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Challenges in the development of an M_4 PAM *in vivo* tool compound: The discovery of VU0467154 and unexpected DMPK profiles of close analogs

Michael R. Wood[a,c,†], **Meredith J. Noetzel**[a,b,†], **Michael S. Poslusney**[a,†], **Bruce J. Melancon**[a,b], **James C. Tarr**[a], **Atin Lamsal**[a], **Sichen Chang**[a], **Vincent B. Luscombe**[a,b], **Rebecca L. Weiner**[a,b], **Hyekyung P. Cho**[a,b], **Michael Bubser**[a], **Carrie K. Jones**[a,b,e], **Colleen M. Niswender**[a,b,e], **Michael W. Wood**[d], **Darren W. Engers**[a,b], **Nicholas J. Brandon**[d], **Mark E. Duggan**[d], **P. Jeffrey Conn**[a,b,e], **Thomas M. Bridges**[a,b,*], and **Craig W. Lindsley**[a,b,c,*]

[a]Vanderbilt Center for Neuroscience Drug Discovery, Vanderbilt University Medical Center, Nashville, TN 3 7232, USA

[b]Department of Pharmacology, Vanderbilt University School of Medicine, Nashville, TN 37232, USA

[c]Department of Chemistry, Vanderbilt University, Nashville, TN 37232, USA

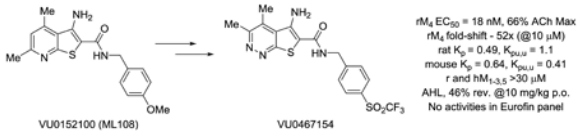
[d]Neuroscience Innovative Medicines, Astra Zeneca, 141 Portland Street, Cambridge, MA 02139, USA

[e]Vanderbilt Kennedy Center, Vanderbilt University School of Medicine, Nashville, TN 37232, USA

Abstract

This letter describes the chemical optimization of a novel series of M_4 positive allosteric modulators (PAMs) based on a 5-amino-thieno[2,3-*c*]pyridazine core, developed via iterative parallel synthesis, and culminating in the highly utilized rodent *in vivo* tool compound, VU0467154 (**5**). This is the first report of the optimization campaign (SAR and DMPK profiling) that led to the discovery of VU0467154, and details all of the challenges faced in allosteric modulator programs (steep SAR, species differences in PAM pharmacology and subtle structural changes affecting CNS penetration).

Graphical abstract



*To whom correspondence should be addressed: thomas.m.bridges@vanderbilt.edu and craig.lindsley@vanderbilt.edu.
†These authors contributed equally

Keywords

M_4; Muscarinic acetylcholine receptor; Positive allosteric modulator (PAM); Schizophrenia; Structure-Activity Relationship (SAR)

M_4 (muscarinic acetylcholine receptor subtype 4) positive allosteric modulators (PAMs) represent an exciting therapeutic strategy to treat multiple domains of schizophrenia,[1-18] as well as other CNS disorders,[19,20] via a new molecular mechanism.[21] However, the great potenital has been hampered by limited chemical diversity centered on a 3-amino-thieno[2,3-*b*]pyridine core, as in **1**-**4**, (Figure 1), which engenders steep SAR, species differences (rat versus human M_4 PAM potency, affinity/cooperativity and subtype selectivity), poor solubility, and/or low CNS penetration.[1-18] Early *in vivo* tool compounds, such as **2**,[1,2] energized the field but were not optimal. Recently, we disclosed VU0467154 (**5**) based on a novel 5-amino-thieno[2,3-*c*]pyridazine core that has proven to be a valuable rodent *in vivo* M_4 PAM tool compound with robust efficacy in a broad range of preclinical mouse and rat models of psychosis and cognition, as well as Huntington's disease.[11,17,18] Here, we describe for the first time the optimization campaign (SAR and intriguing DMPK profiles) that advanced PAMs **2**-**4**, into **5** with exceptional rodent M_4 PAM potency, selectivity, DMPK profile and CNS penetration; however, a significant species disconnect (35x less potent on human M_4) precluded advancement as a clinical candidate.[11,17,18]

Within the 3-amino-thieno[2,3-*b*]pyridine series, balancing M_4 PAM potency and solubility was a distinct challenge;[1-18] therefore, initial efforts focused on replacements for the pyridine ring, as very few substituents were tolerated. Regioisomeric pyridines were evaluated, along with isomeric pyrimidines, but all lost considerable M_4 PAM potency at both human and rat M_4. Based on the attractive physiochemical properties and high dielectric constant of the pyridazine ring, we prepared a 5-amino-thieno[2,3-*c*]pyridazine congener **6** of **2** (Figure 2). This modification proved favorable (M_4 PAM potency enhanced 4-fold, free fraction increased up to 9-fold, CL_{hep} improved as well as $K_{p,uu}$ and cLogP with no change in molecular weight), but the PMB amide proved to be a metabolic ‘hot spot’ (CYP-mediated oxidative demethylation) and would need to be replaced with an alternate amide.[22] At this point, multiple avenues of inquiry were pursued, and here we will focus on amide moieties containing sulfur (thioethers, sulfoxides, sulfones and SF_5) moieties.

The synthesis of analogs **9** proved to be straightforward. Condensation of commercially available 3-chloro-5,6-dimethylpyridazine-4-carbonitrile **7** with methyl thioglycolateunder basic conditions smoothly affords the sodiumcarboxylate **8** in 78% yield.[11] A HATU-mediated amide coupling with various benzyl amines then delivered analogs **9**in yields ranging from 45-92%. In all cases, either the desired benzylamine or the corresponding nitrile, which was easily reduced to the requisite benzyl amine, was commercially available.

SAR was driven on rat M_4, as the objective was an *in vivo* POC tool compound, but key compounds were assessed on human M_4 as well. As shown in Table 1, many potent rat M_4 PAMs were discovered, and several displayed excellent CNS pentration (K_p and $K_{p,uu}$). The direct thioether analog **9a** of **6** displayed an ~10-fold increase in rat M_4 PAM potency (EC_{50}= 11.2 nM), but diminished CNS exposure (K_p = 0.13). Oxidation to the

corresponding sulfoxide **9b** lost potency(EC_{50} = 139 nM), which was restored by the methyl sulfone**9c** (EC_{50} = 51.3 nM; however, CNS penetration was poor, K_p= 0.04). Moving the sulfone from the 4-position to the 3-position, as in **9d**, was poorly tolerated (EC_{50} = 417 nM), while steric bulk, as in **9e**-**g**, retained good rat M_4 PAM potency (EC_{50}s 22-48 nM), but with low, variable K_ps (0.05 to 0.11). Thus, steric bulk alone was not sufficient to balance PAM potency and K_p, therefore increased lipophilicity, in the form of fluorine atoms, was then evaluated. Addition of a single fluorine atom alpha to the methyl sulfone (**9h**) maintained PAM potency (EC_{50} = 58 nM), and CNS penetration improved (K_p = 0.51, $K_{p,uu}$ = 2.78). Consecutive addition of fluorines to the methyl moiety of **9c** afforded various fluoromethyl sulfones **9i**-**j** with potent rat M_4 PAM activity (EC_{50}s 17 to 23 nM), but unexpected and highly variable CNS exposure. The mono-fluoromethyl analog **9i** was not detected in the CNS, while brain distribution of the difluoromethyl congener **9j** was modest (K_p = 0.11, $K_{p,uu}$ = 0.10) and that of the trifluoromethyl derivative **5** was exceptional (K_p = 0.49, $K_{p,uu}$ = 1.1). Finally, an unusual pentaflurosulfur (SF_5) analog, **9l**, was a potent rat M_4 PAM (EC_{50} = 30 nM) with excellent CNS distribution (K_p = 1.4, $K_{p,uu}$ = 1.0). Of these, **5** and **9l** stood out as potential candidates as rodent *in vivo* tool compounds, but we wanted to assess their activity at human M_4 (as species differences are common amongst M_4 PAM ligands) to determine if these could translate into clinical candidates.[8-11,13,14] Unfortunately, as shown in Table 2, there was a 6-to 35-fold rightward shift in human PAM potency, precluding these analogs from consideration as clinical candidates.

Next, we assessed physiochemical properties and DMPK profiles of **9j**, **5** and **9l** in battery of *in vitro* and *in vivo* assays (Table 2). Molecular weights were all similar and both the difluoromethyl sulfone **9j** and the trifluoromethyl sulfone **5** had similar TPSAs, but the SF_5 analog, **9l**, had greatly reduced TPSA. *In vitro* clearance (predicted CL_{hep} for both human and rat) was similar across the three PAMs, but plasma protein binding did differentiate the PAMs. **9j**, with a cLogP of 1.98, had the most favorable fraction unbound (f_u (rat, human) of 0.066 and 0.053) as well as fraction unbound in brain (f_u rat brain, 0.062). In contrast, the highly lipophilic SF_5 (cLogP = 5.13) congener **9l**, displayed poor fraction unbound (f_u (rat, human) of 0.004 and 0.010). **5** represented the middle ground with moderate fractionun bound in plasma (f_u (rat, human) of 0.031 and 0.019) and in brain (f_u rat brain, 0.067) with a n optimal cLogP (2.49) predictive of good CNS exposure. The CYP_{450} profiles were very clean, except for weak inhibition of 2D6 by **9j** and **9l**. *In vivo* rat PK (1 mg/kg i.v.) again distinguished **5**, with low CL_p (7.8 mL/min/kg), a long half-life ($t_{1/2}$ = 5.7 hours), excellent oral bioavailability (61%F, from 3 mg/kg suspension dose) and with excellent brain distribution (K_p = 0.49 and $K_{p,uu}$ = 1.1. Interestingly, and despite varying *in vivo* DMPK profiles, all three afforded robust reversal of amphetamine-induced hyperlocomotion (AHL) when evaluated in single-point oral dosing at either 10 mg/kg (**9j**, 49.3% and **5**, 55.3%) or at 30 mg/kg (**9l**, 66.3%) – our standard pharmacodynamic assay for M_4 PAM optimization. While we have recently reported results from dose-response AHL (as well as MK-801-induced hyperlocomotion) studies with **5** in rat and mouse (K_p for mouse of 0.64), here we show full dose response AHL for **9j** and **9l** (Fig. 3), and, while not ideal tools, both **9j** and **9l** are efficacious *in vivo*. Data for **5** was previously reported, with 46% reversal at 10 mg/kg p.o, 53% reversal at 30 mg/kg p.o. and a minimum effective dose (MED) at 3 mg/kg p.o. (33% reversal).[11]

To vet these analogs as potential rodent *in vivo* tool compounds, we next evaluated selectivity across the other muscarinic receptors ($M_{1\text{-}3,5}$) and broader ancillary pharmacology in Eurofin 's Lead Profiling panel (68 GPCRs, ion channels and transporter radioligand binding assay panel).[23] All three M_4 PAMs, **9j**, **5** and **9l**, had no activity (EC_{50}s >30 μM) at both rat and human $M_{1\text{-}3,5}$ and **5** displayed no ancillary pharmacology at any of the 68 targets in the Eurofin panel (no % inhibition >50%@10 μM). Both **9j** and **9l** showed a less clean profile, showing significant off-target activities at multiple targets (>50% inhibition@10 μM which were then confirmed in full CRC). **9j** proved to be a ligand for $hCav_{1\ 2}$ (5.7 μM), DAT (175 nM), SERT (600 nM) and 5-HT_3 (1.6 μM). Similarly, **9l** proved to be a ligand for DAT (150 nM), SERT (520 nM), Ghrelin receptor (6.5 μM), BDZ (1.5 μM), GABA (2.1 μM) and 5-HT_3 (1.5 μM) Therefore, **5** emerged as the ideal rodent *in vivo* M_4 PAM tool compound with clean ancillary pharmacology and excellent rodent PK. A subsequent, broader ancillary pharmacology panel identified single off-target activity for **5**, the human adenosine transporter, with an IC_{50} of 240 nM. The full *in vitro* and *in vivo* pharmacological profile of **5** (VU0467154) has been described in detail.[11]

In summary, we have detailed for the first time the progression from the prototypical 3-amino-thieno[2,3-*b*]pyridine core M_4 PAM core to the 5-amino-thieno[2,3-*c*]pyridazine core, which imparts compounds with improved potency, physiochemical properties and DMPK profiles. However, the sulfone series detailed here still suffers from significant species differences in M_4 PAM potency, in favor of rat, and unpredictable variations in CNS penetration based on fluorine content and/or lipophilicity. This effort led to the study of an unusual SF_5 congener with potent PAM activity and *in vivo* efficacy, but the high cLogP proved problematic. Importantly, this gave rise to the highly valuable rodent M_4 PAM *in vivo* tool compound **5** (VU0467154), with a balanced profile and demonstrated utility in target validation studies. Further optimization efforts en route to M_4 PAM clinical candidates, with equivalent human and rat M_4 PAM potencies within this series, will be reported in due course.

Acknowledgments

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22. Manuscript in preparation.
23. For information on the Eurofin LeadProfiling Screen see: www.eurofin.com

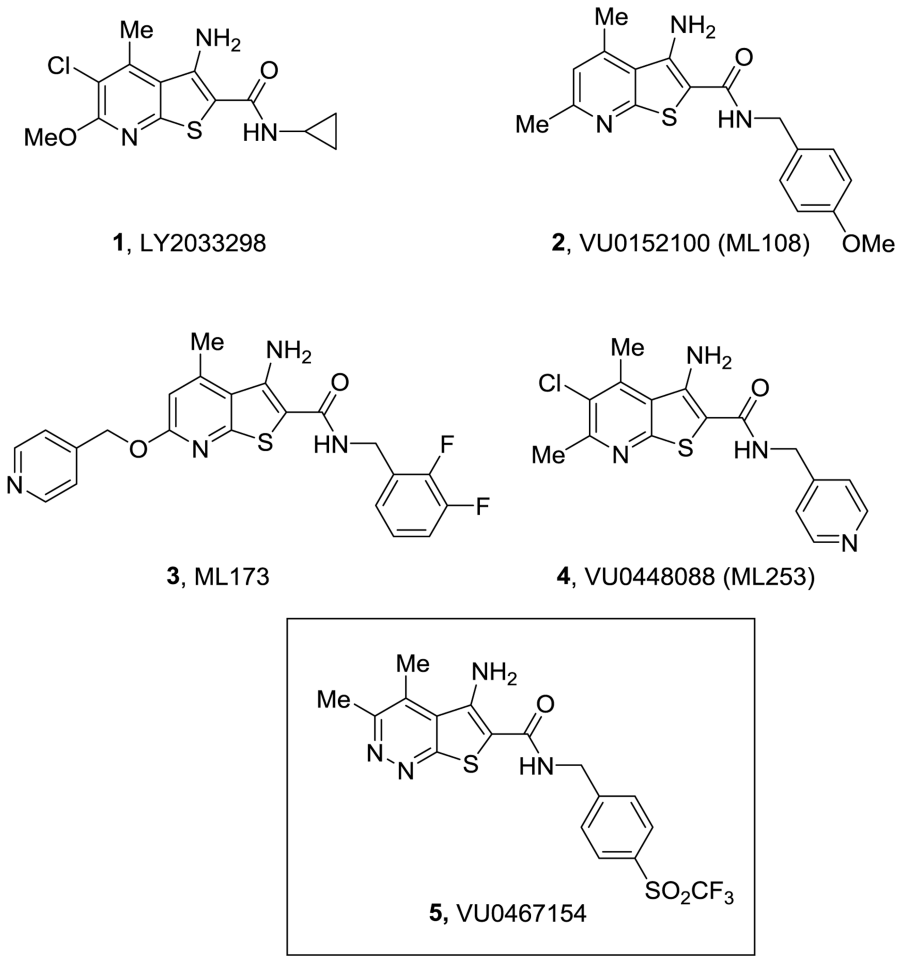
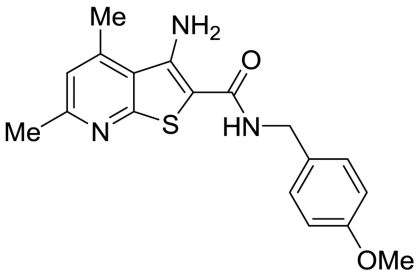
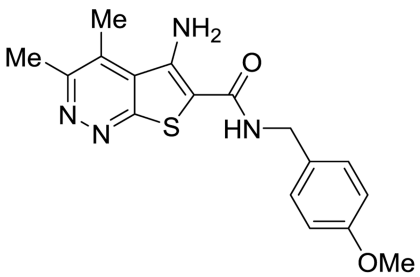


Figure 1.

Structures of representative M_4 PAMs **1**-**4**, highlighting the conserved 3-amino-thieno[2,3-*b*]pyridine chemotype, and the optimized rodent *in vivo* tool M_4 PAM, VU0467154 (**5**) with a novel pyridazine core.



2, VU0152100
hM_4 EC_{50} = 520 nM, 80% ACh Max
K_p = 0.38, $K_{pu,u}$ = 1.1
rCL_{hep} = 58 mL/min/kg
hCL_{hep} =16 mL/min/kg
cLogP = 2.94
f_u (r, h) = 0.015, 0.004



6, VU0464090
hM_4 EC_{50} = 150 nM, 83% ACh Max
K_p = 0.28 $K_{pu,u}$ = 2.98
rCL_{hep} = 37 mL/min/kg
hCL_{hep} =13 mL/min/kg
cLogP = 1.88
f_u (r, h) = 0.022, 0.035

Figure 2.

Structures of the first in vivo M4 PAM tool compound **2**, and the new 5-amino-thieno[2,3-*c*]pyridazine congener **6**, which displayed improved physiochemical, pharmacological and DMPK properties.

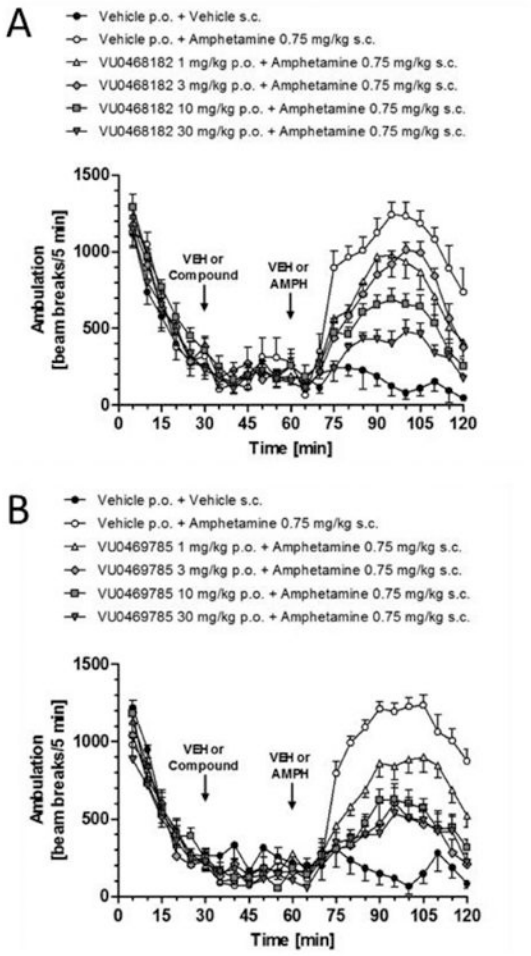
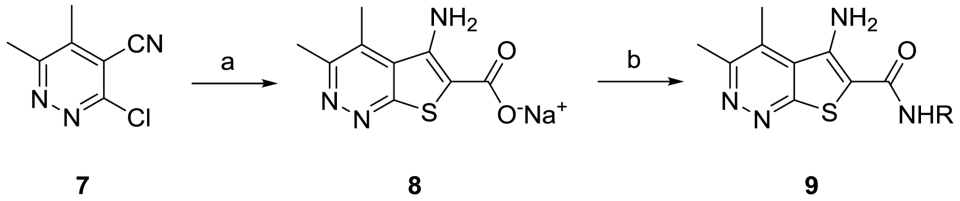


Figure 3.

Reversal of amphetamine-induced hyperlocomotion with A) **9j** (VU0468182), B) **9l** (VU0469785). The M_4 PAMs were administered 30 minutes after habituation in the chamber, and either vehicle or 0.75 mg/kg amphetamine administered s.c. at t = 60 min. For each dose group, n = 7-8 rats.



7 **8** **9**

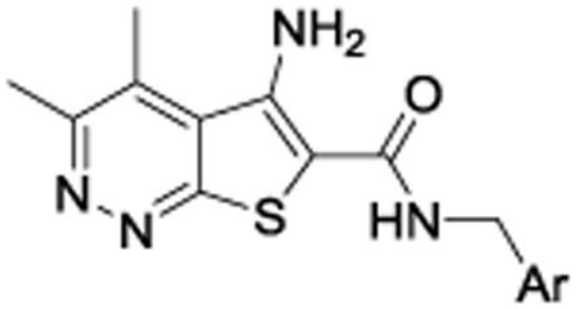
Scheme 1.

Synthesis of M_4 PAM analogs **9**.[a]

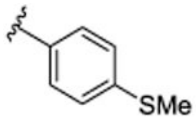
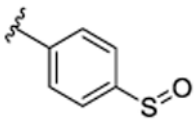
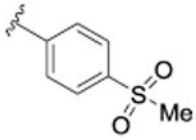
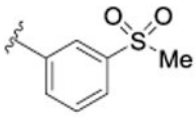
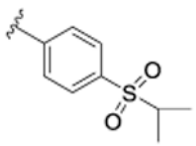
[a]Reagents and conditions: (a) Methyl thioglycolate, MeOH, 1M aq. NaOH, 150 °C, microwave, 30 min, 78%; (b) NH_2CH_2Ar, HATU, DMF, DIEPA, 2 h, 45-92%.

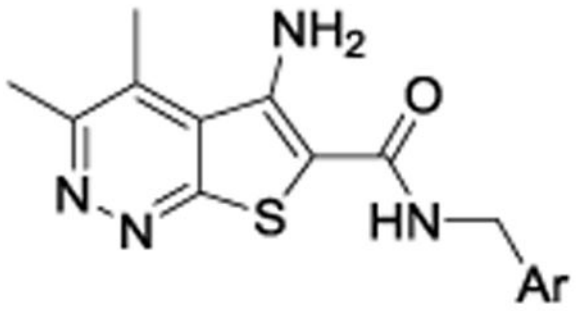
Table 1

Structures and activities for rat M_4 PAM **5** and analogs **9**.

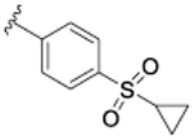
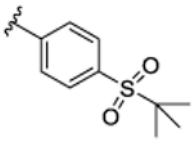
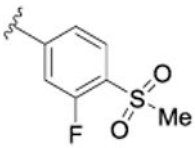
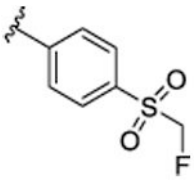
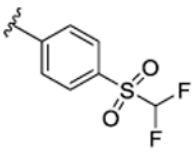


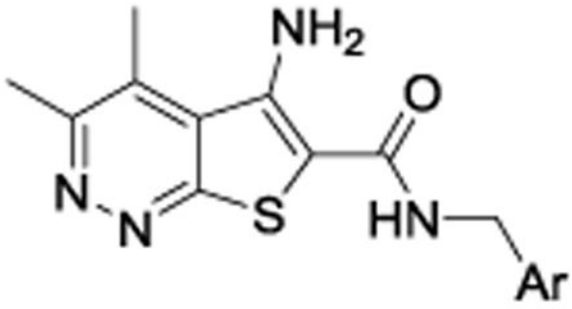
9

Cpd	Ar	rM$_4$EC$_5$0 (nM)[a][% ACh Max ±SEM]	rM$_4$ pEC$_{50}$ (±SEM)	Rat K$_p$(Kp,uu)[b]
9a		11.2 [75.6±1.7]	7.95±0.09	0.13 (0.48)
9b		139 [74.2±1.9]	6.86±0.04	ND
9c		51.3 [72.6±2.2]	7.29±0.08	0.04 (0.14)
9d		417 [74.6±2.1]	6.38±0.17	ND
9e		47.9 [76.8±2.7]	7.32±0.06	0.07 (0.67)

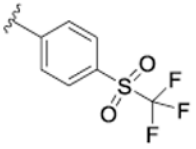
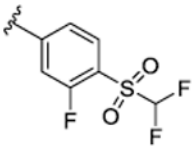
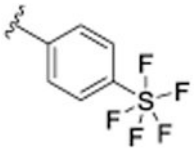


9

Cpd	Ar	rM$_4$EC$_5$0 (nM)[a][% ACh Max ±SEM]	rM$_4$ pEC$_{50}$ (±SEM)	Rat K$_p$(Kp,uu)[b]
9f		22.4 [67.1±3.1]	7.65±0.05	0.05 (0.29)
9g		44.7 [72.1±2.2]	7.35±0.06	0.11 (0.34)
9h		57.7 [69.2±3.5]	7.24±0.10	0.51 (2.8)
9i		21.9 [62.5±5.5]	7.66±0.04	BLQ
9j		17.8 [65.2±2.4]	7.75±0.14	0.11 (0.10)



9

Cpd	Ar	rM$_4$EC$_5$0 (nM)[a] [% ACh Max ±SEM]	rM$_4$ pEC$_{50}$ (±SEM)	Rat K$_p$(Kp,uu)[b]
5		17.8 [68.1±1.6]	7.75±0.06	0.49 (1.1)[c]
9k		23.4 [76.7±2.6]	7.63±0.05	BLQ
9l		30.2 [75.7±1.6]	7.52±0.03	1.4 (1.0)

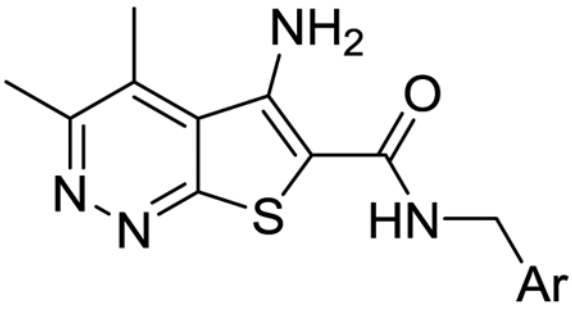
[a] Calcium mobilization assays with rM4/Gqi5-CHO cells performed in the presence of an EC20 fixed concentration of acetylcholine; values represent means from three (n=3) independent experiments performed in triplicate.

[b] Total and calculated unbound brain:plasma partition coefficients determined at 0.25 hr post-administration of an IV cassette dose (0.20-0.25 mg/kg) to male, SD rat (n=1); in conjunction with *in vitro* rat plasma protein and brain homogenate binding assay data. ND = not determined. BLQ = below limit of quantitation.

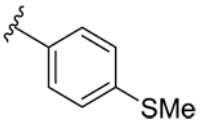
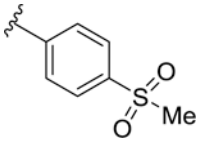
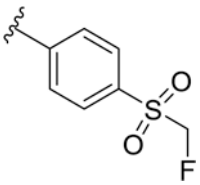
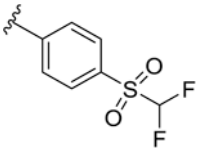
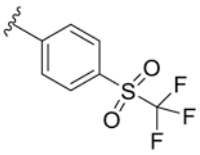
[c] mean values obtained from discrete studies using a 1.5 hr sample time point.[11,13,14]

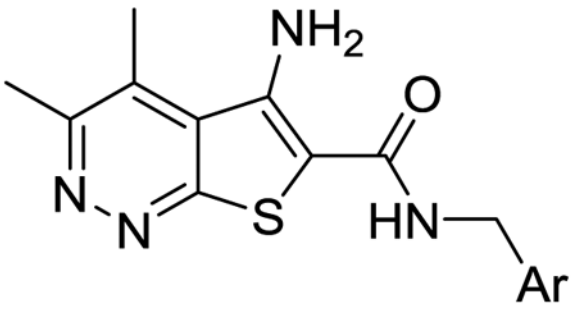
Table 2

Structures and human activities for M_4 PAM analogs **9**.

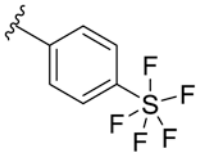


9

Cpd	Ar	hM_4EC_{50} (nM)[a] [% ACh Max ±SEM]	hM_4 pEC_{50} (±SEM)
9a		60.2 [71.9±1.7]	7.22±0.09
9c		363 [73.8±2.2]	6.44 ±0.08
9i		209 [62.5±5.5]	6.68±0.04
9j		251 [72.8±2.4]	6.60±0.14
5		631 [56.4±2.4]	6.20±0.06



9

Cpd	Ar	hM$_4$EC$_{50}$ (nM)[a] [% ACh Max ±SEM]	hM$_4$ pEC$_{50}$ (±SEM)
9l		550 [66.1±1.6]	6.26±0.03

[a]Calcium mobilization assays with hM4/Gqi5-CHO cells performed in the presence of an EC20 fixed concentration of acetylcholine; values represent means from three (n=3) independent experiments performed in triplicate.

Table 3

In vitro and *in vivo* DMPK properties of **9j**, **5** and **9l**.

Property	**9j**	**5**	**9l**
MW	426.4	444.4	438.4
cLogP	1.98	2.49	5.13
TPSA	114	114	79.8
In vitro PK parameters			
CL_{INT} (mL/min/kg), rat	48.1	47.7	95.9
CL_{HEP} (mL/min/kg), rat	28.1	28.4	40.5
CL_{INT} (mL/min/kg), human	15.6	19.8	52.8
CL_{HEP} (mL/min/kg), human	9.34	10.2	15.0
Rat fu_{plasma}	0.066	0.031	0.004
Human fu_{plasma}	0.053	0.019	0.010
Rat fu_{brain}	0.062	0.067	0.004
Cytochrome P_{450} (IC_{50}, μM)			
1A2	>30	>30	>30
2C9	>30	>30	>30
2D6	20.8	>30	23.9
3A4	>30	>30	>30
In vivo PK (Sprague-Dawley rats)			
IV (1 mg/kg)			
CL_p (mL/min/kg)	16	7.8	12
t ½ (h)	2.4	5.7	3.6
V_{ss} (L/kg)	2.9	3.1	3.1
Tissue Distribution (1 mg/kg, IP) Sample time (0.25 h)			
$K_{p,\ brain}$	0.11	0.49	1.0
$K_{puu,\ brain}$	0.10	1.1	0.75
% reversal in AHL (10 mg/kg, p.o.)	49.3	55.3	66.3