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Chemical Switch of a Metabotropic Glutamate Receptor 2 Silent Allosteric Modulator into Dual Metabotropic Glutamate Receptor 2/3 Negative/Positive Allosteric Modulators

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Using an mGluR2 FRET-based binding assay, binders of the transmembrane region devoid of functional activity were identified. It is reported that slight chemical modifications of these SAMs can dramatically change activity of the resulting analogues without altering their affinities. Starting from compound **1**, three mGluR2 NAMs showing also mGluR3 PAM activities were obtained. SAMs therefore represent a useful approach to explore the chemical space for GPCR allosteric modulator identification.

Introduction

Metabotropic glutamate receptors (mGluRs)[a] belong to class C of G-protein coupled receptors (GPCRs), the best family of drug targets with nearly half of the registered therapeutic agents. They have been divided into three groups according to their sequence homologies, pharmacological properties, and signal transduction pathways.[1] Group II mGluRs (composed of mGluR2 and mGluR3) are largely expressed in central regions such as cortex, hippocampus, striatum, and amygdala.[2] Their activation by group-selective orthosteric agonists leads to anxiolytic-like and antipsychotic-like activities in rodents and is currently under clinical evaluations for schizophrenia treatment.[3,4] However, due to high homology between mGluR2 and mGluR3 orthosteric binding sites, subtype-selective agonists or antagonists were never described. Current efforts toward the selective regulation of group II mGluRs are focused on positive allosteric modulators (PAMs) and negative allosteric modulators (NAMs) for the treatment of schizophrenia and Alzheimer's disease respectively.[5−8]

Allosteric modulation represents an emerging and promising approach for the regulation of GPCRs. Compared with activation or inhibition with orthosteric ligands, allosteric modulation is associated with major advantages such as subtype selectivity, saturable effects, mimicking of physiological response, and tractable chemistry. To date, two GPCR allosteric modulators (AMs) have reached the market: Cinacalcet, a calcium-sensing receptor PAM for the treatment of hyperparathyroidism, and Maraviroc, a chemokine receptor CCR5 NAM for the treatment of HIV.[9,10] One possible explanation for this relatively low number of AM drugs lies in the lack of technology specifically dedicated for their discovery. Indeed, the standard screening process in the pharmaceutical industry, based on functional assays set up with specific conditions and a single readout, undersamples the chemical space surrounding potential therapeutic candidates for a chosen GPCR.[11]

Screening of a diverse 10000 compound library with an mGluR2 FRET-based binding assay led to the identification of several micromolar mGluR2 binders, including **1**, that turned out to be inactive in a Ca^{2+}-based functional assay (Table 1). Slight chemical modifications of **1** could dramatically affect its functional activity changing it into NAMs for mGluR2 and PAMs for mGluR3. To our knowledge, the molecules described in this article are the first group II mGluR AMs able to functionally distinguish between the two close subtypes. They constitute novel pharmacological tools to study the respective roles and therapeutic potentials of mGluR2 and mGluR3. In addition, this is the first report describing the use of a silent allosteric modulator (SAM) as a chemical source for the identification of novel PAMs and NAMs.

Results and Discussion

To selectively identify AMs of the human mGluR2, a FRET-based binding assay was developed using a truncated form of the receptor where the N-ter region was replaced by green fluorescent protein (GFP).[12] This truncation results in a receptor that no longer contains the glutamate orthosteric binding site (Figure 1). The fluorescent probe used was derived from nanomolar ligands known to interact with the 7 transmembrane (7TM) region of group II mGluRs.[13]

Screening of this GFP-receptor with a diverse 10000 compound library led to the identification of several 7TM binders that were further characterized using a functional assay with the native human mGluR2 cotransfected with an hybrid G-protein coupling to the Ca^{2+} pathway.[14] PAMs and NAMs were identified together with binders exhibiting no functional activity such as **1**, a close derivative of the mGluR4 PAM PHCCC,[15] that was recently shown to exert weak mGluR4 PAM activity.[16] Compound **1** showed micromolar affinity for mGluR2, with a K_i value of 6.6 μM but no activity

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[a]Abbreviations: AM, allosteric modulator; FRET, fluorescence resonance energy transfer; GFP, green fluorescent protein; GPCR, G-protein-coupled receptor; mGluR, metabotropic glutamate receptor; NAM, negative allosteric modulator; PAM, positive allosteric modulator; SAM, silent allosteric modulator; 7TM, 7 transmembrane.

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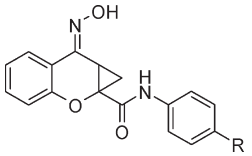
Table 1. Binding Affinities on mGluR2 and Functional Activities on mGluR2 and mGluR3[a]



		human mGluR2[b]		human mGluR3[b]
compd	R	binding affinity K_i (μM)	Ca^{2+} functional test IC_{50} (μM)	Ca^{2+} functional test EC_{50} (μM)
PHCCC	H	NA	NA	NA[c]
1	F	6.6	NA	NA
2	Cl	1.0	0.8 (NAM)	13.4 (PAM)
3	Me	0.6	1.5 (NAM)	8.9 (PAM)
4	OMe	0.8	1.0 (NAM)	10.4 (PAM)

[a] All values are the mean of three separate assay determinations performed in duplicate. Standard deviations for these values were within 15% of the mean value. [b] NA: not active. [c] See ref 16.

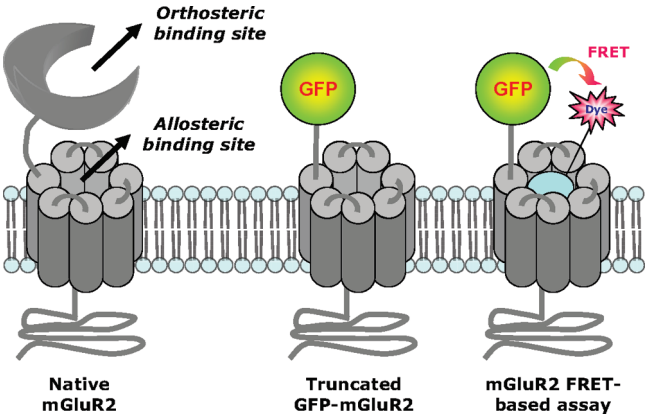


Figure 1. Description of the engineered mGluR2 used for the FRET-based binding assay.

(agonist, PAM or NAM) in the Ca^{2+} functional assay up to 100 μM (see Figure 2A).

Such silent binders were reported previously for another mGluR subtype, mGluR5.[17−19] Interestingly, these reports also described very close analogues displaying PAM or NAM activities. Key modifications distinguishing mGluR5 silent binders from PAMs or NAMs were described as aromatic substitutions on phenyl or pyridine rings. We therefore decided to focus on fluorine replacements in **1** to modulate the functional activity in this series of mGluR2 compounds. The corresponding analogues of **1** were synthesized using the procedure described in Scheme 1.

PHCCC's analogues **1**−**4** were prepared following a synthetic route that slightly differs from the one previously used by Annoura et al.[20] Indeed, in order to explore the phenyl amide moiety, it was found more convenient to start with the cyclopropanation of the chromene-methyl-ester **5** with trimethylsulfoxonium iodide. In situ saponification of the corresponding product leads to the carboxylic acid key intermediate **6** that was used for the synthesis of all compounds of this paper. Coupling reactions were done using EDCI in DMF, and the corresponding amides were treated, without further purification, with hydroxylamine using microwave irradiations in pyridine to lead to compounds **1**−**4**. All compounds were characterized by ^{1}H NMR and LCMS before pharmacological evaluations.

We found that replacement of fluorine by bulkier chlorine (**2**), methyl (**3**), or methoxy (**4**) has positive influence on mGluR2 affinities as the corresponding compounds bind the receptor with K_i of respectively 1, 0.6, and 0.8 μM (Table 1). Functional activities of these compounds were evaluated using the hybrid G-protein G_{qi9} that couples mGluR2 with the intracellular Ca^{2+} transduction pathway. Results show that replacement of fluorine atom of **1** leads to dramatic changes of functional activity. Analogues **2**, **3**, and **4** exert NAM activities, with IC_{50} values in the low micromolar range (Table 1 and Figure 2a). Compounds **2**−**4** were also effective in right-shifting glutamate EC_{50} and decreasing glutamate E_{max} on human mGluR2 (see Figure 3a for an illustration with **3**). Very interestingly, it was recently reported that another close analogue of **1**, the (−) enantiomer derivative having a 4-fluoro-pyridin-2-yl moiety instead of the 4-fluorophenyl moiety, showed weak NAM activity on mGluR2.[16] This further reinforces the observation that slight chemical modifications can switch functional activity of mGluR2 ligands.

The switch in functional activity of **2**−**4** did not seem to be electronically driven as it resulted from the replacement of a fluorine atom by either electron-donating or electron-withdrawing groups. It was more likely the result of the increased lipophilicity found in **2**, **3**, and **4** and the accommodation of the corresponding new substituents in a lipophilic pocket.

To determine their group II mGluR selectivity profile, **1**−**4** were also evaluated against mGluR3 in a functional assay setup with $G_{\alpha 15}$ to couple the Ca^{2+} pathway. Compound **1** was inactive up to 100 μM similarly to that on mGluR2, but surprisingly analogues **2**, **3**, and **4** were shown to be PAMs with respective EC_{50} of 13.4, 8.9, and 10.4 μM (Table 1 and Figure 2b). Tested against a dose−response of glutamate, **2**−**4** also left-shifted EC_{50} and increased E_{max} of glutamate (see Figure 3b for an illustration with **3**). The interesting opposite activities of **2**−**4** on mGluR2 and mGluR3 can be explained by the difference in amino acid composition of the two close group II mGluR 7TM regions. For instance, it was described that the three amino acids involved in the active site of the mGluR2 PAM LY487379 differ in mGluR3 demonstrating that these sites, in opposition to the orthosteric binding site, are subject to less evolutionary pressure.[21]

The close structural relationship between silent binders and PAMs/NAMs was previously described for class A muscarinic receptors,[22] class C mGluR5,[17−19] and was observed for other GPCR members such as the mGluR4 subtype or the peptidic class A Neurotensin NTSR1. We propose here to use the term silent allosteric modulator or SAM to define this new class of AMs that fulfill the following two criteria: (1) binding to the receptor of interest, (2) being inactive in a given

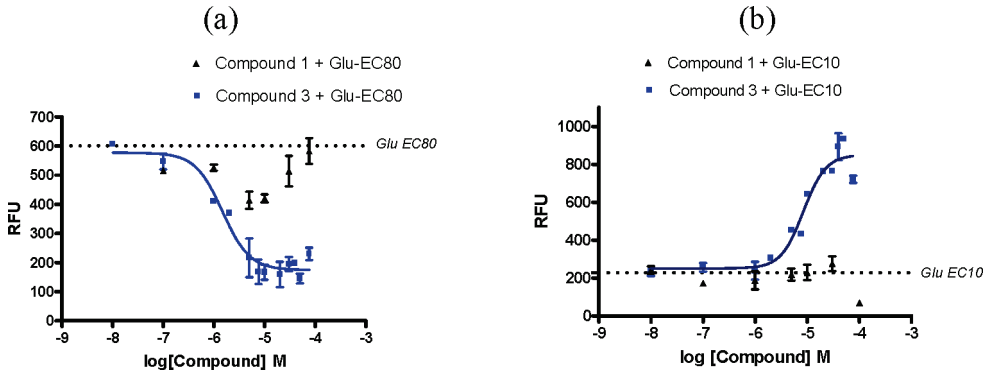
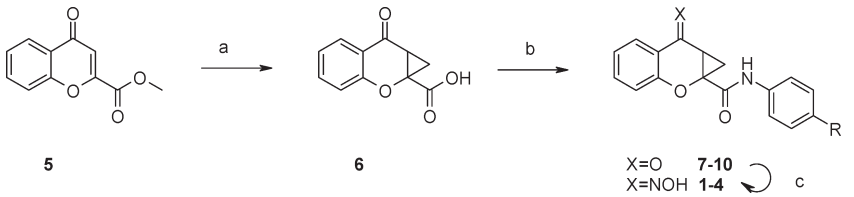


Figure 2. Activities of compounds **1** and **3** tested in dose−response experiments in the presence of a glutamate EC_{80} on mGluR2 (a) and in the presence of a glutamate EC_{10} on mGluR3 (b).

Scheme 1. Synthesis of Compounds **1**−**4**[a]



[a] Reagents and conditions: (a) (1) Me_3SOI, NaH, DMF, 0 °C to RT, 19 h, (2) HCl 1N; (b) aniline derivatives, EDCI-HCl, DMAP, DMF, 15 h; (c) NH_2OH-HCl, pyridine, 150 °C 5 min. (microwave).

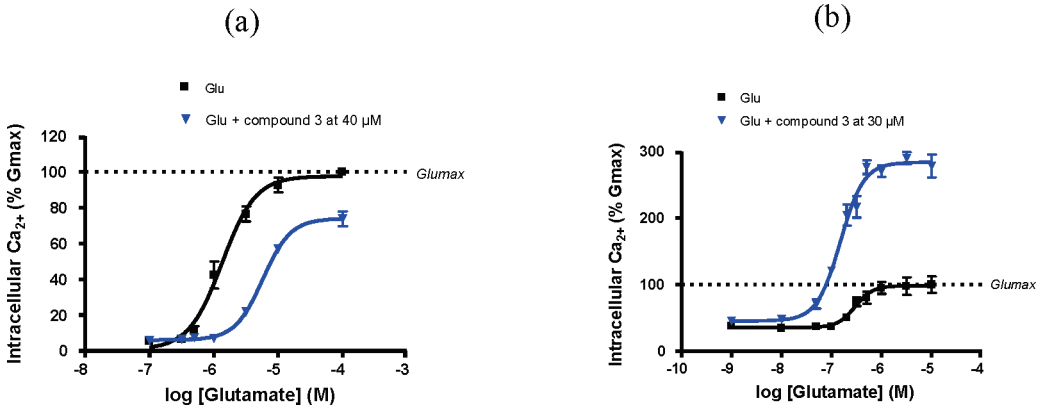


Figure 3. Concentration−response curves for glutamate in absence or presence of **3**. Compound **3** was tested at 40 μM on mGluR2 (a) and at 30 μM on mGluR3 (b).

functional test, in which close derivatives are active. The silent feature of the ligand will therefore refer to a particular test in which analogues of the same chemical series will behave either as a PAM or a NAM. Moreover, as an AM can be qualified as silent under specific functional test conditions, but positive or negative under different conditions,[23−26] we are recommending using the term SAM only if accompanied by details of the functional tests performed to characterize it.

This SAM to PAM/NAM change seems to be a generic phenomenon that can be exploited by medicinal chemists as a new source of chemical diversity for GPCR modulator identification. Indeed, current screening technologies require an active member for the identification of a chemical family. Detecting SAMs can enlarge the chemical space available by enabling the discovery of novel chemical scaffolds not accessible through standard functional tests. SAMs therefore represent a unique source of compounds that can be modified by medicinal chemists to obtain active AMs or that can be further functionally characterized to unveil their activity.

Conclusion

In summary, using a FRET-based binding assay, we have revealed a novel category of ligands for mGluR2, silent allosteric modulators that bind the receptor but exert no activity in a calcium-based functional test. Such SAMs represent a unique source of novel chemical diversity for identification of GPCR modulators as they can be transformed into PAMs or NAMs by simple chemical modifications. Moreover, we have obtained compounds that differentiate the close mGluR2 and mGluR3 in functional tests. This is, to our knowledge, the first report of allosteric modulators functionally displaying opposite activities on the two group II mGluRs.

Experimental Section

Chemistry. General. All reagents and starting materials were commercial grade and used without further purification. Commercially available anhydrous solvents were used for reactions conducted under inert atmosphere (argon). Microwave assisted

reactions were run in a Biotage microwave initiator. Silica gel used for chromatography columns was SDS silica gel (60AAC 40−63 μM). All reactions were followed by thin layer chromatography (TLC plates: precoated silica gel F-254) or liquid chromatography mass spectometry (LCMS). Concentration referred to evaporation under vacuum using a rotary evaporator. The reaction yields were not optimized. ^{1}H NMR spectra were recorded on a Bruker 400 MHz spectrometer. Proton chemical shifts (δ in ppm) are listed relative to residual $CDCl_3$ (7.27 ppm) or DMSO (2.50 ppm). Splitting patterns are designated as s (singlet), d (doublet), dd (double−doublet), t (triplet), q (quartet), m (multiplet), or br (broad). Electrospray mass spectometry spectra were obtained on a Waters micromass platform LCMS spectrometer. All mass spectra were full-scan experiments (mass range 100−1500 amu) obtained using electrospray ionization. The HPLC system was a Waters platform with a 2767 sample manager, a 2525 pump, a photodiode array detector (190−400 nM). The column used was an Xterra C_{18} 3.5 μM (4.6 m × 50 mm). The mobile phase consisted in an appropriate gradient of A and B. A was water with 0.05% of TFA, and B was acetonitrile with 0.05% of TFA. Flow rate was 1 mL per min. All LCMS were performed at room temperature. The purities of final compounds **1**−**4** were measured by HPLC and were found to be above 98%.

7-Oxo-7,7a-dihydrocyclopropa[*b*]chromene-1a(1*H*)-carboxylic Acid (6). To a suspension of trimethylsulfoxonium iodide (2.85 g, 1.5 equiv) in DMSO (30 mL) cooled at 15 °C, NaH (500 mg, 1.4 equiv) was added portion by portion over 10 min. This suspension was stirred 1 h, and then a solution of chromenone **5** (1.766 g) in DMSO (15 mL) was added dropwise over 10 min. Reaction mixture was allowed to warm to room temperature and was stirred 19 h. The deep-yellow mixture was hydrolyzed with aqueous HCl solution (1N, 200 mL) and was extracted with EtOAc (200 mL). Organic layer was washed with aqueous NaOH solution (1N, 100 mL). This basic aqueous layer was acidified to pH 1 by addition of concentrated aqueous HCl and was extracted with EtOAc (200 mL). This organic layer was washed with brine, dried over $MgSO_4$, and was concentrated under reduce pressure to afford **6** as a beige solid in 30% yield. ^{1}H NMR (400 MHz, DMSO): 1.69 (t, J = 6.7 Hz, 1H), 2.12 (dd, J = 11.1 Hz, J = 6.7 Hz, 1H), 2.64 (dd, J = 11.1 Hz, J = 6.7 Hz, 1H), 7.14−7.18 (m, 2H), 7.63−7.67 (m, 1H), 7.79 (d, J = 8.2 Hz,1H).

General procedures for amidation of (**6**) with anilines and for reaction of corresponding amides with hydroxylamine. To a solution of 7-oxo-7,7a-dihydrocyclopropa[*b*]chromene-1a(1*H*)-carboxylic acid (40 mg, 0.19 mmol) in DMF (1 mL), EDCI-HCl (58 mg, 1.6 equiv), DMAP (35 mg, 1.5 equiv), and the selected amine (2.0 equiv) were added. The resulting mixture was stirred at R.T. until carboxylic acid **6** was consumed base on LCMS analysis (around 16 h). Reaction mixture was then hydrolyzed with aqueous HCl (1N, 25 mL) and extracted with EtOAc (25 mL). Organic layer was washed with brine (20 mL), aqueous NaOH (1N, 20 mL), brine (15 mL), dried over $MgSO_4$, and concentrated. Residue was used in the next step without further characterization.

To a solution of ketone **7**−**10** (0.19 mmol) in pyridine (1.0 mL), hydroxylamine hydrochloride (25 mg, 1.9 equiv) was added. The resulting mixture was heated through microwave irradiation for 5 min at 150 °C. Reaction mixture was then hydrolyzed with aqueous HCl (1N, 25 mL) and extracted with EtOAc (25 mL). Organic layer was washed with brine (15 mL), dried over $MgSO_4$, and concentrated. Residue was triturated in Et_2O to afford **1**−**4** in 21% to 51% yields.

***N*-(4-Fluorophenyl)-7-oxo-7,7a-dihydrocyclopropa[*b*]chromene-1a(1*H*)-carboxamide (1). 1** was obtained as a beige solid in 21% yield. ^{1}H NMR (400 MHz, DMSO): 1.44 (dd, J = 7.0 Hz, J = 5.8 Hz, 1H), 1.96 (dd, J = 10.8 Hz, J = 5.8 Hz, 1H), 3.07 (dd, J = 10.8 Hz, J = 7.0 Hz, 1H), 7.03−7.08 (m, 1H), 7.16−7.22 (m, 3H), 7.39−7.44 (m, 1H), 7.70−7.77 (m, 3H), 10.19 (s, 1H, D_2O exchange), 11.46 (s, 1H, D_2O exchange). MS m/z $(M + H)^+$ = 313.

***N*-(4-Chlorophenyl)-7-oxo-7,7a-dihydrocyclopropa[*b*]chromene-1a(1*H*)-carboxamide (2). 2** was obtained as a beige solid in 51% yield. ^{1}H NMR (400 MHz, DMSO): 1.45 (dd, J = 7.0 Hz, J = 5.9 Hz, 1H), 1.97 (dd, J = 10.8 Hz, J = 5.9 Hz, 1H), 3.08 (dd, J = 10.8 Hz, J = 7.0 Hz, 1H), 7.04−7.07 (m, 1H), 7.17 (dd, J = 8.3 Hz, J = 1.1 Hz, 1H), 7.39−7.43 (m, 3H), 7.73−7.77 (m, 3H), 10.24 (s, 1H, D_2O exchange), 11.47 (s, 1H, D_2O exchange). MS m/z $(M\ [^{35}Cl] + H)^+$ = 329.

***N*-(4-Methylphenyl)-7-oxo-7,7a-dihydrocyclopropa[*b*]chromene-1a(1*H*)-carboxamide (3). 3** was obtained as an orange solid in 31% yield. ^{1}H NMR (400 MHz, DMSO): 1.42 (dd, J = 7.0 Hz, J = 5.8 Hz, 1H), 1.95 (dd, J = 10.8 Hz, J = 5.8 Hz, 1H), 2.28 (s, 3H), 3.07 (dd, J = 10.8 Hz, J = 7.0 Hz, 1H), 7.03−7.07 (m, 1H), 7.14−7.19 (m, 3H), 7.38−7.43 (m, 1H), 7.57−7.60 (m, 2H), 7.75 (dd, J = 7.9 Hz, J = 1.6 Hz, 1H), 10.03 (s, 1H, D_2O exchange), 11.45 (s, 1H, D_2O exchange). MS m/z $(M + H)^+$ = 309.

***N*-(4-Methoxyphenyl)-7-oxo-7,7a-dihydrocyclopropa[*b*]chromene-1a(1*H*)-carboxamide (4). 4** was obtained as a white solid in 34% yield. ^{1}H NMR (400 MHz, DMSO): 1.41 (dd, J = 6.8 Hz, J = 5.8 Hz, 1H), 1.94 (dd, J = 10.7 Hz, J = 5.8 Hz, 1H), 3.06 (dd, J = 10.7 Hz, J = 6.9 Hz, 1H), 3.74 (s, 3H), 6.90−6.93 (m, 2H), 7.02−7.07 (m, 1H), 7.16−7.18 (m, 1H), 7.38−7.42 (m, 1H), 7.57−7.61 (m, 2H), 7.74 (dd, J = 7.9 Hz, J = 1.7 Hz, 1H), 10.0 (s, 1H D_2O exchange), 10.38 (bs, 1H, D_2O exchange). MS m/z $(M + H)^+$ = 325.

FRET Assay on Human mGluR2 Receptor. Compounds were evaluated on human mGluR2 receptor using a fluorescence resonance energy transfer (FRET) binding assay, enclosing an appropriate dyed probe and a HEK cellular model stably expressing the human mGluR2 receptor truncated from its amino terminal extremity and fused to the enhanced green fluorescent protein (eGFP; GE HealthCare). The HEK GFP-hmGluR2 cells were seeded onto poly-D-lysine precoated black-walled 96-well plates in Modified Eagle's Medium (MEM) supplemented with 10% FCS (0.8×10^5 cells per well). After 24 h of culture at 37 °C, the cell media was removed and cells were washed with assay buffer (137.5 mM NaCl, 1.25 mM $MgCl_2$, 1.25 mM $CaCl_2$, 6 mM KCl, 5.6 mM glucose, 10 mM HEPES, 0.4 mM NaH_2PO_4, 0.1% bovine serum albumin (w/v), pH 7.4). The tested compounds were coapplied with the probe on cells by the FLIPR^{TETRA} (Molecular Devices). Binding of a tested compound on the receptor is detected by a modification of the FRET signal between the dyed probe and the GFP-fused receptor, as measured by the variation of GFP fluorescence at 510 nm. Time curves of ligand binding were recorded during 2000 s (excitation: light emitting diode 470−495 nm, emission: 510 ± 10 nm). Dose-dependent FRET inhibition curves were fitted with variable slope, using GraphPad Prism software, in order to determine IC_{50} and K_i values. Experiments were all performed in duplicate, three times independently.

Ca^{2+} Functional Assays on Human mGluR2 and mGluR3. HEK cells were cultured in Modified Eagle's Medium (MEM) supplemented with 10% fetal calf serum (FCS) and transfected by electroporation as previously described.[27,28] Plasmids encoding mGlu receptors were constructed from a pRK backbone and human mGluR2 (cloned from SK-NSH mRNA extract) or mGluR3 (purchased from BioXTal) cDNAs. Construction of the plasmids encoding the chimeric Gqi9 protein or the promiscuous G protein Gα15 (used to deviate the natural coupling of the receptors from inhibition of cAMP production to Ca^{2+} release pathway) has been described previously.[14,27] Receptor activity was detected by changes in intracellular calcium, as measured using the fluorescent Ca^{2+} sensitive dye, Fluo4AM (Molecular Probes). Cells were plated after transfection onto poly ornithine coated, clear bottom, black-walled, 96-well plates and cultured for 24 h. The day of the screening, cells were washed with freshly prepared buffer (1× HBSS supplemented with 20 mM HEPES, 1 mM $MgSO_4$, 3.3 mM Na_2CO_3, 1.3 mM

$CaCl_2$, 2.5 mM Probenecid, and 0.5% BSA) and loaded at 37 °C, 5% CO_2 for 1 h 30 min with buffer containing 1 μM Fluo4AM and 0.1 mg/mL pluronic acid. After washing, cells were incubated with 50 μL of buffer; 50 μL of 3× drug solution (prepared in buffer) and then 50 μL of 3× glutamate solution (prepared in buffer) were successively added by the fluorescence microplate reader FlexStation II384 (Molecular Devices) in each well after 20 s of recording. Fluorescence signals (excitation, 485 nm; emission, 525 nm) were then measured at sampling intervals of 1.5 s for 60 s after each injection. Dose−inhibition curves were fitted with variable slope, using GraphPad Prism software, in order to determine EC_{50} or IC_{50} values. Compounds **1**−**5** were tested in positive allosteric modulators (PAM) conditions in the presence of a glutamate EC_{10} concentration and in negative allosteric modulators (NAM) conditions in presence of a glutamate EC_{80} concentration. Experiments were all performed in triplicate, three times independently.

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Supporting Information Available: ^{1}H NMR of compounds **1**−**4** and **6**, and LCMS analyses of final compounds **1**−**4**. This material is available free of charge via the Internet at http://pubs.acs.org.

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