}\right)$$

Where *D* is the dilution factor in brain homogenate and fu (apparent) is the measured free fraction of diluted brain tissue.

Mouse pharmacokinetic study

Compound **18** was formulated as a 10% Tween 80 microsuspension in sterile water at the concentration of 0.5 mg/ml and administered intraperitoneally to male CD-1 mice weighing around 30 g at the dose of 10 mg/kg. The volume of administration used was 20 ml/kg. The mice blood and brain samples were collected at 15, 30, 60, 180 and 360 min after dose administration. Animals were euthanized and decapitated, and the brains were removed, thoroughly washed in cold PBS, and immediately frozen on dry ice. Blood (cardiac puncture) was collected in EDTA Vacutainer tubes, and plasma was separated by centrifugation and stored at -80 °C until analysis. Three animals were used for each time point. On the day of analysis, frozen whole brains were weighed, and homogenized in 1:5 (w/w) volumes of ice-cold PBS (pH 7.4). The sample extraction of plasma (100μl) and brain homogenate (100 μl) was performed by a method based on protein precipitation, using three volumes of cold acetonitrile containing 0.1% formic acid and an internal standard having final concentration of 50 ng/ml. Extracts were vortex mixed for 5 min followed by centrifugation at 14,000 rpm for 10 min. The supernatants of plasma and brain homogenate extracts were analyzed by means of HPLC/MS/MS, using a ThermoFinnigan TSQ Quantum Ultra (Thermo Fisher Scientific, Waltham, MA) mass spectrometer in positive ion mode. The chromatographic separation was achieved on an Acquity UPLC BEH C18 column (1.7um; 2.1×50mm) at a flow rate of 0.8 ml/min. The gradient program was used with the mobile phase, combining solvent A (95: 5: 0.1% formic acid in water: acetonitrile) and solvent B (95: 5: acetonitrile: 0.1% formic acid in water) as follows: 20% B (0.5 min), 20–95% B (0.5 min), 95% B (1 min), 95–20% B (0.2 min), 20% B (2.8 min). The column temperature was set at 50 °C. The software Xcalibur version 2.0 was used to control the instrument and collect data. The electrospray ionization source was fitted with a stainless steel capillary (100 μm i.d.). Nitrogen was used as both the sheath gas and the auxiliary gas. The ion transfer tube temperature was 300 °C. The spray voltage, tube lens voltage, and pressure of sheath gas and auxiliary gas were optimized to achieve maximal response using the test compounds mixing with the mobile phase A (50%) and B (50%) at a flow rate of 0.8 ml/min. Collision-induced dissociation was performed on compound and internal standard under 1.0 mTorr of argon. Selected reaction monitoring was carried out using the transitions from *m*/*z* 269.3 to 184.3 for test compound @ collision energy of 36eV, and *m*/*z* 310 to 223 for internal standard @ collision energy of 25eV. The calibration curves were constructed and linear response was obtained in the range of 10 - 2000 ng/ml by spiking known amounts of test compound in blank brain homogenates and plasma. Brain concentrations were corrected for dilution in PBS. The final PK parameters were calculated by noncompartmental analysis using WinNonlin software (version 5.1, Pharsight Inc.).

Marble burying experiment

Compounds

The $mGlu_5$ NAMs MTEP and **18** (prepared in-house) were dissolved in 10% Tween 80, vortexed vigorously, heated gently with a Master Heat Gun (Master Appliance Corp., Racine, WI), and sonicated at 37°C for 30 min. The pH was checked using 0-14 EMD strips and adjusted to approximately 7. All doses were administered at 10 ml/kg ip.

Five dose groups

vehicle, 15 mg/kg MTEP (positive control), 3 mg/kg **18**, 10 mg/kg **18**, 30 mg/kg **18**.

Subjects

This study was conducted using male Harlan CD-1 mice (Harlan Sprague Dawley, Indianapolis, IN), weighing 30 to 35 grams. Subjects were housed in a large colony room under a 12-h light/dark cycle (lights on at 6:00 a.m.) with food and water provided *ad libitum*. Test sessions were performed between 10:00 a.m. and 4:00 p.m. All dose groups consisted of 12 mice. All experiments were conducted in accordance with the National Institute of Health regulations of animal care covered in Principles of Laboratory Animal Care (National Institutes of Health publication 85-23, revised 1985) and were approved by the Institutional Animal Care and Use Committee.

Procedure

Eight small Plexiglass cages (32 × 17 × 14 cm) were arranged in two rows of four cages on top of a large, round table. Mice were transported from the colony room to the testing room and allowed to habituate for 30 minutes. Mice were pretreated with a dose of MTEP or **18** for 15 min and individually placed in the cages in which 12 black glass marbles (14 mm diameter) had been evenly distributed (spaced 6.4 cm vertically and 4.25 cm horizontally from each other and the walls of the cage) on top of 2.5 cm Diamond Soft Bedding (Harlan Teklad, Madison, WI). The compound and comparator were evaluated in a counterbalanced design, in which all doses of compounds were tested in each session. Mice receiving the same dose were placed in cages on opposite sides of the table to control for effects of lighting and context. Clear, perforated plastic lids were set on top of each cage and the amount of marble burying was recorded over a 30 min interval. The mice were then removed from the cages and the number of buried marbles was counted using the criteria of greater than 2/3 covered by bedding. Each session was videotaped with a Sony MiniDV camcorder equipped with a Sony wide-angle lens mounted on a 1.5 m tripod.

Data Analysis

The data for the dose-response studies were analyzed by a between-group analysis of variance. If there was a main effect of dose, then each dose group was compared with the vehicle control group using a Dunnett's comparison. The calculations were performed using JMP IN 8 (SAS Institute, Cary, NC) statistical software and graphed using SigmaPlot9 (Sasgua, MA).

Operant sensation seeking (OSS) experiments

Animal care

Male C57Bl/6J mice (3-5 weeks old) were housed in Vanderbilt Animal Care Facilities in groups of four with lights on from 3:00 p.m. to 3:00 a.m. Food and water was available *ad libitum* for the duration of the experiments. All procedures were approved by the Animal Care and Use Committee at Vanderbilt University. Experiments took place between 9:00 a.m. and 2:00 p.m. and began after 3 days of handling.

Operant chambers

Operant sensation seeking (OSS) was performed as previously reported.[63,64,67] Operant training chambers are housed inside sound-attenuating cubicles containing an exhaust fan (MED Associates). Chambers (21.6×17.8×12.7 cm) are equipped with two levers, one on each side of the right wall as described.[70,71,72] Levers are mounted 2.2 cm above the grid floor, with cue lamps (yellow LEDs) mounted 2 cm above them, and a house lamp mounted

on the opposite wall. At the beginning of each session, the house light is illuminated and the exhaust fan is turned on. For OSS, a compound visual/auditory stimulus is presented after completion of the required ratio, while presses on the inactive lever are counted but have no programmed consequence. The stimulus is a presentation of flashing cue lights with random duration of 1, 2, 4, or 8 sec and random flash rate of 0.625, 1.25, 2.5 or 5 Hz. Each light flash is randomly on the right or left side of the chamber and the house light is turned off during the visual stimuli. The auditory stimulus is activation of an infusion pump located within the cubicle (no infusion is made).

Fixed Ratio (FR)

Parallel experiments using separate mice (n=8 per group) were run using either OSS or food reinforcer. Experiments began with daily 1 hour operant sessions without any prior training. OSS mice received varied visual and auditory stimuli as a reinforcer, while food mice received diluted Vanilla Ensure® (~40 μl of a 10% solution) as a reinforcer. Experiments began with 10-14 sessions using an FR-1 schedule of reinforcement. Mice that did not meet criteria (≥20 active lever presses, ≥60% lever accuracy for the final two sessions) within the first 14 sessions continued FR-1 training until meeting criteria or until the maximum of 25 sessions had been run. One animal (food reinforced) was excluded for not meeting criteria after 25 sessions.

Progressive Ratio (PR)

After FR training, mice responded for reinforcers on a progressive ratio (PR) in 2 hour sessions. The schedule of reinforcement was increased in the following pattern: 1, 2, 4, 6, 9, 12, 16, 20, 25, 30, etc.[63,67] To facilitate acquisition of PR, only the active lever was available. Mice had five initial PR sessions prior to the drug testing phase of the experiment. During these 5 sessions, mice were habituated to the injection procedure (20 ml/kg saline, i.p., 10 min prior to sessions).

Testing Phase

Testing of the compound was done on a PR schedule of reinforcement as described above. Compound **18** was prepared and given 10 min prior to operant sessions (vehicle: 10% Tween-80, 20 ml/kg i.p.). Mice received each dose (0, 3, 10, 30 mg/kg) 48 hours apart using a within-subjects Latin square design, with two non-drug sessions (saline pretreatment) between each dose. Operant responding (reinforcers earned) following drug treatment was analyzed by one-way repeated measures ANOVA followed by Dunnett's post hoc tests comparing vehicle to each drug dose. Mice were excluded if they didn't earn ≥ 3 reinforcers following vehicle injection. One mouse (food reinforced group) was excluded for this reason. Testing of was done as described above with the exception that all animals received 12 FR sessions prior to the five PR sessions. MTEP-HCl (Ascent Scientific, Princeton, NJ) was prepared and given 10 min prior to operant sessions (vehicle: 0.9% saline, 20 ml/kg i.p.) and mice received each dose (0, 1.5, 5, 15 mg/kg) as described for compound **18**. Data were analyzed by one-way repeated measures ANOVA followed by Dunnett's post hoc tests comparing vehicle to each drug dose. One data point from the final day of experiments was removed due to injury during injection.

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Abbreviations

mGlu	metabotropic glutamate receptor
GPCR	G-protein-coupled receptor
NAM	negative allosteric modulator
GERD	gastroesophageal reflux disease
PD	Parkinson's Disease
7TM	seven transmembrane
cAMP	cyclic adenosine monophosphate
MPEP	2-methyl-6-(phenylethynyl) pyridine
MTEP	3-[(2-methyl-1,3-thiazol-4-yl)ethynyl]pyridine
PDLID	Parkinson's Disease levodopa-induced dyskinesia
MAOS	microwave-assisted organic synthesis
LC	liquid chromatography
MS	mass spectrometry
SAR	structure-activity relationship(s)
NIDA	National Institute on Drug Abuse
FDA	Food and Drug Administration
OSS	operant sensation seeking
THF	tetrahydrofuran
DCM	dichloromethane
DMEM	Dulbecco's Modified Eagle's medium
FBS	fetal bovine serum
HEPES	*N*'-2-hydroxyethylpiperazine-*N*'-2 ethanesulphonic acid
DMSO	dimethylsulfoxide
Tris	tris(hydroxymethyl)aminomethane
methoxyPEPy	3-methoxy-5-(pyridin-2-ylethynyl)pyridine
FDSS	Functional Drug Screening System
NADPH	nicotinamide adenine dinucleotide phosphate

HPLC	high performance liquid chromatography
RED	rapid equilibrium dialysis
PBS	phosphate buffered saline
Tween 80	polyoxyethylene (20) sorbitan monooleate
EDTA	ethylenediaminetetraacetic acid
PK	pharmacokinetics
LED	light-emitting diode
FR	fixed ratio
PR	progressive ratio
ANOVA	analysis of variance
RMANOVA	repeated measures analysis of variance
dppf	1,1'-bis(diphenylphosphino)ferrocene

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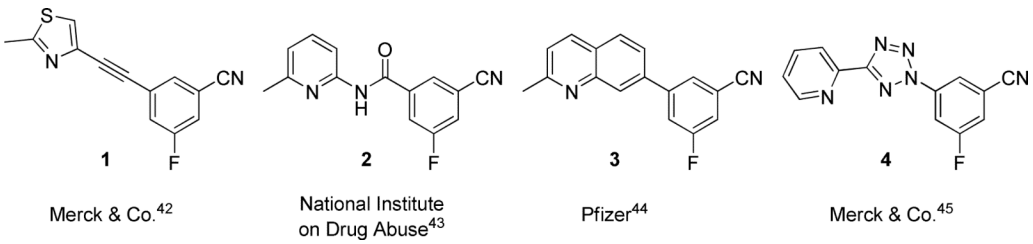
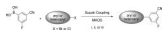


Figure 1.
3-Cyano-5-fluorophenyl ring containing non-competitive antagonists of $mGlu_5$.



Scheme 1.

(i) $Pd(^tBu_3)_2$, 1M Cs_2CO_3/THF (1:1), μw, 150 °C, 10 min. (ii) $Pd(PPh_3)_4$, 1M Cs_2CO_3/THF (1:1), μw, 120 °C, 20 min. (iii) $PdCl_2$(dppf), 1M Na_2CO_3/DMF (1:3), μw, 140 °C, 20 min.

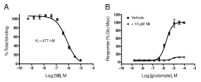


Figure 2.

Radioligand binding and blockade of glutamate response in rat cortical astrocytes. A) Compound **18** potently inhibits binding of [^{3}H]3-methoxy-5-(pyridin-2-ylethynyl)pyridine. B) 10 μM of compound **18** produces a near complete blockade of the response to glutamate in rat cortical astrocytes.

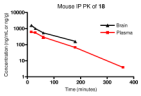


Figure 3.
Pharmacokinetics of **18** following intraperitoneal dosing in mice demonstrated good exposure in the brain out to one hour.

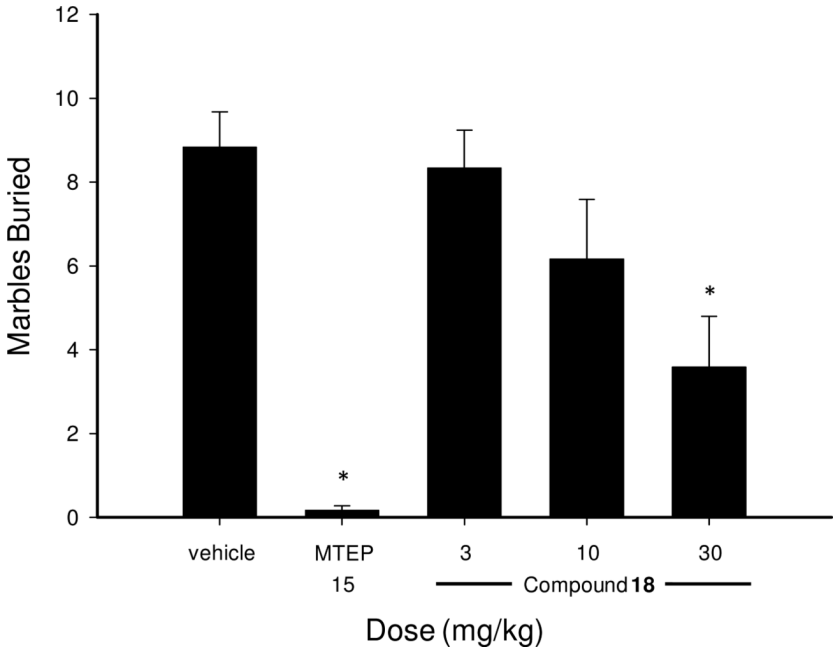


Figure 4.
Inhibition of marble burying by compound **18** in mice.

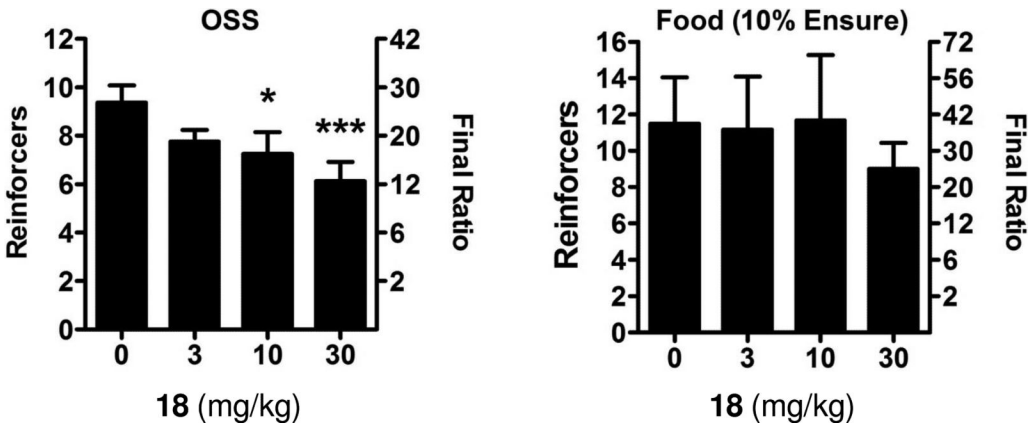


Figure 5.

Dose dependent reduction of progressive ratio responding for OSS stimuli, but not for food, by compound **18** in mice. OSS: n=8, Food: n=6, *p<0.05,***p<0.001.

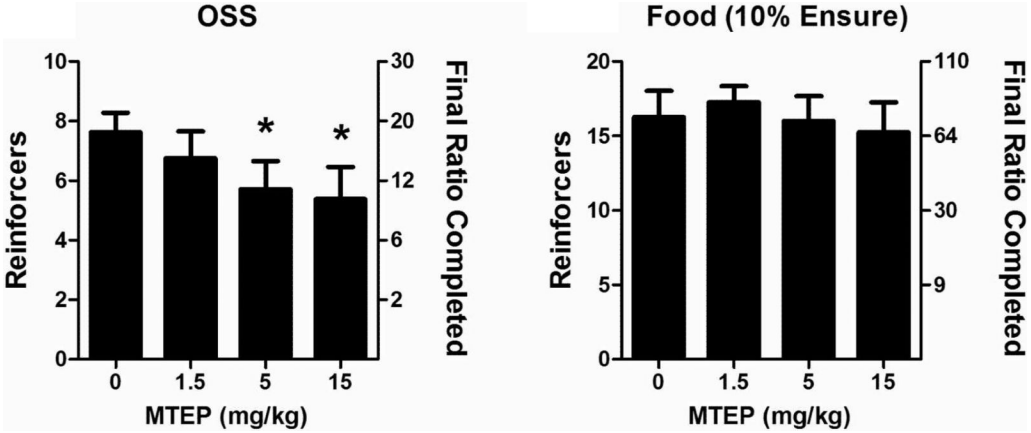
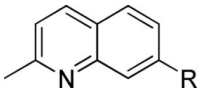
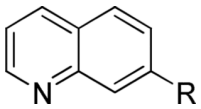
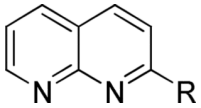
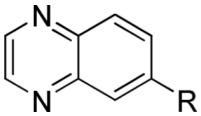
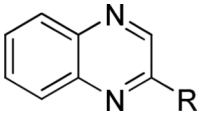
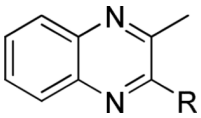
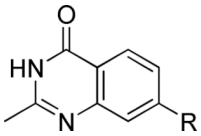
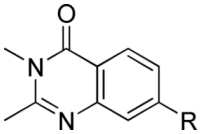
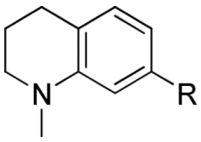
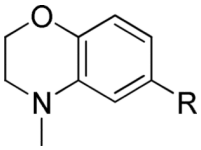


Figure 6.

Dose dependent reduction of progressive ratio responding for OSS stimuli, but not for food, by MTEP in mice. OSS: n=8, Food: n=8, *p<0.05.

Table 1

SAR of 6,6 fused ring heterocycles

Compound	Structure[a]	mGlu$_5$ IC$_{50}$ (nM)[b]	% Glu Max[c]	Synthetic Conditions
3		9.5 ± 1.6	1.2 ± 0.3	i
5		182 ± 114	1.8 ± 0.2	ii
6		133 ± 63	2.6 ± 0.6	iii
7		>30,000	–	ii
8		1190 ± 61	20.1 ± 4.1	i
9		6000 ± 663	12.4 ± 1.6	i
10		>30,000	–	i
11		>10,000[d]	54.0 ± 6.4	i
12		>30,000	–	ii
13		>10,000[d]	66.6	ii

[a] R = 3-cyano-5-fluorophenyl

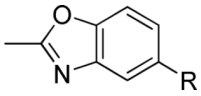
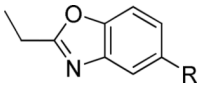
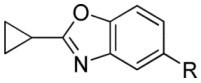
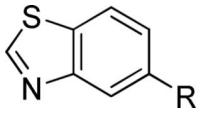
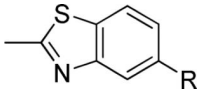
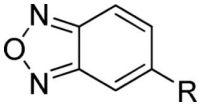
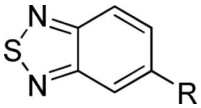
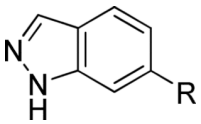
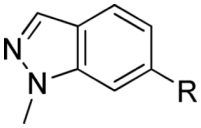
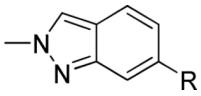
[b] Calcium mobilization mGlu5 assay; values are average of n ≥ 3

[c] Amplitude of response in the presence of 30 μM test compound as a percentage of maximal response (100 μM glutamate); values are average of n ≥ 3

[d] CRC does not plateau

Table 2

SAR of 5,6 fused ring heterocycles

Compound	Structure[a]	mGlu$_5$ IC$_{50}$ (nM)[b]	% Glu Max[c]	Synthetic Conditions
14		>30,000	–	i
15		2460 ± 226	26.3 ± 14.4	iii
16		>10,000[d]	46.5 ± 7.8	i
17		>30,000	–	i
18		61 ± 7	0.63 ± 0.12	i
19		>30,000	–	iii
20		>30,000	–	iii
21		>10,000[d]	60.4 ± 2.5	iii
22		>30,000	–	from **21**[e]
23		1520 ± 296	4.8 ± 2.3	from **21**[e]

[a] R = 3-cyano-5-fluorophenyl

[b] Calcium mobilization mGlu5 assay; values are average of n ≥ 3

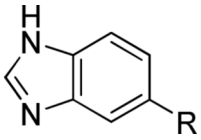
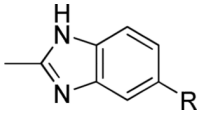
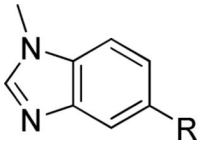
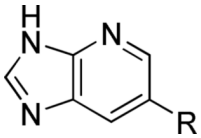
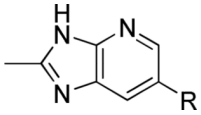
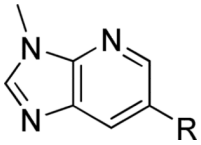
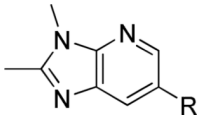
[c] Amplitude of response in the presence of 30 μM test compound as a percentage of maximal response (100 μM glutamate); values are average of n ≥ 3

[d] CRC does not plateau

[e] Reaction of **21** with K_2CO_3 and MeI in DMF afforded a separable mixture of **22** and **23**

Table 3

SAR of benzimidazole analogs

Compound	Structure[a]	$mGlu_5$ IC_{50} (nM)[b]	% Glu Max[c]	Synthetic Conditions
24		>30,000	–	iii
25		7010 ± 916	7.9 ± 2.5	i
26		>10,000	53.8 ± 6.2	i
27		>30,000	–	iii
28		>30,000	–	iii
29		555 ± 56	1.3 ± 0.4	i
30		>30,000	–	i

[a] R = 3-cyano-5-fluorophenyl

[b] Calcium mobilization mGlu5 assay; values are average of n ≥ 3

[c] Amplitude of response in the presence of 30 μM test compound as a percentage of maximal response (100 μM glutamate); values are average of n ≥ 3

[d] CRC does not plateau

Table 4

In vitro DMPK profile of **18**

Metabolic stability in liver microsomes	
Species	**% Parent Remaining**
mouse	18
human	20

Protein binding	
Sample	**% Bound**
mouse brain homogenate	99.9
mouse plasma	99.5
human plasma	99.4