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Discovery of novel positive allosteric modulators of the metabotropic glutamate receptor 5 ($mGlu_5$)

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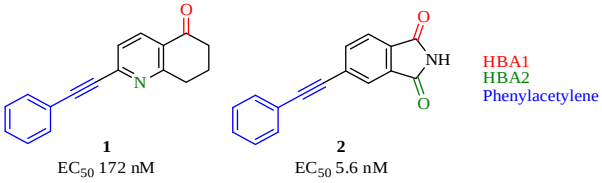
ABSTRACT

Novel in vitro $mGlu_5$ positive allosteric modulators with good potency, solubility, and low lipophilicity are described. Compounds were identified which did not rely on the phenylacetylene and carbonyl functionalities previously observed to be required for in vitro activity. Investigation of the allosteric binding requirements of a series of dihydroquinolinone analogs led to phenylacetylene azachromanone **4** (EC_{50} 11.5 nM). Because of risks associated with potential metabolic and toxicological liabilities of the phenylacetylene, this moiety was successfully replaced with a phenoxymethyl group (**27**; EC_{50} 156.3 nM). Derivation of a second-generation of $mGlu_5$ PAMs lacking a ketone carbonyl resulted in azaindoline (**33**), azabenzimidazole (**36**), and *N*-methyl 8-azaoxazine (**39**) phenylacetylenes. By scoping nitrogen substituents and phenylacetylene replacements in **39**, we identified phenoxymethyl 8-azaoxazine **47** (EC_{50} 50.1 nM) as a potent and soluble $mGlu_5$ PAM devoid of both undesirable phenylacetylene and carbonyl functionalities.

Approximately 1% of the world's population is affected by schizophrenia. Current therapies, however, are associated with unwanted side effects and also do not provide sufficient relief across all symptom domains.[1,2] Hypofunction of the *N*-methyl-D-aspartate receptor (NMDAr) has been implicated in the symptoms of schizophrenia, which makes activation of GPCRs with the ability to normalize NMDAr signaling a promising approach for the development of antipsychotics.[3]

One receptor with the ability to potentiate NMDAr glutamatergic postsynaptic neurotransmission is the metabotropic glutamate receptor 5 ($mGlu_5$).[4] Achieving mGlu receptor subtype selectivity with orthosteric agonists remains challenging due to highly conserved extracellular binding sites. As a result, current strategies to selectively enhance $mGlu_5$ signaling in the presence of its endogenous ligand glutamate have focused on the discovery of compounds which bind to the less well conserved allosteric modulatory site.[5]

In 2008, Merz Pharmaceuticals and Vanderbilt University reported dihydroquinolinone **1**[6] and phthalimide **2**[7] (Fig. 1), respectively, as $mGlu_5$ positive allosteric modulators (PAMs). Both compounds possess a phenylacetylene which is also found in well known $mGlu_5$ negative allosteric modulators such as MPEP and MTEP.[8] Based on published data, we utilized a working hypothesis



1
EC_{50} 172 nM

2
EC_{50} 5.6 nM

Figure 1. Pharmacophore features and reported EC_{50}'s of **1** and **2**.

that one or both of the oxygen lone pairs in **1** (HBA1 as in Fig. 1) could possibly serve as a rigid hydrogen-bond acceptor. Furthermore, evidence also suggested that another possible hydrogen-bond acceptor (HBA2) could be accommodated in the form of either an aromatic nitrogen (**1**) or a second carbonyl (**2**). The similarity of these features suggests that both compounds potentially interact with the same or similar spatial regions of the allosteric binding pocket. Our efforts to discover novel $mGlu_5$ PAMs have focused on exploiting these common pharmacophore features using hypothesis-driven design to enhance our understanding of allosteric binding requirements while also optimizing physiochemical profiles for in vivo testing.

Compounds were prepared through synthesis or purchase of heteroaryl halides, alcohols, or triflates, which were then elaborated into final compounds using known literature conditions (Fig. 2; see Supplementary data).

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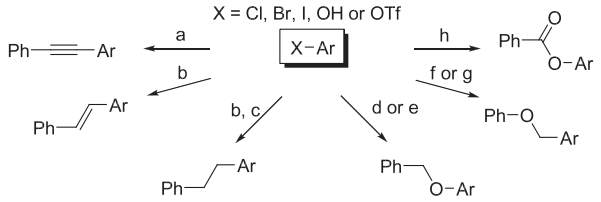
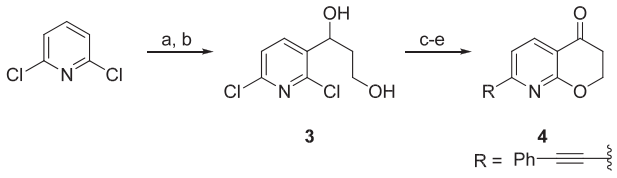


Figure 2. Installation of phenylacetylene and replacement side chains on heteroaromatic/aromatic (Ar) cores.

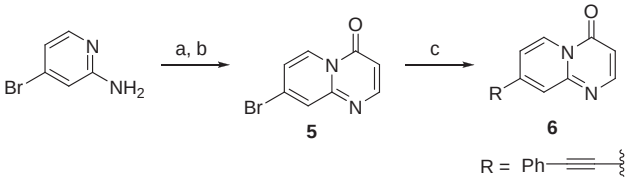
The syntheses of unique heteroaryl cores and their corresponding final compounds are described in Schemes 1 and 2. Azachromanone **4** was synthesized in five steps from 2,6-dichloropyridine (Scheme 1). Lithiation, followed by quenching with TBS-protected 3-hydroxypropanal, exclusively afforded addition at the 3-position of the pyridyl ring. Silyl cleavage then yielded diol **3**, which cyclized upon exposure to potassium *tert*-butoxide in *tert*-butanol at elevated temperature. Incorporation of the phenylacetylene group then proceeded smoothly under either Stille or Sonogashira conditions. Higher yields were obtained if this coupling was performed prior to (rather than following) Swern oxidation.

To prepare azaquinolizinone **6**, commercially available 4-bromo-2-aminopyridine (Scheme 2) was reacted with the methoxymethylene derivative of Meldrum's acid, followed by heating to 240 °C to form azaquinolizinone bromide **5**. Stille coupling conditions were then used to append the phenylacetylene group.

Initial efforts to develop novel $mGlu_5$ PAMs focused on modification of the heteroaryl cores of **1** and **2** while maintaining the putative HBA1 interaction with the allosteric binding pocket (Table 1). Specifically, we examined the impact of subtle changes in ring size, ring saturation, and carbonyl C–O bond polarization to evaluate the geometric and electronic constraints of this hydrogen bond. For example, known lactams **12**[7] and **13**[7] were >13-fold more potent than ketones **7** and **9**. While the potential impact of a newly introduced amide N–H as a hydrogen bond donor should not be discounted, the observed potency increase supports the concept of a stronger hydrogen bond between the allosteric binding pocket and an amide oxygen versus that with a ketone oxygen. Changes in ring size (**7** to **9**, **8** to **10**, and **12** to **13**) had very little

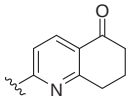
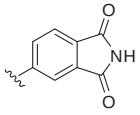
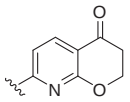
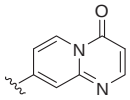
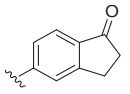
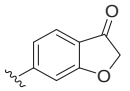
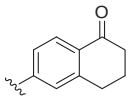
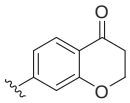
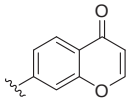
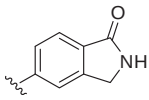
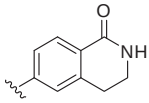
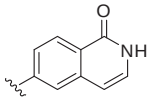


Scheme 1. Reagents and conditions: (a) LDA, $HCOCH_2CH_2OTBS$, THF, −78 °C, 75%; (b) TBAF, THF, 0 °C, 92%; (c) *t*-BuOK, *t*-BuOH, 85 °C, 96%; (d) $Bu_3SnCCPh$, $Pd(PPh_3)_4$, PhMe, 110 °C, 71%; (e) $(COCl)_2$, DMSO, Et_3N, CH_2Cl_2, −78 °C, 84%.



Scheme 2. Reagents and conditions: (a) $(CH_3)_2C(OCO)_2CHCH_2OCH_3$, *i*PrOH, 82 °C, 85%; (b) Ph_2O, 240 °C, 86%; (c) $Bu_3SnCCPh$, $Pd(PPh_3)_4$, PhMe, μW, 110 °C, 47%.

Table 1
Core modifications to probe HBA1 and HBA2

Compd	Ar	pEC_{50}[a]	EC_{50} (nM)	Sol[b] (μM)	clog*P*[c]
	Ph—≡—Ar				
1		7.85 ± 0.02	14.0	22	3.7
2		7.94 ± 0.16	11.6	>600	3.8
4		7.94 ± 0.03	11.5	23	3.4
6		7.27 ± 0.13	53.2	16	3.0
7		6.29 ± 0.09	518.8	<1	4.4
8		7.04 ± 0.10	91.9	nt[d]	4.0
9		6.23 ± 0.10	588.8	<1	4.8
10		7.53 ± 0.07	29.9	1	4.4
11		6.93 ± 0.14	117.5	<1	4.0
12[7]		7.53 ± 0.08	29.5	7	3.5
13[7]		7.35 ± 0.09	44.3	1	3.6
14		6.74 ± 0.01	182.0	<1	3.5

[a] Data are the average of at least two experiments. For details on pEC_{50} and EC_{50} determination, Ref. 9.
[b] Equilibrium solubility (pH 7.4).[10]
[c] Calculated log*P*.
[d] nt = not tested.

effect, while the introduction of unsaturation (**10** to **11** and **13** to **14**) consistently decreased potency by approximately 4-fold. If it is assumed that the rigid phenylacetylene occupies the same region of space for the compounds in question, these results suggest that some movement of the carbonyl within the allosteric binding pocket is tolerated. What is not clear is if increased planarity introduced by unsaturation decreases potency due to steric constraints, decreased movement of the carbonyl (thereby limiting the ability to form a hydrogen bond) or both. We had expected the vinylogous

ester in **11** to increase electron density on the carbonyl oxygen, thereby making it a better hydrogen bond acceptor. Although **11** is still a reasonably potent compound, this hypothesis did not translate to dramatic potency shifts, perhaps due to other newly introduced negative interactions with the added functionality.

We also evaluated the proposed HBA2 interactions of **1** and **2**. In particular, we were keen to determine if the pyridyl nitrogen of **1** and the basal carbonyl of **2** were forming hydrogen bonds of equal importance, potentially with the same region(s) of the $mGlu_5$ allosteric binding pocket. This theory was supported by molecular overlays but not substantiated by in vitro data. Thus, there is a >40-fold potency difference between **1** and its des-aza analog **9**. In contrast, there is only a 2- to 3-fold decrease in potency going from phthalimide **2** to isoindolone **12**. These results suggest that the contributions to potency of the pyridyl nitrogen of **1** and the basal carbonyl of **2** are not equivalent.

Much like the nitrogen of dihydroquinolinone **1**, the aryl ether oxygens of **8** and **10** also contributed significantly to $mGlu_5$ potentiation. Thus, benzofuranone **8** was >5-fold more potent than indenone **7**. Similarly, chromanone **10** was >19-fold more active than α-tetralone **9**. To determine if the potency contributions exemplified by the ring heteroatoms of **1** and **10** were additive, we designed azachromanone **4** with a secondary goal of improving physiochemical properties (Sol >10 μM; *clogP* ⩽ 3.5). While a slight increase in potency was observed compared to **10**, azachromanone **4** was equipotent with dihydroquinolinone **1**. One hypothesis that might explain these results is that both **1** and **10** are participating in the same HBA2 interaction, which exists as a bifurcated hydrogen bond for azachromanone **4** (Fig. 3).

Due to the perceived potential metabolic and toxicological liabilities of the acetylene linker, we chose to proactively develop a strategy to identify an acetylene surrogate rather than run the risk of retaining an intractable series flaw in the drug discovery process. Using a matrix approach, acetylene replacements (e.g., styrenyl, phenethyl, 3-phenylazetidine, benzyloxy, phenoxymethyl, 3-phenylbicyclo[1.1.1]pentyl, etc.) were systematically surveyed across multiple cores from Table 1.[11] While many cores lost most, if not all, activity once the acetylene was removed, the chromanone core of **10** maintained good potency for several acetylene surrogates (Table 2). Thus, both benzyloxy (**15**) and phenoxymethyl (**21**) chromanones exhibited good $mGlu_5$ potentiation and had improved solubility and lower *clogP*s compared to **10**. Additionally, both solubility and *clogP* could be further modulated through substitution of the terminal aromatic ring of **15** without appreciable loss of activity (**16**–**18**). We were also intrigued to find that potency could be maintained if the phenylacetylene of chromanone **10** was replaced with a simple benzoate moiety (**22**). Due to the potential liability of the phenyl ester in vivo, we regard **22** as a tool compound that has helped reinforce the possibility of finding acetylene surrogates.

Both benzyloxy and phenoxymethyl azachromanones (**27** and **28**) also maintained good activity with respect to $mGlu_5$. As seen when comparing **4** and **10**, solubilities and *clogP*s were improved

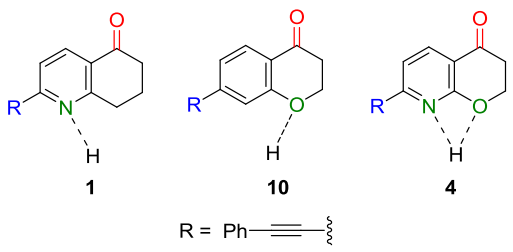
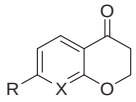


Figure 3. Proposed HBA2 interactions for compounds **1**, **4**, and **10**.

Table 2
Chromanone and azachromanone phenylacetylene analogs



#	R	X	pEC_{50}[a]	EC_{50} (nM)	Sol[b] (μM)	*c*Log*P*[c]
10	PhCC	C	7.53 ± 0.07	29.9	1	4.4
15	$PhCH_2O$	C	6.78 ± 0.25	167.9	7	3.5
16	3-Cl-$PhCH_2O$	C	6.68 ± 0.09	208.9	1	4.2
17	3-F-$PhCH_2O$	C	6.59 ± 0.18	285.5	2	3.7
18	3-CN-$PhCH_2O$	C	6.53 ± 0.18	295.1	7	2.9
19	3-Me-$PhCH_2O$	C	6.24 ± 0.27	572.1	3	4.0
20	4-F-$PhCH_2O$	C	6.11 ± 0.21	776.2	5	3.7
21	$PhOCH_2$	C	6.46 ± 0.18	349.4	12	3.4
22	PhC(O)O	C	7.53 ± 0.30	29.9	1	4.4
4	PhCC	N	7.94 ± 0.03	11.5	23	3.4
23	C_5H_9CC	N	6.97 ± 0.20	107.2	400	3.1
24	$C_6H_{11}CC$	N	6.69 ± 0.14	206.5	57	3.7
25	(*E*)-PhCHCH	N	6.62 ± 0.18	242.7	14	3.5
26	$PhCH_2CH_2$	N	6.28 ± 0.36	524.8	>480	3.2
27	$PhCH_2O$	N	6.81 ± 0.16	156.3	64	3.0
28	$PhOCH_2$	N	6.34 ± 0.09	453.6	100	2.5

[a] Data are the average of at least two experiments. For details on pEC_{50} and EC_{50} determination, Ref. 9.
[b] Equilibrium solubility (pH 7.4).[10]
[c] Calculated log*P*.

for all azachromanones relative to their chromanone counterparts. It was also found that positive modulator activity persisted with (*E*)-styrenyl (**25**) and phenethyl (**26**) derivatives, which was not observed for the chromanone core (data not shown).

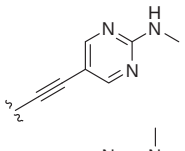
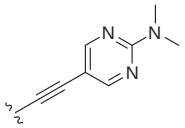
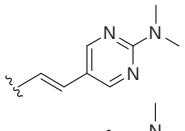
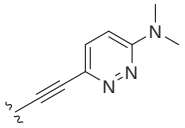
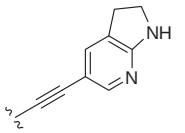
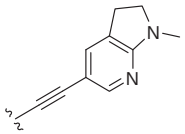
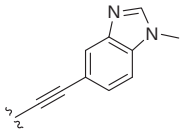
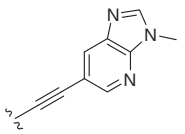
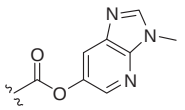
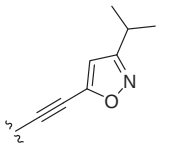
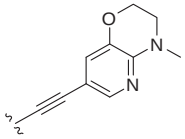
Despite our success with the compounds of Table 2, we had concerns about the inherent reactivity of a ketone carbonyl and its in vivo disposition. In agreement with literature results for **1**,[6] the racemic alcohol analog of **4** (EC_{50} 3.0 μM) was much less active than **4** itself. We also discovered that the azachromanone core had moderate stability under acidic conditions, poor stability under basic and photolytic conditions, and significant microsomal degradation in vitro.[12] While not prohibitive, taken together these factors did adversely impact our ability to interpret in vitro/in vivo data and effectively advance this series in a suitable time frame.

To develop a second generation of non-phenylacetylene, non-carbonyl containing $mGlu_5$ PAMs, we investigated a number of potent pyrimidine phenylacetylenes disclosed by Vanderbilt University.[13] One example is pyrimidine **29** (Table 3), which compares favorably with both compounds of Figure 1 but lacks a carbonyl (HBA1) and hydrogen bond acceptor directly analogous to the HBA2 of either **4** or **10**. We modified the pyrimidine core of **29** to determine the scope of allosteric binding requirements and the tolerance for changes in physiochemical properties. Solubility was a property we were particularly keen to improve. Conversion of the pyrimidine ring to a pyridazine (**32**) or pyridine (not shown) decreased or abolished activity, respectively. In contrast, cyclization of the aniline nitrogen to form azaindoline **33** improved potency by twofold while subsequent alkylation (**34**) weakened potency by >6-fold. Interestingly, benzimidazole **35** also exhibited good solubility and potentiation of $mGlu_5$ but had a higher than desired *clogP*. These properties could be improved through reintroduction of a pyridine nitrogen analogous to that of **33** to afford **36**. The observed potency improvement is consistent with the proposed role of the pyridine nitrogen as a hydrogen bond acceptor. Of the pyrimidine, azaindoline, and azabenzimidazole series, very little tolerance for phenylacetylene modification was observed (**31**, **37**).

A chemotype that also exhibited potent positive modulation of $mGlu_5$ was *N*-methyl 8-azaoxazine **39**. While initially designed

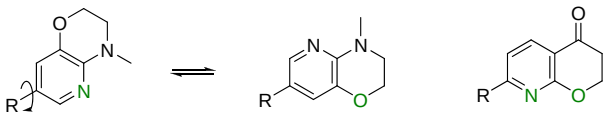
Table 3
Pyrimidine-derived $mGlu_5$ PAMs

Ph−X−Ar

#	X-Ar	pEC_{50}[a]	EC_{50} (nM)	Sol[b] (μM)	*c*log*P*[c]
29[13]		7.51 ± 0.18	30.7	<1	3.1
30		6.95 ± 0.07	113.1	<1	3.2
31		6.25 ± 0.33	566.7	nt[d]	3.3
32		6.40 ± 0.14	401.2	nt[d]	3.0
33		7.80 ± 0.13	15.8	nt[d]	3.9
34		6.97 ± 0.02	108.4	nt[d]	4.3
35		7.43 ± 0.14	37.2	10	4.2
36		7.76 ± 0.09	17.4	42	3.1
37		6.67 ± 0.24	216.3	>550	2.5
38		6.51 ± 0.10	307.8	nt[d]	4.0
39		7.52 ± 0.20	30.2	4	4.5

[a] Data are the average of at least two experiments. For details on pEC_{50} and EC_{50} determination, Ref. 9.
[b] Equilibrium solubility (pH 7.4).[10]
[c] Calculated log*P*.
[d] nt = not tested.

because of structural similarity to pyrimidine **29**, it also shares common features with azachromanone **4**. As with several of the cores in Table 3, rotation about the aryl-phenylacetylene bond of **39** is expected to be facile due to a low energy barrier to rotation. Thus, as shown in Figure 4, it can easily be observed that the aryl



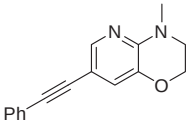
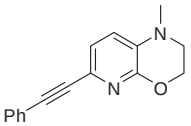
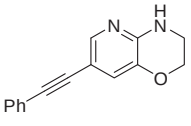
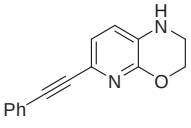
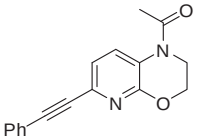
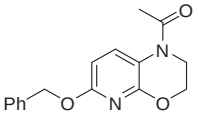
39 **39b** **4**

Figure 4. Rotation about the aryl-phenylacetylene bond of **39**.

ether oxygen of **39b** is potentially analogous to that of **4**. It is also clear that a carbonyl oxygen (HBA1) is not required for strong $mGlu_5$ allosteric modulation within this series.

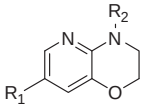
As demonstrated with chromanone **10**, we hypothesized that shifting the position of the pyridine nitrogen to afford 5-azaoxazine **40** might improve potency (Table 4). Gratifyingly, **40** maintained roughly equivalent potency to **39** and also had significantly improved solubility. However, when the des-methyl analogs were compared, 8-azaoxazine **41** maintained activity but 5-azaoxazine **42** did not. Analysis in a membrane binding assay in the presence of a proprietary $mGlu_5$ NAM radioligand competitive with MPEP revealed that **42** binds only weakly to the allosteric site (data not shown). The discrepancy in activity between **41** and **42** underscores the potential difference in binding mode between two very similar scaffolds. Acylation of **42** restored potency (**43**), and the resulting acetamide also met our criteria for solubility and *c*log*P*, prompting us to examine phenylacetylene replacements. Of the surrogates examined, benzyloxy **44** maintained moderate potency with respect to $mGlu_5$.

Table 4
Oxazine analogs as $mGlu_5$ PAMs

#	Structure	pEC_{50}[a]	EC_{50}(nM)	Sol[b] (μM)	*c*Log*P*[c]
39		7.52 ± 0.20	30.2	4	4.5
40		7.25 ± 0.15	56.7	100	4.5
41		7.69 ± 0.07	20.3	nt[d]	4.1
42		4.60	>25,000	nt[d]	4.1
43		7.21 ± 0.28	61.7	210	2.7
44		6.13 ± 0.08	749.9	>570	2.3

[a] Data are the average of at least two experiments. For details on pEC_{50} and EC_{50} determination, Ref. 9.
[b] Equilibrium solubility (pH 7.4).[10]
[c] Calculated log*P*.
[d] nt = not tested.

Table 5
Potentiation of 3,4-dihydro-2*H*-pyrido[3,2-*b*][1,4]oxazines



#	R^1	R^2	pEC_{50}[a]	EC_{50}(nM)	Sol[b](μM)	clog*P*[c]
41	PhCC	H	7.69 ± 0.07	20.3	nt[d]	4.1
45	$PhOCH_2$	H	6.43 ± 0.08	371.5	nt[d]	3.2
39	PhCC	Me	7.52 ± 0.20	30.2	4	4.5
46	PhC(O)O	Me	7.91 ± 0.03	12.3	130	3.8
47	$PhOCH_2$	Me	7.30 ± 0.20	50.1	18	3.5
48	$PhOCH_2$	CH_2CH_3	7.22 ± 0.12	60.3	169	4.0
49	$PhOCH_2$	$(CH_2)_2OMe$	6.34 ± 0.20	460.6	nt[d]	3.4
50	$PhOCH_2$	C(O)OMe	6.32 ± 0.13	475.0	>520	2.2
51	$PhOCH_2$	SO_2Me	6.22 ± 0.18	609.5	10	2.3
52	$PhOCH_2$	C(O)Me	6.15 ± 0.14	713.4	40	1.8
53	(*E*)-PhCHCH	Me	6.16 ± 0.18	696.0	<1	4.5
54	$CyOCH_2$	Me	6.08 ± 0.04	831.8	>440	3.5

[a] Data are the average of at least two experiments. For details on pEC_{50} and EC_{50} determination, see Ref. 9.
[b] Equilibrium solubility (pH 7.4).[10]
[c] Calculated log*P*.
[d] nt = not tested.

We revisited 3,4-dihydropyrido-oxazines **39** and **41** with the goal of investigating acetylene replacements and decreasing series lipophilicity (Table 5). In contrast to previous efforts, benzyloxy surrogates were weakly active or completely inactive (data not shown). For des-methyl analogs (R_2 = H), only the phenoxymethyl side chain maintained any kind of potent positive modulation of $mGlu_5$. Similar to chromanone **21** in Table 2, benzoate **46** was the most potent non-phenylacetylene derivative of Table 5. Weaker activity was exhibited by styrenyl and cyclohexyloxymethyl oxazines **53** and **54**, respectively. When N–H oxazine **45** was alkylated to afford tertiary amine **47**, potency improved (>6-fold). Extending the alkyl side chain (**48**) maintained activity and increased solubility at the cost of increased lipophilicity. Attenuating the capacity for electron donation (**49**–**52**) decreased activity, suggesting that the electronic character of the oxazine nitrogen is important or that the increased steric bulk is not well tolerated. Whether this nitrogen plays a role similar to the carbonyl of compounds in Table 1 remains to be seen.

In summary we have described the discovery of several novel in vitro $mGlu_5$ positive allosteric modulators with good potency, solubility, and low lipophilicity. Investigation of the allosteric binding requirements of a series of dihydroquinolinone analogs disclosed by Merz Pharmaceuticals led to phenylacetylene azachromanone **4** (EC_{50} 12 nM; 110% effect). Because of the perceived liability of the acetylene side chain, we investigated replacing this moiety. This was accomplished with a number of different functional groups, of which phenoxymethyl (**27**; EC_{50} 156.3 nM) was the most potent. To derive a second-generation $mGlu_5$ PAM that did not contain a ketone carbonyl and offered better stability, we also explored the SAR of a number of pyrimidine-derived compounds resulting in azaindoline (**33**), azabenzimidazole (**36**), and *N*-methyl 8-azaoxazine (**39**) phenylacetylenes as potent $mGlu_5$ positive allosteric modulators. Oxazine **39** (EC_{50} 30.2 nM) was observed to be very similar to azachromanone **4**, leading to the discovery of 5-azaoxazines **40** and **43**. Both **40** and **43** exemplify dihydroquinolinone analogs that do not contain a carbonyl previously proposed as being critical for potent activity. Lastly, through the scoping of nitrogen substituents and phenylacetylene replacements for **39**, we identified 8-azaoxazine **47** as a potent (EC_{50} 50.1 nM) and soluble $mGlu_5$ PAM with low lipophilicity (clog$P \leqslant 3.5$) devoid of both undesirable phenylacetylene and carbonyl functionalities.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.01.027.

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9. $mGlu_5$ FLIPR Assay: A Ca^{2+} Flux FLIPR assay similar to that described by Andreas Ritzén et al. (Discovery of a potent and brain penetrant mGluR5 positive allosteric modulator. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 3275) was used to detect $mGlu_5$ positive allosteric modulator (PAM) activity in an HEK293 cell line stably expressing the d isoform of human $mGlu_5$. PAM activity was assessed by measuring potentiation of the EC_{20} response to L-glutamate in the presence of test compound. *Procedure*: The day before the experiment 25,000 cells/well were plated in DMEM containing 10% dialyzed FBS (Hyclone) in 384 well poly-D-lysine coated plates (Becton Dickinson). After removal of the plating medium the following day, cells were labeled for 1 h at 37 °C in 4.3 μM Fluo-4 AM (In Vitrogen) containing 10% Pluronic F-127 in assay buffer (HBSS (CellGro), 20 mM HEPES, 1 mM Probenecid (Sigma), pH 7.4). Cells were washed at rt to remove excess dye prior to addition of test compounds serially diluted from 10 mM in 100% DMSO into assay buffer. Compounds were assayed for any underlying agonist activity by addition of test compounds to the cells on the FLIPR instrument (first addition: 13 μL test compound to 25 μL assay buffer/well) and the response was recorded for 1 min. 11 different concentrations were tested for each compound. Fifteen minutes after the 1st addition, positive modulator activity was assayed by challenge with EC_{20} (200–300 nM) L-glutamate (second addition: 14 μL (740–1100 nM), final concentration 200–300 nM L-glutamate) and the response was recorded for 1 min. Positive modulator activity was calculated from the fluorescence max–min data normalized to yield responses for no modulation (EC_{20} response) and full stimulation (10 μM L-glutamate) as 0% and 100% modulation, respectively. Concentration–response data were fitted to the four-parameter logistic equation to estimate compound potency (EC_{50}) and efficacy (E_{max}).
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