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Letter

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Discovery of VU0467485/AZ13713945: An M_4 PAM Evaluated as a Preclinical Candidate for the Treatment of Schizophrenia

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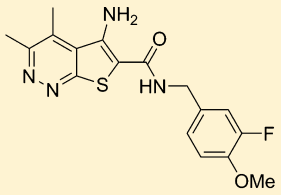
Supporting Information

ABSTRACT: Herein, we report the structure−activity relationships within a series of potent, selective, and orally bioavailable muscarinic acetylcholine receptor 4 (M_4) positive allosteric modulators (PAMs). Compound **6c** (VU0467485) possesses robust *in vitro* M_4 PAM potency across species and *in vivo* efficacy in preclinical models of schizophrenia. Coupled with an attractive DMPK profile and suitable predicted human PK, **6c** (VU0467485) was evaluated as a preclinical development candidate.



6c/VU0467485/AZ13713945

KEYWORDS: *Positive allosteric modulator (PAM), muscarinic acetylcholine receptor 4 (M_4), VU0467485, schizophrenia*

The rapid onset of clinical efficacy of the M_1/M_4 preferring agonist xanomeline in both schizophrenic and Alzheimer's patients led to a revolution in muscarinic receptor drug discovery efforts targeting allosteric mechanisms to afford highly subtype selective M_1 and M_4 positive allosteric modulators (PAMs).[1−6] Of these, M_4 has emerged as the favored mAChR subtype responsible for antipsychotic-like efficacy as well as modest cognitive enhancement in multiple preclinical rodent models via a fundamentally new molecular mechanism.[7−15] While M_1 PAMs with diverse chemotypes (and without major species differences in pharmacology) have rapidly advanced to potentially address cognitive dysfunction and negative symptoms,[5,6,16,17] M_4 PAMs have progressed more slowly for many reasons, including species differences in M_4 PAM potency (i.e., affinity and cooperativity), challenges with respect to M_2 selectivity, and P-gp efflux as well as limited chemical diversity (Figure 1).[9−14,18−20] Clearly, these are significant roadblocks en route to an M_4 PAM preclinical candidate. In this Letter, we detail our navigation of these issues within the VU0467154 series of M_4 PAMs, leading to the discovery of a potent, selective, and orally bioavailable M_4 PAM **6c** (VU0467485) with robust efficacy in behavioral models that was evaluated as a preclinical candidate. This is the first disclosure of the structure−activity relationships (SAR) and preclinical profile of **6c**.

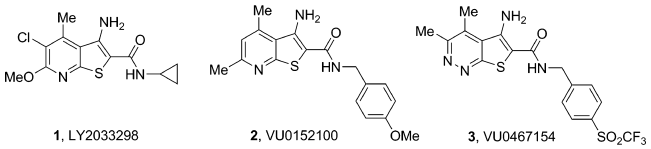


Figure 1. Structures of reported M_4 PAMs. LY2033298 (**1**) was the first reported M_4 PAM but was human-preferring in potency. VU0152100 (**2**) was the first centrally active M_4 PAM in rodents, and VU0467154 (**3**) has proven to be the best-in-class M_4 PAM rodent *in vivo* tool compound.

Our discovery of **6c** originated from an optimization campaign centered on **2**, and from which **3** was identified, but discontinued due to a human and rat M_4 PAM potency disconnect that precluded development.[10,12−14] A major thrust of the initial lead optimization effort was to survey alternatives for the pyridine ring in **2**, and while other pyridine regioisomers and pyrimidines afforded modest M_4 potentiation, the pyridazine core, found in **3**, proved optimal for imparting both potency, metabolic stability, and favorable physiochemical properties to the series.[21] SAR was steep and divergent across

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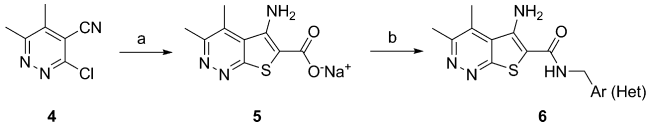
rat and human M_4, and CNS penetration was low in this series; however, exceptional DMPK properties could be achieved.[21]

The synthesis of analogue **6** was straightforward and required only two steps from known materials (Scheme 1).[12−14]

Scheme 1. Synthesis of Analogues 6[a]



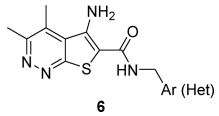
[a]Reagents and conditions: (a) methyl thioglycolate, MeOH, 1 M aq. NaOH, 150 °C, microwave, 30 min, 78%; (b) NH_2CH_2Ar(Het), HATU, DMF, DIEPA, 2 h, 45−92%.

Condensation of 3-chloro-5,6-dimethylpyridazin-4-carbonitrile **4** with methylthioglycolate under microwave irradiation delivered the core sodium 5-amino-3,4-dimethylthieno[2,3-*c*]pyridazine-6-carboxylate **5** in yields averaging 78%. Next, a HATU-mediated amide coupling reaction with various amines provides analogues **6** in yields ranging from 45 to 92% after HPLC purification.[21] The direct congener of **2**, VU0464090 (**6a**), demonstrated a ∼3-fold increase in M_4 PAM potency (human M_4 PAM EC_{50} = 130 nM, pEC_{50} = 6.89 ± 0.06, 83.7 ± 3.5 ACh Max and rat M_4 PAM EC_{50} = 59.7 nM, pEC_{50} = 7.22 ± 0.06, 78.1 ± 1.7 ACh Max), increased fraction unbound in plasma (fu_{plasma}(r,h) = 0.022, 0.035) and reduced hepatic microsomal intrinsic clearance (CL_{int}(r,h) = 81 and 36 mL/min/kg; predicted CL_{hep}(r,h) = 37 and 13 mL/min/kg) by virtue of the pyridazine ring system; however, metabolic instability of the PMB group precluded further advancement of **6a**.[14] As shown in Table 1, application of the fluorine walk strategy[22−24] for allosteric modulator optimization proved fruitful, affording a number of potent M_4 PAMs (**6c−h**). Heteroatom incorporation into the benzyl ring was met with limited success, with only 3-pyridyl congeners (**6i** and **6j**) retaining PAM activity (other regioisomeric pyridines, pyrimidines, pyrazines, and pyridazines were inactive, M_4 EC_{50}s > 10 μM). Moreover, we suspected CYP_{450}-mediated oxidative dealkylation of **6a** led to **6b**, an active putative metabolite (human M_4 PAM EC_{50} = 96.7 nM, pEC_{50} = 7.01 ± 0.01, 69.8 ± 2.2 ACh Max), but with high clearance *in vivo* (rat) and undesired activity at hM_2. As a battery of *in vitro* and *in vivo* DMPK assays (*vide infra*) quickly identified **6c** as the most attractive PAM in the series, we prepared its putative dealkylated metabolite **6k**, which also proved to be active (human M_4 EC_{50} = 59 nM), but with no activity at hM_2, suggesting the *ortho*-fluoro moiety enhances selectivity versus hM_2. Incorporation of chiral methyl groups at the benzylic position of **6c** led to (*R*)-**6l** (human M_4 EC_{50} > 10 μM) and (*S*)-**6m** (human M_4 EC_{50} = 508 nM), where enantiospecific activity was noted, but with unacceptable loss in human M_4 PAM potency. Finally, based on previous beneficial disposition by virtue of the kinetic isotope effect, we evaluated a deuterated congener, but in this instance, there was no benefit to stability.

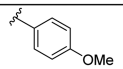
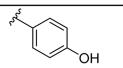
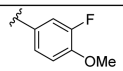
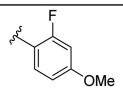
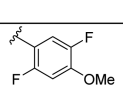
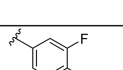
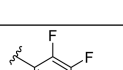
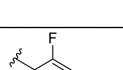
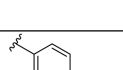
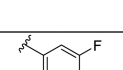
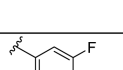
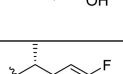
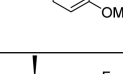
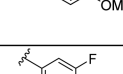
Thus, efforts focused on **6c** as a potential M_4 PAM preclinical candidate with minimal species differences in PAM potency.

The molecular pharmacology profile of M_4 PAM **6c** is shown in Figure 2. PAM **6c** is inactive in the absence of acetylcholine (ACh), but in the presence of an EC_{20} concentration of ACh (Figure 2A), **6c** potentiates M_4 in a concentration-dependent manner, affording potent activity at both human and rat M_4 (human M_4 PAM EC_{50} = 78.8 nM, pEC_{50} = 7.10 ± 0.01, 80.6 ±

Table 1. Structures and Activities of Analogues 6



6

Entry	Ar (Het)	hM_4 EC_{50} (nM)[a] [% ACh Max ±SEM]	hM_4 pEC_{50} (±SEM)
6a		130 [83.7±3.5]	6.89±0.06
6b		96.7 [69.8±2.2]	7.01±0.08
6c		78.8 [80.6±0.7]	7.10±0.01
6d		90.6 [75.5±2.4]	7.04±0.14
6e		41.4 [68.5±1.4]	7.38±0.05
6f		84.1 [75.3±1.1]	7.07±0.03
6g		43.4 [71.2±3.6]	7.36±0.02
6h		100 [87.1±1.9]	6.99±0.06
6i		239 [76.9±2.8]	6.62±0.11
6j		142 [80.8±1.5]	6.85±0.08
6k		59.1 [83.1±2.9]	7.22±0.07
6l		>10	>5.0
6m		508 [62.4±2.5]	6.29±0.09
6n		55.7 [87.1±0.8]	7.25±0.04

[a]Calcium mobilization assays with hM_4/G_{qi5}-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.

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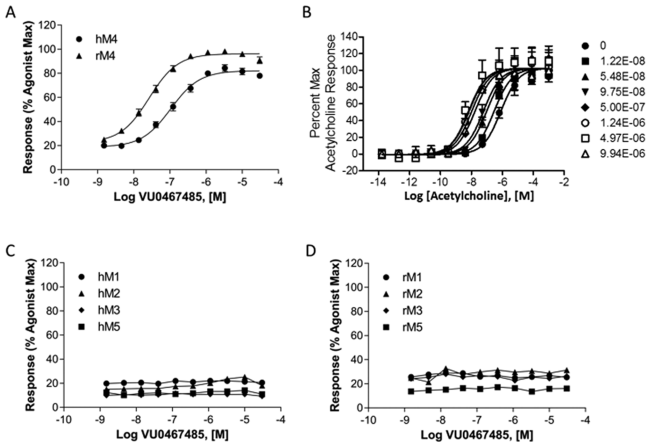

Figure 2. Molecular pharmacology profile of M_4 PAM **6c**. (A) Enhanced intracellular calcium release induced by a subthreshold concentration of acetylcholine (EC_{20}), a PAM CRC on both rat and human M_4, with EC_{50}s of 26.6 and 78.8 nM, respectively. (B) Compound **6c** induces a ∼45-fold maximal leftward shift of the hM_4 acetylcholine response curve at 10 μM. (C) Compound **6c** is highly selective for hM_4 over $hM_{1-3,5}$. (D) Compound **6c** is highly selective for rM_4 over $rM_{1-3,5}$. Data represent means from at least three independent determinations performed in triplicate using CHO cells stably transfected with the indicated mAChR.

0.7 ACh Max and rat M_4 PAM EC_{50} = 26.6 nM, pEC_{50} = 7.57 ± 0.05, 68.7 ± 3.4 ACh Max). For a preclinical candidate, comparable activity across species is essential for IND-enabling toxicology studies, and this was a major issue for previously reported M_4 PAMs for which the species disconnect averaged 10−50-fold.[9−14,18−20] Thus, we also evaluated **6c** against dog and cynomolgous monkey M_4 and the key antitarget, M_2. Beyond rat and human, **6c** displayed no major species differences in potency (dog M_4 EC_{50} = 87 nM, 49% ACh Max, dog M_2 > 30 μM and cyno M_4 EC_{50} = 102 nM, 74% ACh Max, cyno M_2 > 30 μM). In a progressive fold-shift assay with human M_4 (Figure 2B), **6c** afforded a maximal ∼45-fold leftward shift of the human M_4 ACh concentration−response curve (CRC) at 10 μM (∼40-fold shift at rat M_4). Moreover, PAM **6c** was selective versus human and rat $M_{1-3,5}$. The operational model of allosterism was applied to the fold-shift data to better understand the allosteric effects of this promising candidate.[5,22] PAM **6c** displayed robust potentiation of ACh and cooperativity (log $\alpha\beta$ = 2.1, $\alpha\beta$ = 134), significant intrinsic efficacy (log τ_B = 5.1), and an approximately 1 μM estimated affinity (pK_B 6.025, K_B = 944 nM) at human M_4.

Compound **6c** was then evaluated in a panel of *in vitro* DMPK assays[23] where it displayed properties that supported continued progression. Not only did **6c** possess an exceptionally clean CYP_{450} inhibition (3A4, 2D6, 2C9, 1A2 IC_{50}s > 30 μM in human hepatic microsomes) and induction profile (3A4, 1A2, 2B6 EC_{50}s > 50 μM, E_{max}s ≤ 1.0 in cryopreserved human hepatocytes), but also moderate plasma protein binding was noted across species (fu_{plasma}(r,h,c) = 0.031, 0.054, and 0.091) with moderate fraction unbound in rat brain homogenate (fu_{br} = 0.037). Based on hepatic microsomal CL_{int} data, moderate predicted hepatic clearance was also observed for **6c** (predicted CL_{hep}(r,h,c) = 73, 26, and 38 mL/min/kg). Moreover, **6c** was not a P-gp substrate (ER = 1.4 in MDCK-MDR1) and showed good apparent permeability (Caco-2 P_{app} = 31 × 10^{-6} cm/s). In rat and dog brain distribution studies, **6c** displayed moderate to high CNS penetration with K_ps of 0.31 to 1.0 and $K_{p,uu}$s of 0.37 to 0.84. With regard to toxicity evaluation *in vitro*, **6c** was clean in GSH trapping experiments (human hepatic microsomes) and a mini-Ames test (data not shown). *In vivo* PK (Table 2)

Table 2. Pharmacokinetic Parameters of 6c

parameter	rat[a] (SD)	dog[a] (beagle or mongrel)	NHP[a] (cyno)
dose (mg/kg) i.v./p.o.	1/3	1/3	0.2 (iv only)
CL_p (mL/min/kg)	29	21	25
V_{ss} (L/kg)	1.5	1.5	0.88
elimination $t_{1/2}$ (h)	4.2	1.3	0.48
C_{max} (μM) p.o.	1.2	0.22	
T_{max} (h) p.o.	1.5	0.75	
$AUC_{o\text{-}inf}$ (μM·h) p.o.	3.8	0.59	
F (%) p.o.	79	9.5	
total brain/total plasma (K_p)	0.31	1.0	
unbound brain/unbound plasma ($K_{p,uu}$)	0.37	0.84	

[a]Values represent means from two to three animals.

was assessed in three species (rat, dog, and cynomolgous monkey), which revealed moderate clearance, low to moderate volume of distribution at steady-state, and short to moderate elimination half-life with high oral bioavailability in rat but low bioavailability in dog (3 mg/kg suspension dose of a mono-HCl salt). *In vitro* metabolite identification experiments found good coverage of human metabolites in dog and cynomolgous monkey and no evidence for human-unique metabolites (Figure 3). Human CYP_{450} phenotyping experiments revealed

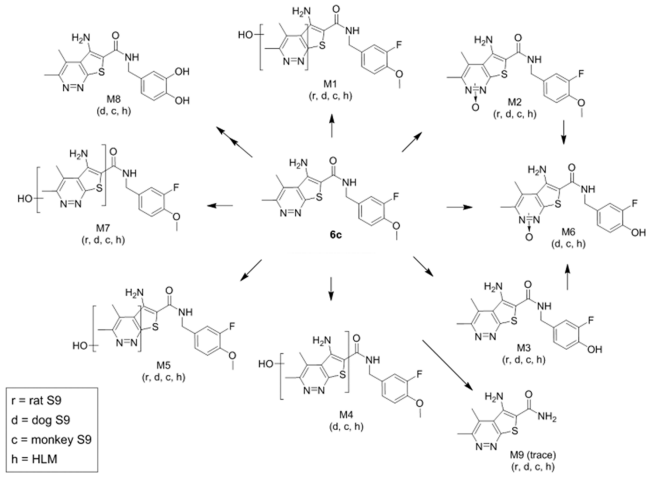

Figure 3. Metabolite identification studies for **6c** across species (rat, dog, cyno monkey, and human).

that multiple CYPs contribute to **6c**'s metabolism (Figure 4) with a generally low potential for drug−drug interactions (no metabolism-related DDI liabilities anticipated in Alzheimer's disease clinical population as concomitant medications (acetylcholinesterase inhibitors possess a 3A4/2D6 phenotype) and, in schizophrenia populations, common antipsychotics (e.g., clozapineolanzapine) possess a 1A2/2D6/3A4 phenotype). Furthermore, in a functional hERG assay, **6c** was inactive when tested at 11 μM, as well as against a larger cardiac ion channel panel (IC_{50}s > 33 μM). Finally, ancillary pharmacology was assessed in an internal AZ/Cerep panel against 200 targets, and no significant off-target activities (IC_{50}s or EC_{50}s > 10 μM)

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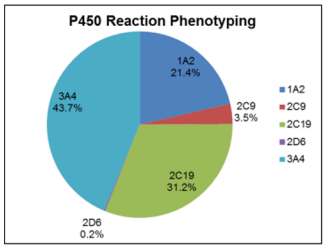

CYP Mapping with Recombinant P450s:

	1A2	2C9	2C19	2D6	3A4
M1	√	√	√	√	√
M2	√	√	√	√	√
M3	√	√	√	√	√
M4	√	√	√	-	√
M5	√	-	-	√	√
M6	√	-	-	√	-
M7	-	-	-	√	√
M8	-	-	-	-	√
M9	√	-	-	-	√

Figure 4. P450 phenotyping and CYP mapping for **6c**, indicating that multiple CYP_{450}s (3A4/2C19/1A2 and to a lesser extent, 2C9) catalyze biotransformation.

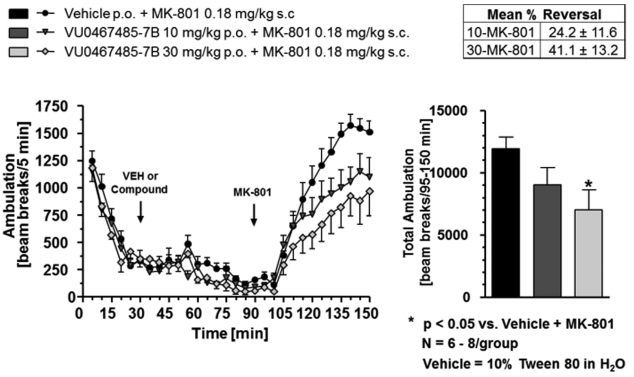

Figure 6. Compound **6c** has antipsychotic-like activity in an MK-801 hyperlocomotion rat model. Compound **6c** dose-dependently (10−30 mg/kg, po) reverses MK-801-induced hyperlocomtion (MK-801, 0.18 mg/kg, s.c., *p < 0.05 vs vehicle + MK-801. N = 6−8 rats /group.

were noted, including both binding and functional assays for cardiac ion channels, with the exception of a 1.2 μM IC_{50} (radioligand binding) at the rat $GABA_A$ receptor.[21]

Evaluation in a rat amphetamine-induced hyperlocomotion (AHL) study, a traditional preclinical model of antipsychotic activity,[10,12−14] revealed that **6c** (Figure 5) showed robust activity with a minimum effective dose (MED) of 10 mg/kg p.o. (43.2% reversal) that correlates with terminal (time = 1.5 h) plasma and brain concentrations of 1.8 μM (0.06 μM unbound) and 0.56 μM (0.02 μM unbound), respectively, and showing greater efficacy than our previously reported rat tool M_4 PAM, VU0467154.[13,14] Beyond hyperdopaminergic states, we also assessed the ability of **6c** to reverse hyperlocomotion induced by the *N*-Methyl-D-Aspartate (NMDA) receptor antagonist MK-801 (Figure 6) to pharmacologically model NMDA receptor hypofunction and the associated prefrontal cortex-mediate impairments.[13,24,25] Here as well, **6c** displayed a dose-responsive reversal of MK-801-induced hyperlocomotion, with an MED of 30 mg/kg p.o. and a 41.1% reversal.

Based on the fact that **6c** was the first M_4 PAM discovered in our program with similar M_4 PAM activity across all preclinical species and man, an attractive DMPK and ancillary pharmacology profile, as well as robust efficacy in a rodent model of antipsychotic activity, **6c** was further profiled as a putative preclinical candidate, including evaluation in a rat modified Irwin test for effects on autonomic and somatomotor functions. In this study, following a single high dose (56.6 mg/kg, p.o., N = 6), no significant effects were observed over a 6 h observation period. However, based on these animal model acute concentration-effect data (rat AHL) and **6c**'s predicted human PK profile (Table 3), efficacious human oral doses projected to provide 12-h daily coverage of the target/mechanism were undesirably high and frequent (e.g., >450 mg, TID).

Table 3. Predicted Human Pharmacokinetics of 6c

parameter	value
CL (mL/min/kg)[a]	3.7−8.9
V_{ss} (L/kg)[b]	1.5−2.1
$t_{1/2}$ (h)	1.9−6.6
F (%)[c]	71
k_a (1/h)[d]	0.55

[a]Predicted by multispecies IVIVE. [b]Predicted by scaling of unbound V_{ss} from rat and dog. [c]Predicted by assumption of an optimized form/formulation providing an f_{abs} of 1.0 with an f_{gut} of 1.0 and f_{hep} of 0.71 (i.e., ER_{hep} = 0.29 based on mean predicted human CL/Q_{hep}). [d]Predicted by the MAT method using rat oral PK data from a 3 mg/kg dose of the mono-HCl salt formulated as a suspension in 0.1% tween80 and 0.5% methylcellulose in water.

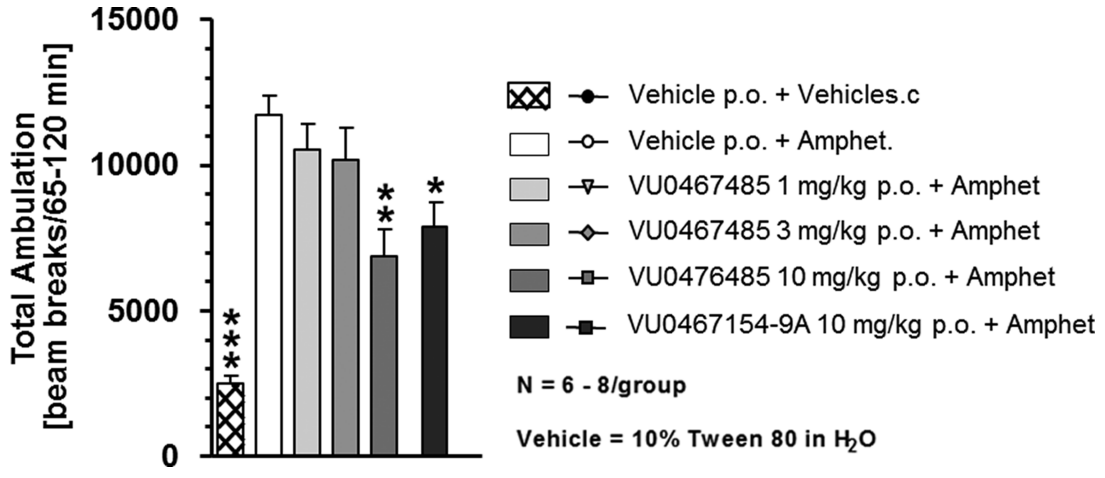

Figure 5. Compound **6c** has antipsychotic-like activity in an AHL rat model. Compound **6c** dose-dependently (1−10 mg/kg, po) reverses AHL (amphetamine, 0.75 mg/kg, s.c., *p < 0.05 vs vehicle + amphetamine, **p < 0.01 vs vehicle + amphetamine, ***p < 0.001 vs vehicle + amphetamine). N = 6−8 rats/group.

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Additionally, **6c** displayed low aqueous solubility (2.4 μM at pH 7.4) and evidence for solubility-limited absorption in dog was observed, even at low doses and when administered as an HCl salt.

Still, **6c** represents a major advance in the field, as the first potent M_4 PAM to overcome major species differences in potency while maintaining high selectivity versus M_2 (rat, dog, cyno, and human EC_{50}s > 30 μM), CNS penetration, and *in vivo* efficacy. However, given the projected human efficacious dosing and solubility issues, together with an anticipated challenge in achieving sufficiently high oral exposure in IND-enabling safety studies to establish acceptable margins, further advancement of **6c** was halted (pending pharmaceutical sciences work), and optimization efforts shifted toward improvement of aqueous solubility and longer predicted human $t_{1/2}$ while retaining all the desirable properties of **6c**. Results from this ongoing work will be reported in due course.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.6b00461.

General methods for the synthesis and characterization of all compounds, and methods for the *in vitro* and *in vivo* DMPK protocols and supplemental tables(PDF)

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Notes

The authors declare the following competing financial interest(s): The authors are developing M4 PAMs for the treatment of schizophrenia and hold patents on the same.

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■ ABBREVIATIONS

DCM, dichloromethane; AHL, amphetamine-induced hyperlocomotion; MED, minimum effective dose; mAChR, muscarinic acetylcholine receptor; PAM, positive allosteric modulator; HATU, *O*-(7-azabenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; DIEPA, *N*,*N*-diisopropylamine; MED, minimum effective dose; AHL, amphetamine-induced hyperlocomotion

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