Challenges in the development of an M4 PAM preclinical candidate: The discovery, SAR, and *in vivo* characterization of a series of 3-aminoazetidine-derived amides

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Abstract

This letter details the continued chemical optimization of a novel series of M_4 positive allosteric modulators (PAMs) based on a 5-amino-thieno[2,3-*c*]pyridazine core by incorporating a 3-amino azetidine amide moiety. The analogs described within this work represent the most potent M_4 PAMs reported for this series to date. The SAR to address potency, clearance, subtype selectivity, CNS exposure, and P-gp efflux are described. This work culminated in the discovery of VU6000918, which demonstrated robust efficacy in a rat amphetamine-induced hyperlocomotion reversal model at a minimum efficacious dose of 0.3 mg/kg. 2009 Elsevier Ltd. All rights reserved.

Graphical Abstract

*To whom correspondence should be addressed: thomas.m.bridges@vanderbilt.edu and craig.lindsley@vanderbilt.edu.
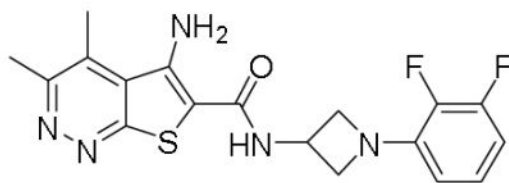
17j (VU6000918)
hM$_4$ EC$_{50}$ = 19 nM
% ACh$_{Max}$ = 88
rat AHL reversal = 74% (3 mg/kg PO)

Keywords

M$_4$; Muscarinic acetylcholine receptor; Positive allosteric modulator (PAM); Schizophrenia; Azetidine

Positive allosteric modulators (PAMs) of the muscarinic acetylcholine receptor (M$_4$) (**1-4**) have emerged as an exciting potential strategy for the treatment of numerous CNS disorders, including schizophrenia,[1–20] Huntington's disease,[21] and Alzheimer's disease.[22] Previous reports from our laboratory have described the discovery and characterization of VU0152100 (ML108, **1**), an *in vivo* tool compound which demonstrated efficacy in rodent models of anti-psychotic efficacy.[3,4] We subsequently reported related congener VU0467154 (**2**), based on a 5-amino-thieno[2,3-*c*]pyridazine core, which, despite its robust *in vivo* activity in multiple preclinical rodent models and a favorable pharmacokinetic (PK) profile, suffered from considerably lower potency at the human M$_4$ receptor as compared to rat.[11,19] In the course of our medicinal chemistry campaign to identify a compound with improved potency at the human M$_4$ receptor while maintaining suitable DMPK properties for a clinical candidate, we encountered steep SAR not only in potency at M$_4$, but in multiple DMPK properties as well.[13,14,19,20] Herein, we describe our efforts to replace the benzylic linker present in compounds **1-4** with substituted 3-amino azetidines.

Observing that small cyclic amides afforded potent analogs in both Eli Lilly's and our M$_4$ PAM programs, we wished to examine the introduction of a cyclic linker between the 5-aminothieno[2,3-*c*]pyridazine amide core and the appended aryl ring. Such a change may serve to decrease the planarity of the molecule, thus reducing its ability to form pi-stacking interactions and thereby improve solubility, restrict the conformations available for the aryl ring to adopt, and remove the benzylic methylene as a potential metabolic soft spot. Diamine linkers would provide a convenient synthetic handle by which to introduce substituents on the cyclic linker. Several potential linkers were examined, including monocyclic and bicyclic diamines; however, 3-amino substituted azetidines yielded the most potent analogs (Figure 2).

Analogs were readily prepared following functionalization of commercially available 3-(Boc-amino)-azetidine via nucleophilic substitution or Buchwald-Hartwig[23,24] cross-

coupling reactions, followed by Boc deprotection and amide coupling to the thieno[2,3-c]pyridazine core (Scheme 1). Our initial library examined the effect of tertiary carbamates, sulfonamides, and amides (Table 1). Basic tertiary azetidine amines were poorly tolerated and led to a sharp decrease in human M_4 (hM_4) potency (data not shown). Carbamates proved to be the most potent compounds in this class, with analog **6b** displaying an EC_{50} of 23 nM. However, upon further profiling, **6b** was found to have weak activity at human M_2 (hM_2, EC_{50} = 2.65 μM) and a short elimination half-life *in vivo* in rat ($t_{1/2}$ < 30 min) due to facile hydrolysis of the carbamate, which proved to be the case in general for the carbamate series and thus precluded their advancement. Azetidine sulfonamides (**6e**), ureas (**6d**), and amides (**6f-h**) were also tolerated, albeit with lower potency as compared to the carbamates. Compound **6h** was selected for further assessment, which gratifyingly found an improved profile compared to the carbamate series with reduced activity at hM_2 (EC_{50} > 10 μM) and low *in vivo* clearance (rat CL_p = 3.1 mL/min/kg). Unfortunately, **6h** was found to have low CNS exposure (rat brain:plasma K_p = 0.03, $K_{p,uu}$ = 0.37 at 0.25 hr post-IV cassette dose) likely due to P-gp efflux (MDCK-MDR1 ER = 96).

Encouraged by our initial results within this series, we sought to further improve the azetidine amides by examining *N*-aryl azetidines. Initial efforts sought to mimic the carbamate functionality by incorporating 1,3-heteroaryl substituents (**16a-c**). Compound **16c** maintained reasonable hM_4 potency (EC_{50} = 106 nM), exhibited moderate *in vitro* clearance (predicted rat and human CL_{hep} = 41 and 12 mL/min/kg, respectively, based on hepatic microsomal CL_{int}), and achieved moderate CNS exposure (rat brain:plasma K_p = 0.17, $K_{p,uu}$ = 1.3). Despite lacking mAChR subtype selectivity (hM_2 EC_{50} = 220 nM), **16c** was advanced to a rat amphetamine hyperlocomotion (AHL) reversal study where it exhibited marginal efficacy (16% reversal following 10 mg/kg PO) but provided initial proof-of-concept for the azetidine amide class of compounds. Broadening our scope of *N*-heteroaryl azetidines led us to identify numerous potent analogs (**16d-q**). A wide variety of heterocycles was tolerated, with pyridyl, pyrimidyl, and pyrazinyl substituents providing EC_{50}s < 100 nM. Substitution of the heteroaryl ring proved capable of imparting dramatic shifts in potency. While the parent 4-pyridyl azetidine analog possessed micromolar PAM activity (data not shown), introduction of halogen substituents on the 4-pyridyl ring provided exquisitely potent analogs (**16p, 16q**). Substitution at the *meta*-position was also tolerated on 3-pyridyl analogs, with methoxy (**16l**) and fluorine (**16m**) providing analogs of comparable potency to **16e**.

While we were able to achieve excellent *in vitro* potencies within this series, obtaining both reasonable CNS exposure and metabolic stability proved more challenging. A number of compounds (**16e-h, 16l**) failed to achieve acceptable CNS exposure (rat brain:plasma K_p < 0.05) and/or were found to be substrates for human P-gp efflux (**16e, 16i, 16q,**). Additionally, rat *in vivo* PK studies revealed evidence for extrahepatic non-CYP_{450} metabolism in certain cases (**16o, 16p**; possibly due to aldehyde oxidase-mediated metabolism *alpha* to the 4-pyridyl nitrogen). Increasing the lipophilicity of analogs by incorporation of halogen atoms generally led to modest increases in rat CNS exposure and reduced P-gp efflux. Compound **16j** was selected for further DMPK profiling, which revealed low predicted clearance (human and rat predicted CL_{hep} = 5.6 and 18 mL/min/kg,

respectively, based on hepatic microsomal CL_{int}) and low potential for CYP_{450} inhibition (3A4, 2D6, 2C9, 1A2 $IC_{50} > 30$ μM). In a rat (male, Sprague-Dawley; $n = 2$) *in vivo* PK study, **16j** demonstrated low clearance ($CL_p = 8.8$ mL/min/kg) with a small volume of distribution ($V_{ss} = 0.89$ L/kg) and moderate elimination half-life ($t_{1/2} = 1.3$ hr). Total and unbound distribution of **16j** to the brain was moderate in rat (brain:plasma $K_p = 0.12$, $K_{p,uu} = 0.33$ at 0.25 hr post-IV cassette dose), and, while it was still a substrate for human P-gp (ER = 8.5 in MDCK-MDR1 cells), its efflux was attenuated compared to related analogs. Given this favorable profile, it was advanced to a dose-response amphetamine hyperlocomotion (AHL) study in rat where it demonstrated robust reversal of AHL (Figure 3). An oral dose of 1 mg/kg provided a 44% reversal of AHL, and a maximal effect of 55% AHL reversal was achieved from 10 and 30 mg/kg dose levels. This level of *in vivo* efficacy was encouraging for the series and comparable to that previously reported for benchmark compounds **3** and **4**. However, due to **16j**'s P-gp liability and potentiation of hM_2 ($EC_{50} = 0.96$ μM, $ACh_{Max} = 43\%$), it was deemed not suitable for further development.

Expanding on the scope of azetidine analogs we had already investigated, we synthesized a library of *N*-aryl analogs (Table 3). Phenyl congener **17a** retained the excellent hM_4 potency that could be attained with *N*-heteroaryl analogs. While **17a** did suffer from higher *in vivo* clearance (rat $CL_p = 45$ mL/min/kg), a consequence of phenyl hydroxylation based on *in vitro* metabolic soft-spot experiments (data not shown), as well as moderately potent potentiation of hM_2 ($EC_{50} = 1.78$ μM, 58% ACh_{Max}), it showed a promising divergence from the *N*-heteroaryl azetidine SAR with improved CNS exposure (rat brain:plasma $K_p = 0.57$, $K_{p,uu} = 0.73$). Fluorine substitution(s) on the phenyl ring (**17b**, **17h-j**) generally maintained good hM_4 potency and reduced *in vivo* clearance (**17i**, **17j**; rat $CL_p = 4.4$ and 16 mL/min/kg, respectively). Notably, the azetidine congeners (**17f**, **17g**) of benzylic-linked M_4 PAMs previously reported by our group[19,20] exhibited significantly weaker hM_4 activity compared to that of **17j** (~10-fold and 40-fold, respectively). In light of its favorable potency and rat PK, compound **17j**, bearing a 2,3-difluorophenyl substituent, was selected for additional characterization. Operational model parameters were determined for **17j** from ACh CRC fold-shift experiments (Ca^{2+} mobilization assays), which revealed a rat M_4 K_B of 120 nM and αβ of 63, a cynomolgus monkey M_4 K_B of 890 nM and αβ of 120, and a human M_4 K_B of 2000 nM and αβ of 380. Gratifyingly, **17j** displayed broadly attractive properties including an absence of P-gp efflux (MDCK-MDR1 ER = 1.3), acceptable selectivity versus hM_2 (~230-fold based on potency), and high brain distribution (rat brain:plasma $K_p = 0.77$, $K_{p,uu} = 0.86$ at 0.25 hr post-IV cassette dose).

Based on these findings, compound **17j** was then evaluated in a rat AHL dose response study (Figure 4) where it demonstrated *s*tatistically significant AHL reversal (18%) from a low oral dose of 0.03 mg/kg and reached maximal reversal (74%) from a 3 mg/kg dose, with a resulting *in vivo* plasma EC_{50} of 74 nM (0.66 nM unbound) based on terminal concentrations measured in the study animals (1.5 hr post-administration of **17j**).

Compound **17j** was further studied to evaluate its suitability for progression into IND-enabling studies. An Ames test found no evidence of mutagenesis, a large secondary pharmacology panel (Cerep) revealed a fairly clean profile (all IC_{50}s/EC_{50}s >25–30 μM except for human DAT binding $IC_{50} = 0.45$ μM), and CYP_{450} inhibition was acceptable

(1A2 IC_{50} = 25 μM, 3A4 IC_{50} = 11 μM, 2D6 IC_{50} = 14 μM, 2C9 IC_{50} = 3.8 μM, 2C19 IC_{50} = 6.3 μM; with no evidence for time-dependent inhibition). IV and PO PK studies with **17j** in rat (male, Sprague Dawley, n = 1–3) and dog (female, mongrel, n = 1–2) found the compound to possess low to moderate *in vivo* clearance (rat and dog CL_p = 16 and 17 mL/min/kg, respectively) with a small to moderate volume of distribution (rat and dog V_{ss} = 0.67 and 1.6 L/kg, respectively), and moderate oral bioavailability (rat and dog F = 38% and 20%, respectively, from a 2–3 mg/kg solution dose). However, lower oral bioavailability in dog was observed (11%) from a suspension dose (2 mg/kg) in a low-excipient vehicle. These findings, coupled with a low kinetic solubility (solubility in FaSSIF at pH 6.5 after 1 hr = 0.015 mg/mL; aqueous solubility at pH 7.4 = 1.8 μM), as well as suboptimal predicted human PK (~1–2 hr $t_{1/2}$ based on moderate and small predicted CL and V_{ss}, respectively) led us to deprioritize **17j** for further evaluation as a preclinical candidate and focus efforts on overcoming the evident solubility-limited absorption.

In summary, substitution of the benzyl linker with a 3-aminoazetidine moiety afforded facile entry into an extremely potent series of M_4 PAMs. By varying the substitution pattern of *N*-aryl and *N*-heteroaryl groups, we were able to optimize subtype selectivity, clearance, CNS exposure, and P-gp efflux. Compound **17j** demonstrated robust AHL reversal in a rodent model; however, an unacceptably short projected human half-life and low oral bioavailability (due in part to solubility-limited absorption) precluded its advancement as a clinical candidate. Further study and optimization within this series is ongoing, and will be reported in due course.

Acknowledgments

References and notes

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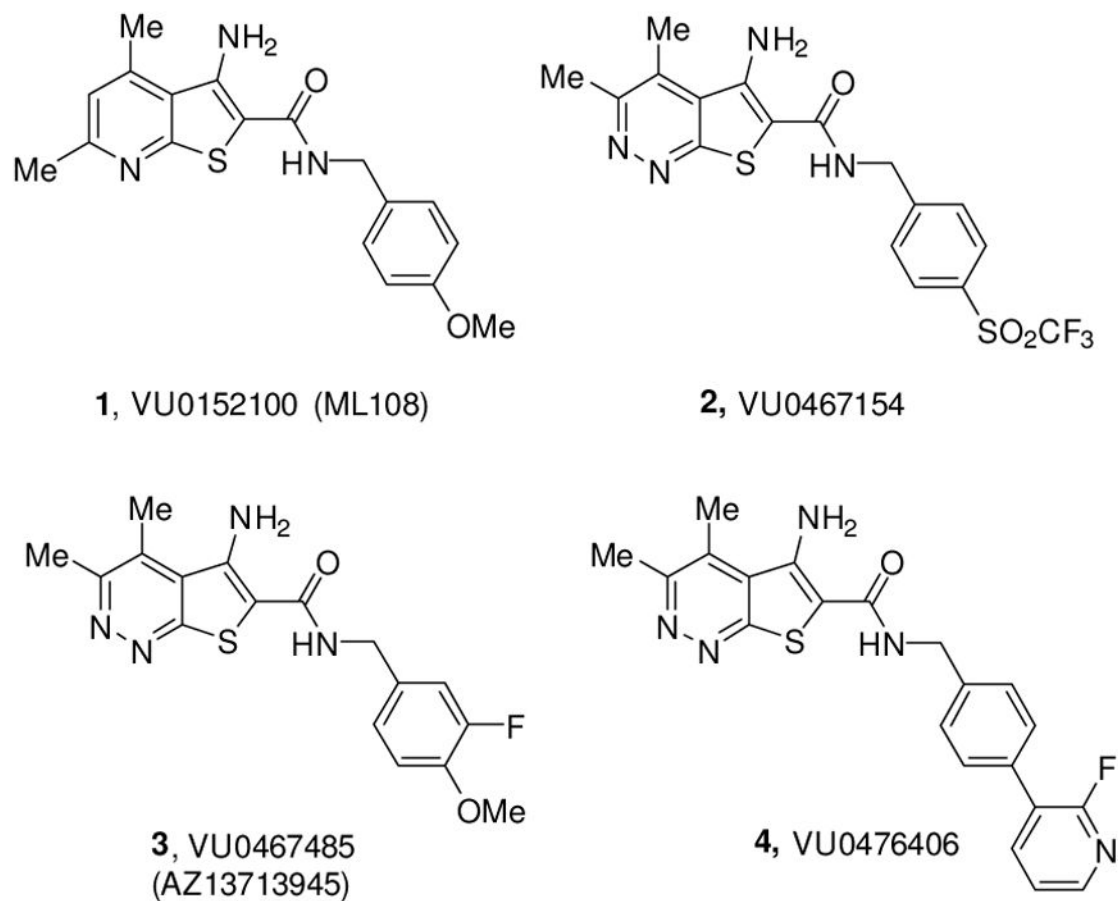24. Hartwig JF. Acc Chem Res. 1998; 31:852.

Figure 1.
Structures of representative M₄ PAMs **1-4**, highlighting the optimized rodent *in vivo* tool M₄ PAM, VU0467154 (**2**), the clinical candidate VU0467485/AZ13713945 (**3**) and the non-human primate *in vivo* tool VU0476406 (**4**).
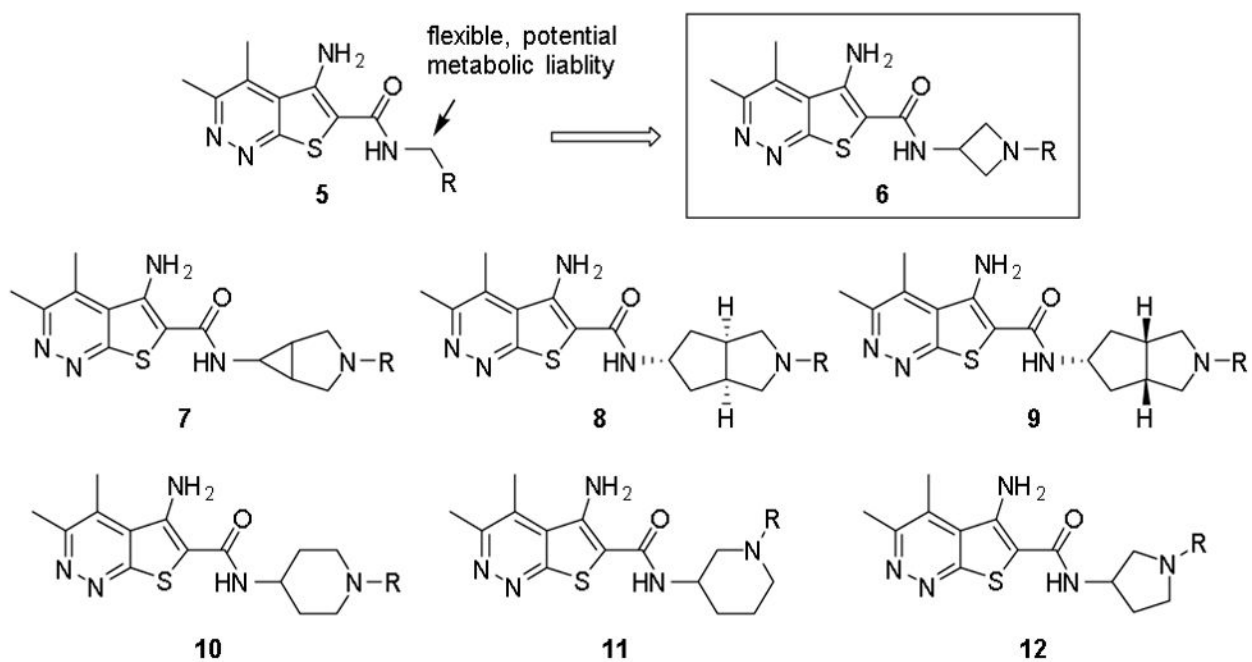
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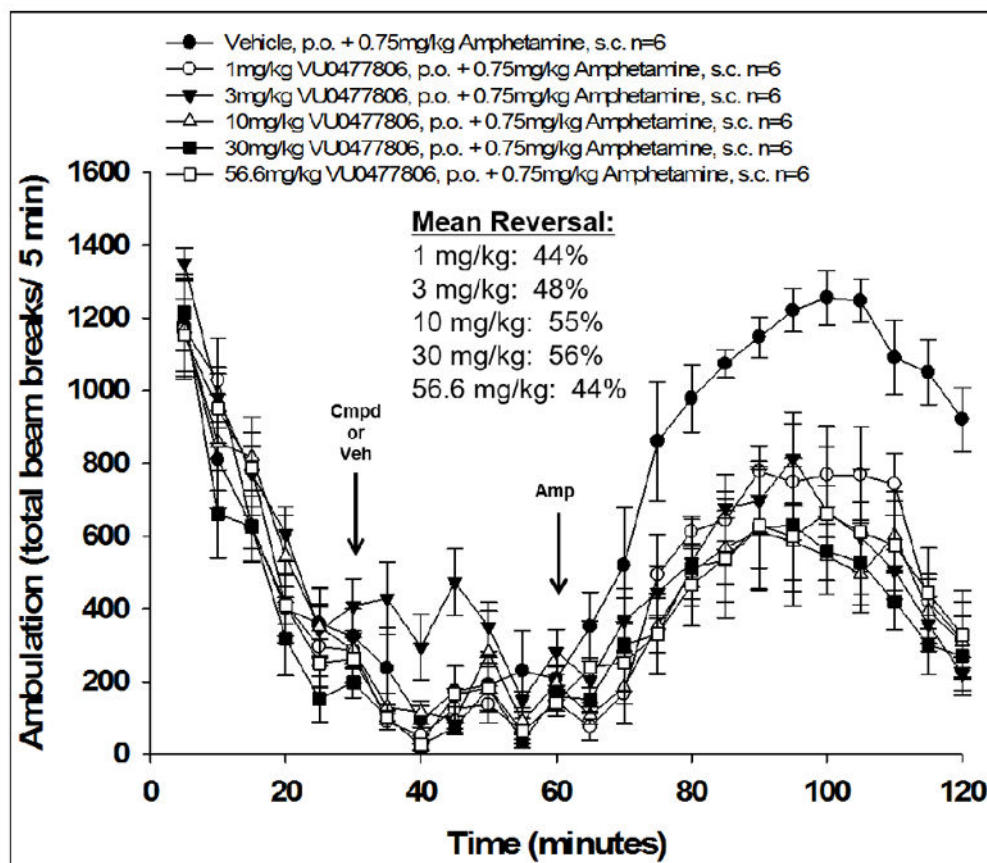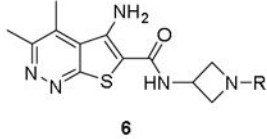Cyclic diamines examined as alternative amide linkers to thieno[2,3-*c*]pyridazine core.

Figure 3.
Reversal of amphetamine-induced hyperlocomotion in rat (male, Sprague Dawley, $n = 6$ per dose group) by **16j** (VU0477806). M_4 PAM or vehicle (10% tween-80 90% water [v/v]) was administered orally 30 min after habituation in the chamber, and then 0.75 mg/kg amphetamine was administered subcutaneously 30 min later (t = 60 min). Total ambulations were measured over the subsequent 1 hr interval (t = 60–120 min) and used to calculate %reversal of AHL for each dose group. Data represent means ± SEM.
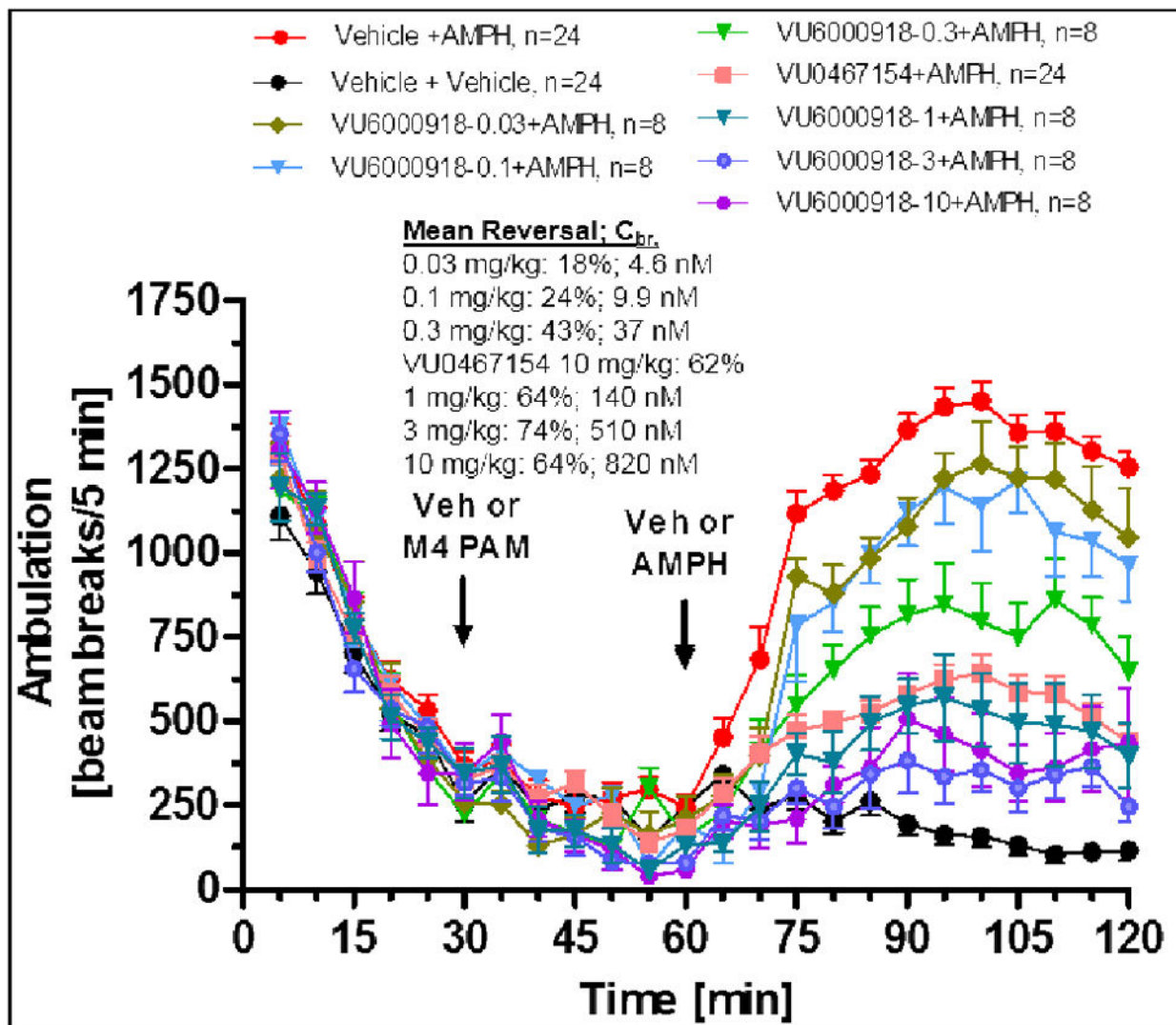
Figure 4.
Reversal of amphetamine-induced hyperlocomotion in rat (male, Sprague Dawley, n = 8–24 per dose group) by **17j** (VU6000918). M_4 PAM, control M_4 PAM (VU0467154) or vehicle (10% tween-80 90% water [v/v]) was administered orally 30 min after habituation in the chamber, and then 0.75 mg/kg amphetamine or vehicle (100% water) was administered subcutaneously 30 min later (t = 60 min). Total ambulations were measured over the subsequent 1 hr interval (t = 60–120 min) and used to calculate % reversal of AHL for each dose group. Data represent means ± SEM.
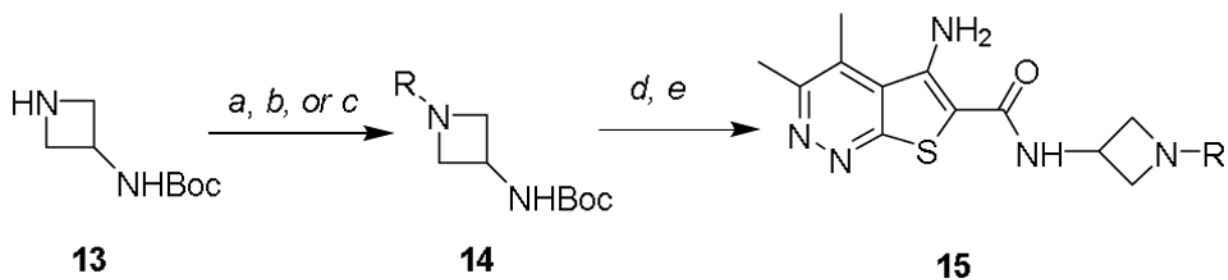
Scheme 1.

Synthesis of M$_4$ PAM analogs **6**, **16**, **17**. Reagents and conditions: (a) R-X, DCM, DIPEA, rt. (b) R-Het-X, Cs$_2$CO$_3$, DMF, heat (c) Ar-X, Pd$_2$(dba)$_3$, *rac*-BINAP, Cs$_2$CO$_3$, toluene, 100 °C (d) TFA, DCM, rt, 3 hr (e) 5-amino-3,4-dimethylthieno[2,3-*c*]pyridazine-6-carboxylic acid, HATU, DIPEA, DMF, 2 hr.
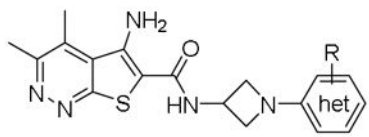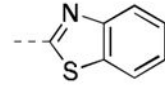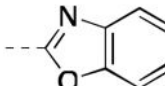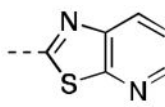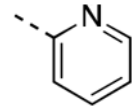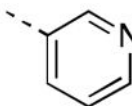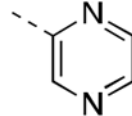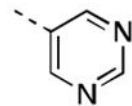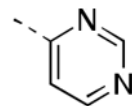
Table 1

Structures and activities for M_4 PAM analogs **6.**

6

Cmpd	R	hM$_4$ EC$_{50}$ (nM)^a [% ACh Max ±SEM]	hM$_4$ pEC$_{50}$ (±SEM)
6a	CO$_2$Bn	30 [81±8]	7.62±0.19
6b	CO$_2$Ph	23 [96±3]	7.65±0.03
6c	CO$_2$(3-Me)Ph	67 [85±9]	7.23±0.17
6d	C(O)NHPh	217 [89±6]	6.66±0.03
6e	SO$_2$Ph	268 [70±8]	6.58±0.07
6f	C(O)Ph	773 [85±6]	6.22±0.25
6g	C(O)2-pyridyl	564 [91±5]	6.25±0.03
6h	C(O)4-pyridyl	179 [85±7]	6.75±0.03

^{a}Calcium mobilization assays with hM4/Gqi5-CHO cells performed in the presence of an EC$_{20}$ fixed concentration of acetylcholine; values represent means from three (n=3) independent experiments performed in triplicate.

Table 2

Structures and activities for M$_4$ PAM analogs **16.**

16

Cmpd	R	hM$_4$ EC$_{50}$ (nM)^a [% ACh Max ±SEM]	hM$_4$ pEC$_{50}$ (±SEM)
16a		439 [87±4]	6.36±0.04
16b		419 [89±6]	6.45±0.19
16c		106 [81±4]	7.04±0.16
16d		124 [81±9]	6.99±0.18
16e		34 [96±5]	7.48±0.09
16f		78 [80±11]	7.13±0.11
16g		41 [89±11]	7.41±0.11
16h		38 [83±10]	7.48±0.17

16
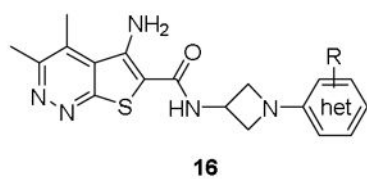
Cmpd	R	hM$_4$ EC$_{50}$ (nM)^a [% ACh Max ±SEM]	hM$_4$ pEC$_{50}$ (±SEM)
16i		29 [82±5]	7.74±0.22
16j		76 [75±6]	7.12±0.08
16k		Inactive	Inactive
16l		17 [88±11]	7.77±0.01
16m		16 [90±7]	7.81±0.03
16n		185 [87±4]	6.80±0.19
16o		51 [86±6]	7.30±0.00
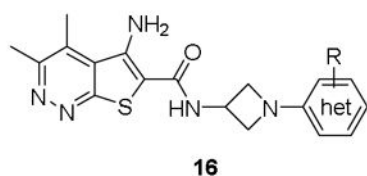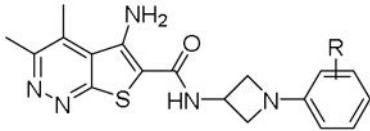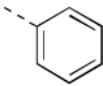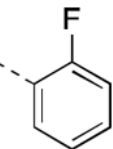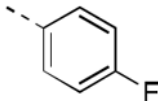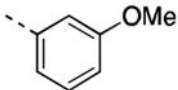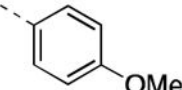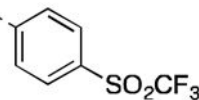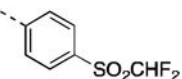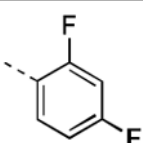
Cmpd	R	hM$_4$ EC$_{50}$ (nM)^a [% ACh Max ±SEM]	hM$_4$ pEC$_{50}$ (±SEM)
16p		8 [86±3]	8.09±0.05
16q		11 [84±5]	7.97±0.09

^{a}Calcium mobilization assays with hM$_4$/Gqi5-CHO cells performed in the presence of an EC$_{20}$ fixed concentration of acetylcholine; values represent means from three (n=3) independent experiments performed in triplicate.

Table 3

Structures and activities for M$_4$ PAM analogs **17.**

17

Cmpd	R	hM$_4$ EC$_{50}$ (nM)^a [% ACh Max ±SEM]	hM$_4$ pEC$_{50}$ (±SEM)
17a		25 [85±8]	7.61±0.08
17b		28 [82±9]	7.56±0.03
17c		282 [77±8]	6.55±0.04
17d		141 [77±11]	6.88±0.11
17e		1289 [81±8]	5.90±0.06
17f		934 [86±1]	6.03±0.04
17g		325 [75±13]	6.63±0.29
17h		37 [94±9]	7.49±0.12

17
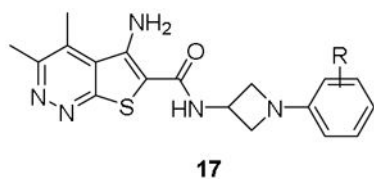
Cmpd	R	hM$_4$ EC$_{50}$ (nM)^a [% ACh Max ±SEM]	hM$_4$ pEC$_{50}$ (±SEM)
17i		30 [84±5]	7.54±0.06
17j		19 [88±5]	7.73±0.06

^{a}Calcium mobilization assays with hM$_4$/Gqi5-CHO cells performed in the presence of an EC$_{20}$ fixed concentration of acetylcholine; values represent means from three (*n*=3) independent experiments performed in triplicate.