ACS Chemical Neuroscience

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ACS Chem. Neurosci., **Just Accepted Manuscript** • DOI: 10.1021/acschemneuro.6b00035 • Publication Date (Web): 13 Apr 2016

ACS Publications

ACS Chemical Neuroscience is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

Discovery, Synthesis and Pre-Clinical Characterization of *N*-(3-chloro-4-fluorophenyl)-1*H*-pyrazolo[4,3-*b*]pyridin-3-amine (VU0418506), a novel positive allosteric modulator of the metabotropic glutamate receptor 4 ($mGlu_4$)

Darren W. Engers[†,€], Anna L. Blobaum[†,€], Rocco D. Gogliotti[†,€], Yiu-Yin Cheng[†,€], James M. Salovich[†,€], Pedro M. Garcia-Barrantes[€], J. Scott Daniels[†,€], Ryan Morrison[†,€], Carrie K. Jones[†,€], Matthew G. Soars[§], Xiaoliang Zhuo[§], Jeremy Hurley[§], John E. Macor[§], Joanne J. Bronson[§], P. Jeffrey Conn[†,€,#], Craig W. Lindsley[†,‡,€], Colleen M. Niswender[†,€,#], Corey R. Hopkins[†,‡,€,*]

[†]Department of Pharmacology, [‡]Department of Chemistry, [€]Vanderbilt Center for Neuroscience Drug Discovery, [#]Vanderbilt Kennedy Center, Vanderbilt University, Nashville, TN 37232. [§]Bristol-Myers Squibb Co., Research and Development, 5 Research Parkway, Wallingford, CT 06492.

Abstract: The efficacy of positive allosteric modulators (PAMs) of the metabotropic glutamate receptor 4 ($mGlu_4$) in pre-clinical rodent models of Parkinson's disease has been established by a number of groups. Here, we report an advanced pre-clinically characterized $mGlu_4$ PAM, *N*-(3-chloro-4-fluorophenyl)-1*H*-pyrazolo[4,3-*b*]pyridin-3-amine (VU0418506). We detail the discovery of VU0418506 starting from a common picolinamide core scaffold and evaluation of a number of amide bioisosteres leading to the novel pyrazolo[4,3-*b*]pyridine head group. VU0418506 has been characterized as a potent and selective $mGlu_4$ PAM with suitable *in vivo* pharmacokinetic properties in three preclinical safety species.

Keywords: metabotropic glutamate receptor 4, $mGlu_4$, CYP induction, Parkinson's disease, pyrazolo[4,3-b]pyridine

INTRODUCTION

Parkinson's disease (PD) is a chronic movement disorder resulting from the loss of dopaminergic neurons in the basal ganglia (BG).[1] The four core motor symptoms of PD are tremor, rigidity, bradykinesia, and postural instability, all of which can impact simple task completion as the disease progresses. At present there is no cure for PD, and there are only medications that provide symptomatic relief. Generally, afflicted individuals are given dopamine receptor agonists or levodopa (L-DOPA) to combat motor symptoms, the latter in combination with carbidopa, which allows L-DOPA to reach the brain before being converted to dopamine.[2] Unfortunately, not all symptoms are responsive to drug treatment (with rigidity and bradykinesia responding the best), leaving a significant portion of the disease population poorly treated; additionally, drug responsiveness wanes over disease progression.[3] Other therapeutics can aid in disease management (e.g., anticholinergics); however, a small, but significant, portion of PD patients remain unresponsive to current treatment options. In certain individuals, deep brain stimulation (DBS), a surgical procedure wherein electrodes are implanted within nuclei of the BG, has been shown to have dramatic results.[4] However, careful patient selection is an important step for DBS success and only a small number of patients qualify for this surgery. Thus, the treatment options for the symptomatic and disease-modifying intervention for PD remain limited, particularly as the disease progresses.

Motor function is controlled by the BG through two distinct pathways from the striatum – the direct and indirect pathways.[5,6] These pathways have opposite effects on motor activity – activation of the direct pathway promotes movement and the indirect pathway inhibits it. In PD patients, the balance between these two pathways is lost, leading to reduced inhibitory input to the internal globus pallidus and substantia nigra pars reticulata via the direct pathway and an

overall increase in the excitatory flow to these output nuclei via the polysynaptic indirect pathway.[5,6] Over the past several years, the metabotropic glutamate receptor 4 ($mGlu_4$) has gained attention as a potential target for pharmacological intervention to normalize the BG circuitry in PD patients.[7,8] This receptor is expressed presynaptically on terminals projecting from the striatum to the globus pallidus external segment (GPe), the first synapse of the BG indirect pathway (the "striato-pallidal" synapse).[9] Activation of $mGlu_4$ at these terminals has been shown to reduce inappropriate GABA release onto the GPe, normalizing motor output.[5,6,9] Additionally, expression of $mGlu_4$ in the striatum and other BG structures has been postulated to be involved in positive responses on motor output induced by activation or potentiation of $mGlu_4$.

Over the past several years, our laboratories and others have disclosed a series of picolinamide-based $mGlu_4$ positive allosteric modulators (PAMs) (**Figure 1**).[10] This scaffold has proven to deliver potent and selective $mGlu_4$ PAMs and several of these compounds have been used in preclinical anti-Parkinsonian animal models.[11,12,13,14] Compounds **3**[15,16] and **7**[16,17] were shown to reverse haloperidol-induced catalepsy after systemic administration (administered either subcutaneously (SC) or via an oral (PO) route). Within the picolinamide series of $mGlu_4$ PAMs, we have extensively characterized compound **7** and have shown that this ligand is effective when administered alone in reversing haloperidol-induced catalepsy, forelimb asymmetry-induced by unilateral 6-hydroxydopamine (6-OHDA) lesions in the median forebrain bundle, and deficits in attention induced by bilateral 6-OHDA nigrostriatal lesions in rats.[17] In addition to activity when administered alone, **7** was shown to enhance the activity of preladenant (an A_{2A} antagonist previously in clinical trials for PD therapy) in the reversal of haloperidol-induced catalepsy.[17] Lastly, when administered with an inactive dose of L-DOPA, **7** potentiated

the effects of L-DOPA in reversing forelimb asymmetry, suggesting that $mGlu_4$ PAMs may also provide L-DOPA sparing activity.[17] Unfortunately, the picolinamide scaffold suffered from poor stability *in vivo* due to, some extent, the instability of the amide bond. Thus, we initiated a medicinal chemistry effort to find a suitable replacement for the picolinamide head group with the goal of identifying a potent and selective $mGlu_4$ PAM with improved pharmacokinetic properties.

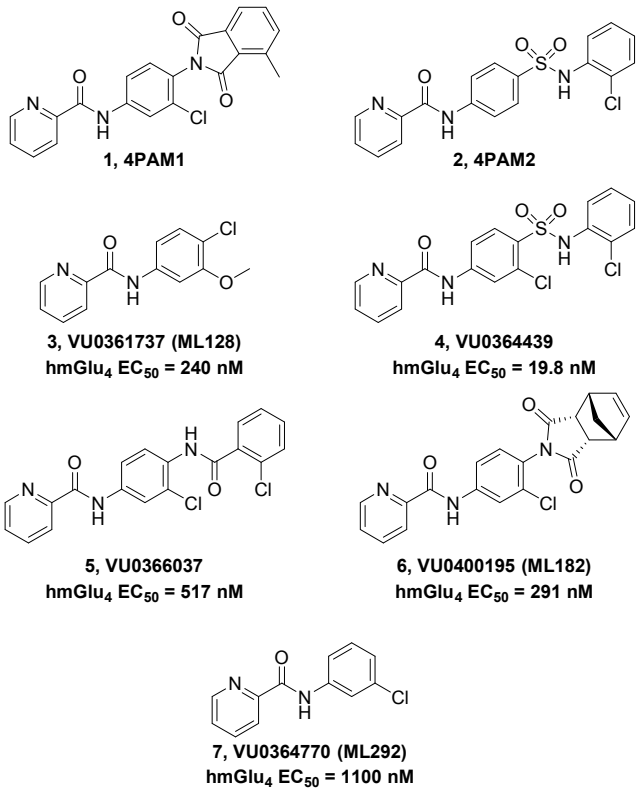


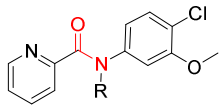
Figure 1. Previously disclosed $mGlu_4$ PAMs from the picolinamide series.

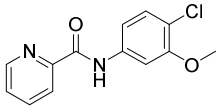
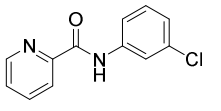
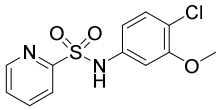
RESULTS AND DISCUSSION

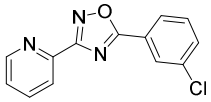
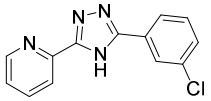
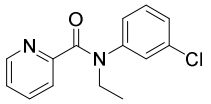
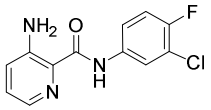
We have previously explored the SAR around the right-hand portion of the molecule and have identified several groups that are tolerated (amides, sulfonamides, imides).[12-14] Thus, our initial SAR optimization in this report started with the evaluation of known amide bioisosteres[18]

while keeping the 2-pyridine moiety intact, as our previous work has shown that this group is optimal for activity (**Table 1**). We have previously evaluated the reduction of the carbonyl group to the benzyl amine, the reversal of the amide, and the introduction of a cyclopropyl group as a carbonyl replacement.[14] However, each of these substitutions led to significant loss of activity. Replacing the picolinamide moiety with the 2-pyridyl sulfonamide, **8**, led to a complete loss of activity against $mGlu_4$. To test the hypothesis that the planarity around this group needed to be maintained, we next evaluated two cyclic amide bioisosteres.[18] First, the 1,2,4-oxadiazole group was introduced, **9**, but this replacement led to an inactive compound. Next, we introduced the triazine moiety, **10**, to keep the hydrogen bond donor group available as in the secondary amide; however, this change led to an inactive compound as well. To introduce steric bulk to the amide bond, we alkylated the secondary amide, **11**; that, too, led to an inactive compound. Lastly, an amino group was introduced to generate an internal hydrogen bond with the carbonyl group, and this was a productive change (**12**, 578 nM). Taking into account our previous attempts,[14,16] as well as this current endeavor, it appears that very little change is tolerated around the picolinamide portion of this scaffold.

Table 1. Amide replacements.



Cmpd		$mGlu_4$ pEC_{50} (± SEM)[a]	$mGlu_4$ EC_{50} (nM)[a]	%GluMax (± SEM)[a,b]
3		6.62[c]	240[c]	
7		5.96[c]	1,100[c]	
8			Inactive[d]	46[d]

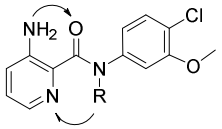
9		<4.5	>30,000	15.3 ± 2.7
10		<4.5	>30,000	25.1 ± 2.7
11		<4.5	>30,000	16.1 ± 1.9
12		6.24 ± 0.03	578	88.1 ± 2.0

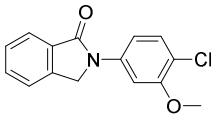
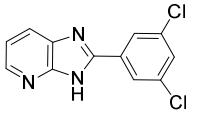
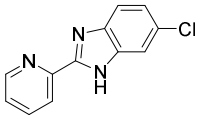
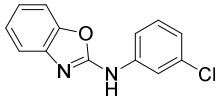
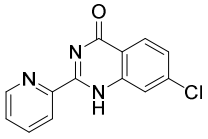
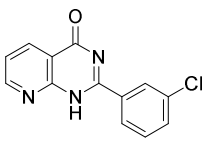
[a]Calcium mobilization $mGlu_4$ assay; values are average of n = 3. [b]Amplitude of response in the presence of 30 μM test compound, normalized to a standard compound, PHCCC (100% GluMax). [c]Data taken from Ref. 16. [d]n = 1.

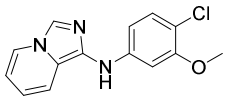
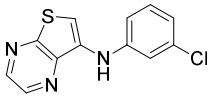
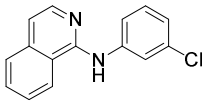
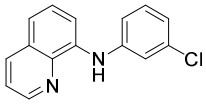
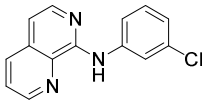
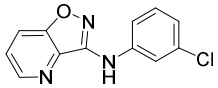
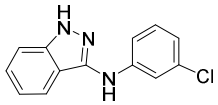
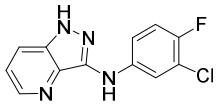
Having established that the aminopicolinamide head group imparted significant $mGlu_4$ PAM activity, we next examined other cyclized head groups to systematically explore the SAR around this portion of the molecule. We first looked to cyclize the amide NH portion with the pyridine moiety as this region was thought to participate in an internal hydrogen bond. However, cyclizing this portion of the molecule, **13**, led to an inactive compound, confirming the previous results observed with alkylated compounds. Efforts were then directed at cyclization of compounds while keeping the NH bond intact. The azabenzimidazole compound, **14,** was inactive; however, the 2-pyridyl benzimidazole, **15**, was active, although 5-fold less potent than **7**. A productive change was introduction of the benzoxazole head group, **16**, which was equipotent with **7**, but 4-fold less active than **3**. The two quinazolin-4-one analogs were not well tolerated (**17** and **18**), even though both compounds possessed the carbonyl and NH components of the amide bond. Other 5- and 6-membered cyclized moieties that either kept the nitrogen of the pyridine constant (**20** and **22**) or the heteroatom of the carbonyl (**19** and **21**), or both, **23**, were also not tolerated. However, when we contracted the 6-membered ring of **23** and introduced an additional heteroatom in the form of an isoxazolo[4,5-*b*]pyridine group[19], we were

pleased to see a significant improvement in $mGlu_4$ PAM activity (**24**, EC_{50} = 339 nM). This compound has the elements of the pyridine and carbonyl; however, it does not contain the additional hydrogen bond found in the aminopyridine. Synthesis of a compound that retains an NH in the form of the indazole group led to a potent PAM (**25**, EC_{50} = 67 nM). Interestingly, this compound does not contain the nitrogen of the putative pyridine group as present in the previous active compounds. Introduction of the pyrazolo[4,3-*b*]pyridine group[20] did not significantly increase activity (**26**, EC_{50} = 68 nM). In addition, inclusion of a 4-fluoro atom in order to block oxidative metabolism in this position was also well tolerated. Overall, however, these compounds represent a novel chemical scaffold for $mGlu_4$ PAMs.

Table 2. Aminopicolinamide replacements.



Cmpd		$mGlu_4$ pEC_{50} (± SEM)[a]	$mGlu_4$ EC_{50} (nM)[a]	%GluMax (± SEM)[a,b]
13		<4.5	>30,000	14.4 ± 2.2
14		<4.5	>30,000	34.8 ± 7.3
15		5.25 ± 0.07	5645	113.6 ± 7.6
16		6.04 ± 0.04	915	35.7 ± 1.8
17		<5	>10,000	25.9 ± 2.6
18		<4.5	>30,000	17.1 ± 2.4

19		<4.5	>30,000	20.5 ± 0.7
20		<4.5	>30,000	16.5 ± 2.7
21		<4.5	>30,000	19.6 ± 2.0
22		<4.5	>30,000	19.2 ± 3.5
23		<5	>10,000	23.5 ± 2.6
24		6.47 ± 0.01	339	106.5 ± 1.9
25		7.18 ± 0.03	67	113.7 ± 3.8
26		7.17 ± 0.07	68	109.3 ± 0.8

[a]Calcium mobilization $mGlu_4$ assay; values are average of n = 3. [b]Amplitude of response in the presence of 30 μM test compound, normalized to a standard compound, PHCCC (100% GluMax).

Having identified replacement head groups for the picolinamide that were potent $mGlu_4$ PAMs, we next determined if these compounds displayed improved pharmacokinetic properties compared to the picolinamides **3** and **7** in a battery of *in vitro* assays (**Table 3**). Compounds **3** and **7**, unsubstituted picolinamides, were unstable in liver microsomes with high intrinsic clearance in rat that approached the rat hepatic blood flow rate.[21,22] However, both compounds displayed moderate free fraction in both rat and human plasma.[22,23] Compounds were also evaluated for their inhibition of the cytochrome P450 (CYP450) enzymes using a cocktail approach in human liver microsomes as a first-tier screen for potential drug-drug interaction liability. Each of these compounds was a potent inhibitor of CYP1A2 (**3**, 130 nM; **7**, 550 nM), but showed no significant activity against CYP2C9, CYP2D6 or CYP3A4 (>30 μM). The

aminopicolinamide compound, **12**, showed a very similar profile to both **3** and **7**, with high predicted clearance in liver microsomes, moderate free fraction in rat and human plasma, and potent inhibition of CYP1A2, but with inactivity against the other CYPs. Neither the isoxazolo[4,5-*b*]pyridine, **24**, nor the indazole, **25**, provided any improvement in the *in vitro* PK properties; in fact, while each of these were more highly protein bound in rat and human, other portions of their overall profile were comparable to the previous compounds profiled. Significant improvement, however, was seen with the pyrazolo[4,3-*b*]pyridine compound, **26**. Compound **26** had significantly lower intrinsic clearance in both rat (CL_{HEP} = 12.1 mL/min/kg) and human (CL_{HEP} = 10.1 mL/min/kg) liver microsomes. In addition, **26** displayed moderate free fraction in both rat (F_u = 0.028) and human (F_u = 0.017) plasma. **26** was a potent inhibitor of CYP1A2 but was much less active against CYP2C9 (IC_{50} = 8.9 μM) and CYP2D6 and CYP3A4 (>30 μM).

To establish an *in vitro*/*in vivo* correlation (IV/IVC), we next evaluated both **24** and **26** in *in vivo* PK experiments to assess clearance (Table 3). Both compounds demonstrated an excellent correlation in rats between *in vitro* and *in vivo* parameters, with **24** showing suprahepatic clearance (CL_p = 117 mL/min/min) and a short half-life ($t_{1/2}$ = 31 min), while **26** showed moderate clearance (CL_p = 29 mL/min/kg) and a moderate half-life ($t_{1/2}$ = 91 min) in rats (**Tables 3 and 4**).[24] To better understand the metabolic instability difference between **24** and **26**, we performed a metabolic soft-spot analysis (**Figure 2**). Analysis of the two compounds revealed that the major metabolite of **24** was N–O bond cleavage of the isoxazolo[4,5-*b*]pyridine moiety, whereas the major metabolite of **26** was oxo-defluorination, albeit in low abundance. Based on these *in vitro* and *in vivo* PK parameters, Compound **26**, VU0418506, was chosen for advancement.

Table 3. *In vitro* and *in vivo* PK properties of selected compounds (predicted hepatic clearance, PPB, CYP inhibition).

Cmpd	Rat CL_{HEP} (mL/min/kg)[a]	Human CL_{HEP}	Plasma Protein Binding (F_u)[b] Rat	Human	CYP450 IC_{50} (μM)[c] 1A2	2C9	2D6	3A4	Rat IV (1 mg/kg) CL (mL/min/kg)	$T_{1/2}$ (min)
3	60.4	17.2	0.024	0.023	0.13	>30	>30	>30		
7	58.7	18.2	0.019	0.021	0.55	>30	>30	>30		
12	64.1	20.3	0.024	0.026	0.2	>30	>30	>30		
24	44	ND	0.004	0.002	<0.1	>30	>30	>30	117	30.6
25	48.6	16.7	0.009	0.009	<0.1	3.6	>30	13.2	71.7	144
26	12.1	10.1	0.028	0.017	<0.1	8.9	>30	>30	29	91

[a]Predicted hepatic clearance based on intrinsic clearance in rat and human liver microsomes. [b]F_u = fraction unbound. [c]IC_{50} determinations of major CYP enzymes in human liver microsomes using specific probe substrate-metabolite pairs for each CYP. ND = not determined.

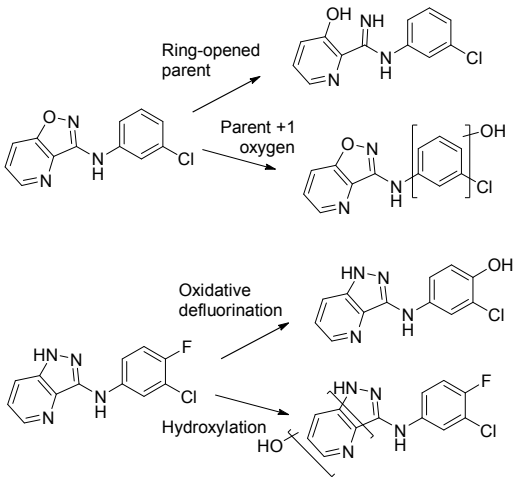
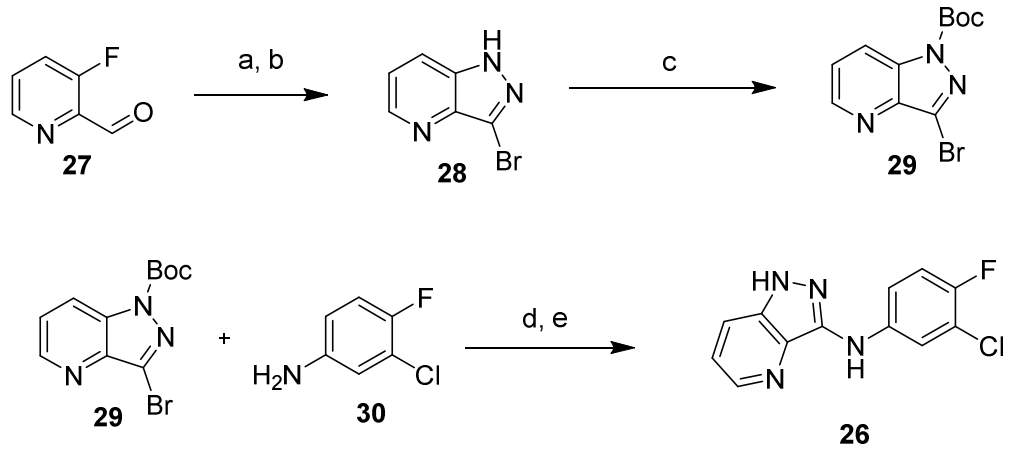


Figure 2. Met ID in Rat Liver Microsomes

To further profile **26**, we prepared >10 grams as outlined in Scheme 1. 3-Fluoro-2-formylpyridine, **27**, was heated in the presence of hydrazine which led to the pyrazolo[4,3-*b*]pyridine which was then brominated (2 M NaOH, Br_2) yielding 3-bromo-1*H*-pyrazolo[4,3-

b]pyridine, **28**, in 29% yield over the two steps. Next, the nitrogen was protected as the Boc amide (Boc_2O, DMAP, Et_3N) in good yield (88%). Finally, the target compound **26** was completed via a Buchwald-Hartwig amination of **29** and **30** ($Pd_2(dba)_3$, X-Phos, Cs_2CO_3) followed by Boc deprotection (TFA, CH_2Cl_2) in good overall yield (70% for two steps).[25,26]



[a]Reagents and conditions: (a) NH_2NH_2, 100 °C; (b) 2 M NaOH, Br_2, 29% over 2 steps; (c) Boc_2O, DMAP, Et_3N, DMF, 88%; (d) Cs_2CO_3, X-Phos, $Pd_2(dba)_3$, 1,4-dioxane, 100 °C; (e) TFA, CH_2Cl_2, rt, 70% over 2 steps.

Scheme 1. Synthesis of **26** (VU0418506).

We next evaluated **26** in a functional assay using the rat $mGlu_4$ protein and demonstrated that its activity was similar to that observed for the human receptor (pEC_{50} = 7.34 ± 0.04, 46 nM). To assess the selectivity of **26** for $mGlu_4$ versus the other mGlu receptors, we performed selectivity assays in fold-shift format for the eight mGlu receptors.[14] **26** was found to be selective versus the Group I and Group II mGlu receptors (**Figure 3 A-C, E**) and against $mGlu_7$ and $mGlu_8$ (**Figure 3, G** and **H**), two of the Group III receptors. The only other mGlu receptor that appears to functionally interact (with equipotency) with **26** is $mGlu_6$ (**Figure 3F**), a receptor primarily expressed in the retina. In addition to mGlu selectivity, **26** was tested against a wider panel of GPCRs, enzymes, ion channels, nuclear hormone receptors and transporters (**Suppl.**

Table 1) and was found to be inactive against all of the targets, except monoamine oxidase-B (MAO-B, 330 nM). A scan against a panel of 96 kinase targets (DiscoveRx, KINOMEscan, www.discoverx.com) revealed no significant activity (<30%). To further de-risk **26** for potential advancement into pre-clinical toxicological studies, we progressed it into a cardiac channel panel IC_{50} screen (ChanTest Cardiac Channel Panel™, www.chantest.com), and tested for activity versus nine cardiac ion channels (including hERG); **26** was inactive up to 100 μM against all nine channels (**Suppl. Table 2**). Lastly, **26** was negative in a mini-AMES test (EuroFins, formerly Ricerca; www.eurofins.com) against TA98 and TA100 with and without S9.

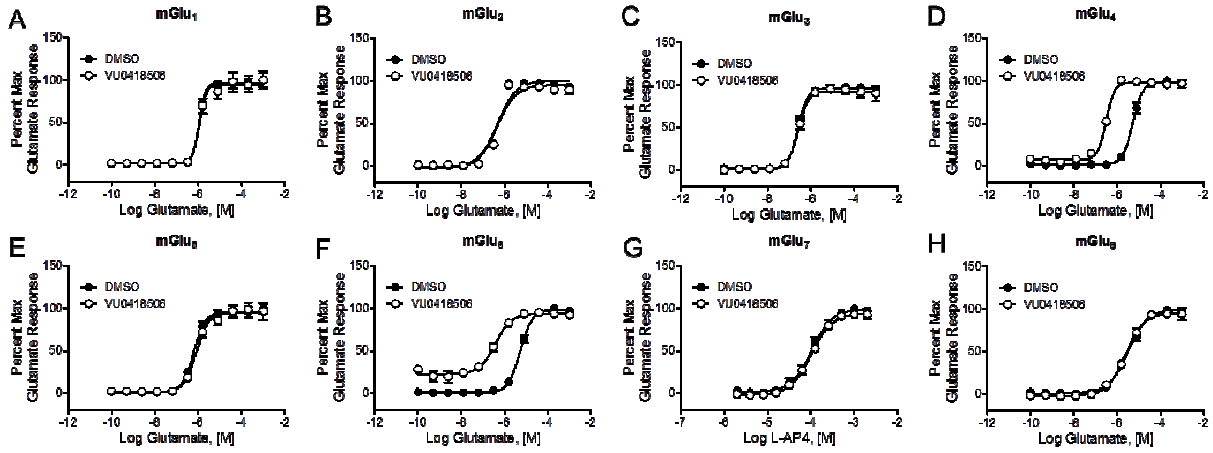
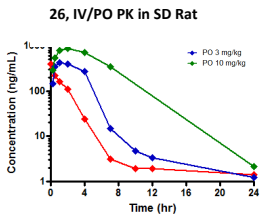
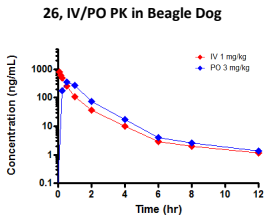
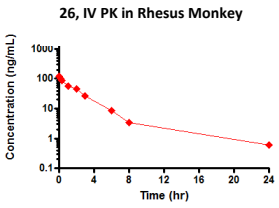


Figure 3. Compound **26** potentiates the glutamate response in $mGlu_4$ expressing cell lines (D) and is inactive against other mGlu receptors, except $mGlu_6$ (F).

Further *in vivo* PK profiling of **26** revealed that the compound had excellent oral bioavailability in both rats (F = 95%) and dogs (F = 36%) (**Table 4**). In addition, evaluation of **26** in a non-human primate (using a rhesus IV cassette) revealed a low clearance molecule (CL_p = 13 mL/min/kg) with a moderate half-life ($t_{1/2}$ = 3 hrs) (**Table 4**). Lastly, a plasma:brain PO snapshot study in rats showed substantial uptake of **26** in both the plasma and brain with a K_p

(brain:plasma ratio (0 – 6 h)) of 2.10 (**Table 4**). A more detailed discussion of the CSF levels and *in vivo* efficacy are detailed in the companion article, Niswender *et al*.

Table 4. *In vivo* PK for lead compounds:

	26			
				
PK Parameters (IV)	**Rat, 1 mg/kg**		**Dog, 1 mg/kg**	**Rhesus, Cassette**
CL_p (mL/min/kg)	29		36	13
V_{ss} (L/kg)	4.0		2.4	2.5
$t_{½}$ (min)	91		221	173
MRT (min)	132		66	184
AUC (hr·ng/mL)	608		515	
PK Parameters (PO)	**Rat**		**Dog, 3 mg/kg**	
	3 mg/kg	**10 mg/kg**		
C_{max} (ng/mL)	429	875	375	
T_{max} (hr)	1	2	0.75	
AUC PO (hr·ng/mL)	1895	7418	556	
%F	100	95	36	
Plasma:Brain (PO, 0 – 6 h, 10 mg/kg)				
$AUCplasma_{0-6}$	8771			
$AUCbrain_{0-6}$	18415			
B:P	2.1			

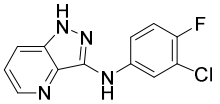
Before moving Compound **26** into extensive *in vivo* testing and chronic dosing paradigms, we investigated the potential of the potent CYP1A2 inhibitory activity observed with Compound **26** to affect dosing strategies. Activity against CYP1A2 is not surprising for these compounds as it is well known that small poly-aromatic groups inhibit this enzyme.[27] However, as CYP1A2 is also the primary enzyme responsible for the metabolism of **26**, we evaluated the potential of **26** to auto-induce CYP1A2 expression or activity in cryopreserved hepatocytes.[28] **26** was shown to be a potent inducer of CYP1A2 activity (EC_{50} = 9.8 μM and E_{max} = 104 fold-induction) and this manifested in a multi-day pharmacokinetic study in rodents where, upon day

4 of dosing, significantly reduced plasma levels of **26** were detected in both male and female Sprague Dawley (SD) rats (15 mg/kg PO, 1x daily) (**Supp. Figure 1**). Total reductions in plasma AUC in male SD rats were from 1148 μM*hr (Day 1) to 200 μM*hr (Day 4); similar results were observed in female SD rats at the same dose. Therefore, due to this CYP1A2 auto-induction profile in rodents, **26** is not suitable for chronic dosing, but still remains a valuable tool compound for acute studies as highlighted in our companion manuscript, Niswender et al.

CONCLUSION

In summary, we report the discovery and characterization of a novel chemical scaffold as an advanced pre-clinical positive allosteric modulator of $mGlu_4$, VU0418506 (**26**), the overall profile of which is shown in Table 5. We have shown that VU0418506 is potent against both the human (EC_{50} = 68 nM) and rat (EC_{50} = 46 nM) receptors and is selective against the other mGlu receptors, except retinally-restricted $mGlu_6$. While VU0418506 auto-induced CYP1A2 activity which limits its use in chronic dosing assays, it is anticipated that it will remain a valuable research tool compound for acute dosing. Extensive *in vivo* PK studies show that VU0418506 displays good bioavailability in two pre-clinical species (rat and dog) and is not a substrate for P-glycoprotein (Pgp, **Table 5**). Further selectivity profiling of VU0418506 revealed that it is selective against a number of kinases and other target families (GPCRs, ion channels, etc.). Initial examination of potential toxicological endpoints showed that VU0418506 is clean against a panel of ion channels associated with cardiac toxicity and it negative in a mini-AMES assay with and without S9. Further *in vivo* examination of VU0418506 in a number of pre-clinical animal models of Parkinson's disease and other CNS disease models is reported in our companion manuscript.

Table 5. Summary of **26** (VU0418506)**.**

	 **26, VU0418506**
Human $mGlu_4$ EC_{50} (nM)	68 nM
Rat $mGlu_4$ EC_{50} (nM)	46 nM
Rat Fold Shift (@ 30 μM)	15.5
MW	262.7
cLogP	3.96
TPSA	53.6
Intrinsic Clearance (mL/min/kg)	
Human CL_{HEP}	10.1
Rat CL_{HEP}	12.1
Dog CL_{HEP}	18.9
Mouse CL_{HEP}	50.1
Rhesus CL_{HEP}	11.5
Plasma Protein Binding (%*Fu*)	
Human	1.7
Rat	2.8
Dog	0.9
Mouse	1.9
Rhesus	1.4
CYP450 Inhibition (μM)	
1A2	<0.1
2C9	8.9
2D6	>30
3A4	>30
2C19	6.1
***In vivo* PK Parameters (Rat IV, 1 mg/kg)**	
CL (mL/min/kg)	29
V_{ss} (L/kg)	4.0
AUC (ng·h/mL)	608
MRT (min)	132
$T_{1/2}$ (min)	91
***In vivo* PK Parameters (Rat PO, 10 mg/kg)**	
Cmax (ng/mL)	875
Tmax (hr)	2
AUC PO (ng·h/mL)	7418
%F	95%
Rat CNS PO PBL (0 – 6 hr)	
Plasma (ng·h/mL)	8771
Brain (ng·h/mL)	18415
B:P	2.1
P_{app}, A-B (10^{-6})	24
P_{app}, B-A (10^{-6})	14
Efflux Ratio	0.6

METHODS

General. All NMR spectra were recorded on a 400 MHz AMX Bruker NMR spectrometer. ^{1}H and ^{13}C chemical shifts are reported in δ values in ppm downfield with the deuterated solvent as the internal standard. Data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, b = broad, m = multiplet), integration, coupling constant (Hz). Low resolution mass spectra were obtained on an Agilent 6120 or 6150 with ESI source. Method A: MS parameters were as follows: fragmentor: 70, capillary voltage: 3000 V, nebulizer pressure: 30 psig, drying gas flow: 13 L/min, drying gas temperature: 350 ºC. Samples were introduced via an Agilent 1290 UHPLC comprised of a G4220A binary pump, G4226A ALS, G1316C TCC, and G4212A DAD with ULD flow cell. UV absorption was generally observed at 215 nm and 254 nm with a 4 nm bandwidth. Column: Waters Acquity BEH C18, 1.0 x 50 mm, 1.7 um. Gradient conditions: 5% to 95% CH_3CN in H_2O (0.1% TFA) over 1.4 min, hold at 95% CH_3CN for 0.1 min, 0.5 mL/min, 55 ºC. Method B: MS parameters were as follows: fragmentor: 100, capillary voltage: 3000 V, nebulizer pressure: 40 psig, drying gas flow: 11 L/min, drying gas temperature: 350 ºC. Samples were introduced via an Agilent 1200 HPLC comprised of a degasser, G1312A binary pump, G1367B HP-ALS, G1316A TCC, G1315D DAD, and a Varian 380 ELSD (if applicable). UV absorption was generally observed at 215 nm and 254 nm with a 4 nm bandwidth. Column: Thermo Accucore C18, 2.1 x 30 mm, 2.6 um. Gradient conditions: 7% to 95% CH_3CN in H_2O (0.1% TFA) over 1.6 min, hold at 95% CH_3CN for 0.35 min, 1.5 mL/min, 45 ºC. High resolution mass spectra were obtained on an Agilent 6540 UHD Q-TOF with ESI source. MS parameters were as follows: fragmentor: 150, capillary voltage: 3500 V, nebulizer pressure: 60 psig, drying gas flow: 13 L/min, drying gas temperature: 275 ºC. Samples were introduced via an Agilent 1200 UHPLC comprised of a G4220A binary pump, G4226A ALS, G1316C TCC, and G4212A DAD with

ULD flow cell. UV absorption was observed at 215 nm and 254 nm with a 4 nm bandwidth. Column: Agilent Zorbax Extend C18, 1.8 μm, 2.1 x 50 mm. Gradient conditions: 5% to 95% CH_3CN in H_2O (0.1% formic acid) over 1 min, hold at 95% CH_3CN for 0.1 min, 0.5 mL/min, 40 ºC. For compounds that were purified on a Gilson preparative reversed-phase HPLC, the system comprised of a 333 aqueous pump with solvent-selection valve, 334 organic pump, GX-271 or GX-281 liquid hander, two column switching valves, and a 155 UV detector. UV wavelength for fraction collection was user-defined, with absorbance at 254 nm always monitored. Method: Phenomenex Axia-packed Luna C18, 30 x 50 mm, 5 μm column. Mobile phase: CH_3CN in H_2O (0.1% TFA). Gradient conditions: 0.75 min equilibration, followed by user defined gradient (starting organic percentage, ending organic percentage, duration), hold at 95% CH_3CN in H_2O (0.1% TFA) for 1 min, 50 mL/min, 23 ºC. Solvents for extraction, washing and chromatography were HPLC grade. All reagents were purchased from Aldrich Chemical Co. and were used without purification.

3-bromo-1*H*-pyrazolo[4,3-*b*]pyridine (28). A mixture of 3-fluoro-2-formylpyridine, **27**, (1.0 eq) and anhydrous hydrazine (8.0 eq) was heated to 110 °C. After 16h, the reaction was cooled to rt and slowly poured onto ice water. After extracting with EtOAc (3 x), the collected organic layers were washed with Brine (100 mL), dried ($MgSO_4$), filtered and concentrated to provide a dark brown oil. LCMS (method A): single peak (214 and 254 nm), R_T = 0.102 min, MS (ESI^+) *m/z* = 120.2 $[M + H]^+$. The crude residue was dissolved in 2 M NaOH (0.5 M) and a solution of Br_2 (1.0 eq) in 2 M NaOH (1.0 M) was added dropwise. After 3 h at rt, $NaHSO_3$ (aqueous) (1 mL) was added followed by 4 N HCl (~1.0M). A solid precipitated formed and was collected by filtration and air dried affording **28** as an off-white solid (29% yield over 2

steps). LCMS (method A): single peak (214 and 254 nm), R_T = 0.270 min, MS (ESI^+) *m/z* = 200.0 $[M + H]^+$.

***tert*-Butyl 3-bromo-1*H*-pyrazolo[4,3-*b*]pyridine-1-carboxylate (29).** To a solution of **28** (1.0 eq), DMAP (10 mg) and Et_3N (1.15 eq) in dry DMF (0.25 M) was added Boc_2O (1.1 eq) at rt. After 16 h at rt, the reaction was added to EtOAc: H_2O (1:1). The separated organic layer was washed with water (2x), brine, dried ($MgSO_4$), filtered and concentrated to provide desired product **29** (88% yield). Material was taken through without further purification. LCMS (method A): single peak (214 and 254 nm), R_T = 0.793 min, MS (ESI^+) *m/z* = 298.2 $[M + H]^+$.

***N*-(3-chloro-4-fluorophenyl)-1*H*-pyrazolo[4,3-*b*]pyridin-3-amine (25):** A mixture of **29** (1.0 eq), Cs_2CO_3 (2.0 eq), $Pd_2(dba)_3$ (5 mol%) and 2-Dicyclohexylphosphino-2′,4′,6′-triisopropylbiphenyl (XPhos) (10 mol%) in a round-bottomed flask were subjected to evacuation and purging with nitrogen (3x). A solution of 3-chloro-4-fluoroaniline, **30**, (1.1 eq) in 1,4-dioxane (0.1 M) was added. The reaction mixture was heated to 100 °C. After 12h, the reaction was added to EtOAc:H_2O (1:1) and the organic layer was separated. The aqueous layer was reextracted with EtOAc (2x). The collected organic layers were washed with brine, dried ($MgSO_4$), filtered and concentrated to afford a crude solid. The material was taken through to the next step. The crude residue was dissolved in DCM (0.1 M) at 0 °C and TFA (1.0 M) was added. The ice bath was removed. After an additional 30 min, LCMS confirmed the loss of starting material. The solvent was removed under reduced pressure and the residue was purified by reverse phase liquid column chromatography (35-65% acetonitrile: water with 0.1% trifluoroacetic acid) to yield **26** as an orange solid (70% over two steps). LCMS (method B): single peak (214 and 254 nm), R_T = 0.753 min, MS (ESI^+) *m/z* = 263.1 $[M + H]^+$; ^1H NMR (400 MHz, d_6-DMSO): δ 9.55 (br s, 1H), 8.49 (s, 1H), 8.11-8.10 (m, 1H), 7.99 (d, J = 8.0 Hz, 1H),

7.66 (m, 1H), 7.46 (m, 1H), 7.30 (dd, J = 9.2, 8.8 Hz, 1H). HRMS, calc'd for $C_{12}H_9N_4FCl$ $[M + H]^+$, 263.0500; found 263.0499.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI:

Chemical synthesis of all analogs, *in vitro* Pharmacology procedures, *in vitro* PK methods, *in vivo* PK methods, Selectivity and initial toxicology evaluation of **26**, 4-day *in vivo* PK study of **26**, human hepatocyte induction methods and *in vitro* biotransformation protocols.

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Author Contributions

C.R.H. and C.W.L. oversaw and designed the chemistry. D.W.E., R.D.G., Y.Y.C., J.M.S., and P.M.B. performed the synthetic chemistry work. A.L.B., J.S.D. and R.M. designed the pharmacokinetic experiments. P.J.C. and C.M.N. designed and analyzed the in vitro pharmacology experiments. C.R.H. and C.M.N. wrote the manuscript with input from all authors.

ACKNOWLEDGMENTS

The authors would like to thank the Michael J. Fox Foundation for Parkinson's Research and Bristol-Myers Squibb for their support of the program to develop $mGlu_4$ PAMs.

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For Table of Contents Use Only

Discovery, Synthesis and Pre-Clinical Characterization of *N*-(3-chloro-4-fluorophenyl)-1*H*-pyrazolo[4,3-*b*]pyridin-3-amine (VU0418506), a novel positive allosteric modulator of the metabotropic glutamate receptor 4 ($mGlu_4$)

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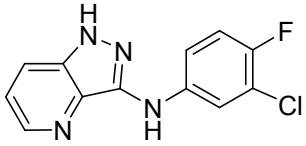

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VU0418506

$hmGlu_4$ EC_{50} = 68 nM

$rmGlu_4$ EC_{50} = 46 nM

B:P = 2.1