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Discovery of VU0467485/AZ13713945: An M₄ PAM Evaluated as a Preclinical Candidate for the Treatment of Schizophrenia

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ABSTRACT: Herein, we report the structure-activity relationships within a series of potent, selective and orally bioavailable muscarinic acetylcholine receptor 4 (M₄) positive allosteric modulators (PAMs). **6c** (VU0467485) possesses robust *in vitro* M₄ 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.

The rapid onset of clinical efficacy of the M₁/M₄ 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 M₁ and M₄ positive allosteric modulators (PAMs).¹⁻⁶ Of these, M₄ 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 M₁ 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} M₄ PAMs have progressed more slowly for many reasons, including species differences in M₄ PAM potency (i.e., affinity and cooperativity), challenges with respect to M₂ 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 M₄ PAM preclinical candidate. In this Letter, we detail our navigation of these issues within the VU0467154 series of M₄ PAMs, leading to the discovery of a potent, selective and orally bioavailable M₄ 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**.

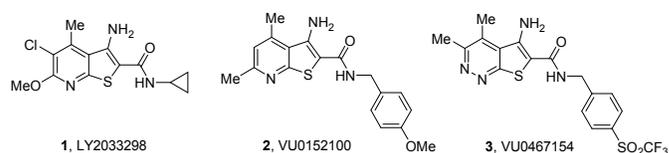


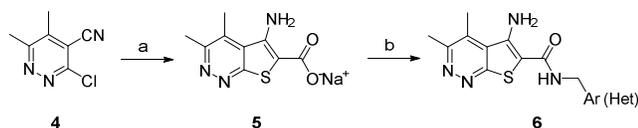
Figure 1. Structures of reported M₄ PAMs. LY2033298 (**1**) was the first reported M₄ PAM, but was human-preferring in potency. VU0152100 (**2**) was the first centrally active M₄ PAM in rodents, and VU0467154 (**3**) has proven to be the best-in-class M₄ PAM rodent *in vivo* tool compound.

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 M₄ 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 M₄ potentiation, the pyridazine core, found in **3**, proved optimal for imparting both potency, metabolic stability, and favorable physicochemical properties to the series²¹. SAR was steep and divergent across rat and human M₄, and CNS penetration was low in this series; however, exceptional DMPK properties could be achieved²¹.

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

Condensation of 3-chloro-5,6-dimethylpyridazin-4-

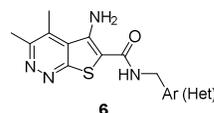
Scheme 1. Synthesis of Analogues **6**^a



^aReagents and conditions: (a) Methyl thioglycolate, MeOH, 1M aq. NaOH, 150 °C, microwave, 30 min, 78%; (b) NH₂CH₂Ar(Het), HATU, DMF, DIEPA, 2 h, 45-92%.

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 45-92% after HPLC purification.²¹ The direct congener of **2**, VU0464090 (**6a**), demonstrated an ~3-fold increase in M₄ PAM potency (human M₄ PAM EC₅₀ = 130 nM, pEC₅₀ = 6.89±0.06, 83.7±3.5 ACh Max and rat M₄ PAM EC₅₀ = 59.7 nM, pEC₅₀ = 7.22±0.06, 78.1±1.7 ACh Max), increased fraction unbound in plasma (*f*_{u,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^{22,23,24} for allosteric modulator optimization proved fruitful, affording a number of potent M₄ 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, M₄ EC₅₀s >10 μM). Moreover, we suspected CYP₄₅₀-mediated oxidative dealkylation of **6a** led to **6b**, an active putative metabolite (human M₄ PAM EC₅₀ = 96.7 nM, pEC₅₀ = 7.01±0.01, 69.8±2.2 ACh Max), but with high clearance *in vivo* (rat) and undesired activity at hM₂. 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 M₄ EC₅₀ = 59 nM), but with no activity at hM₂, suggesting the ortho-fluoro moiety enhances selectivity versus hM₂. Incorporation of chiral methyl groups at the benzylic position of **6c** led to (*R*)-**6l** (human M₄ EC₅₀ >10 μM) and (*S*)-**6m** (human M₄ EC₅₀ = 508 nM), where enantiospecific activity was noted, but with unacceptable loss in human M₄ PAM

Table 1. Structures and Activities of Analogues **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
6o		213 ^b [60.9]	6.72 ^b

^aCalcium mobilization assays with hM₄/G_{q15}-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 unless otherwise noted. ^bvalues from one experiment performed in triplicate.

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 M₄ PAM preclinical candidate with minimal species differences in PAM potency.

The molecular pharmacology profile of M₄ PAM **6c** is shown in Figure 2. PAM **6c** is inactive in the absence of acetylcholine (ACh), but in the presence of an EC₂₀ concentration of ACh (Figure 2A), **6c** potentiates M₄ in a concentration-dependent manner, affording potent activity at both human and rat M₄ (human M₄ PAM EC₅₀ = 78.8 nM, pEC₅₀ = 7.10±0.01, 80.6±0.7 ACh Max and rat M₄ PAM EC₅₀ = 26.6 nM, pEC₅₀ = 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 M₄ PAMs for which the species disconnect averaged 10-50-fold.^{9-14,18-20} Thus, we also evaluated **6c** against dog and cynomolgous monkey M₄ and the key anti-target, M₂. Beyond rat and human, **6c** displayed no major species differences in potency (dog M₄ EC₅₀ = 87 nM, 49% ACh Max, dog M₂ >30 μM and cyno M₄ EC₅₀ = 102 nM, 74% ACh Max, cyno M₂ >30 μM). In a progressive fold-shift assay with human M₄ (Figure 2B), **6c** afforded a maximal ~45-fold leftward shift of the human M₄ ACh concentration-response curve (CRC) at 10 μM (~40-fold shift at rat M₄). Moreover, PAM **6c** was selective versus human and rat M_{1-3,5}. The operational model of allosterism was applied to

rM₄ 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.

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 αβ = 2.1, αβ = 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 M₄.

6c was then evaluated in a panel of *in vitro* DMPK assays²³ where it displayed properties that supported continued progression. Not only did **6c** possess an exceptionally clean CYP₄₅₀ inhibition (3A4, 2D6, 2C9, 1A2 IC₅₀s >30 μM in human hepatic microsomes) and induction profile (3A4, 1A2, 2B6 EC₅₀s >50 μM, E_{max}s ≤ 1.0 in cryopreserved human hepatocytes) profile, but also moderate plasma protein binding was noted across species (f_{u,plasma} r, h, c = 0.031, 0.054 and 0.091) with moderate fraction unbound in rat brain homogenate (f_{u,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 x 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) 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

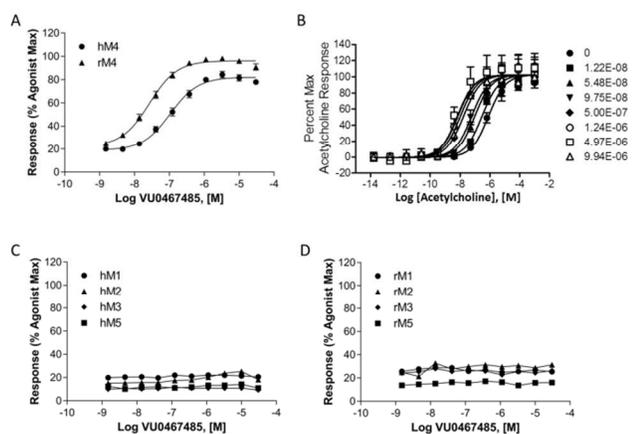


Figure 2. Molecular pharmacology profile of M₄ PAM **6c**. (A) Enhanced intracellular calcium release induced by a subthreshold concentration of acetylcholine (EC₂₀), a PAM CRC on both rat and human M₄, with EC₅₀s of 26.6 nM and 78.8 nM, respectively. (B) **6c** induces an ~45-fold maximal leftward shift of the hM₄ acetylcholine response curve at 10 μM. (C) **6c** is highly selective for hM₄ over hM_{1-3,5}. (D) **6c** is highly selective for

Table 2. Pharmacokinetic Parameters of 6c

Parameter	Rat ^a (SD)	Dog ^a (beagle or mongrel)	NHP ^a (cyno)
Dose (mg/kg) iv/po	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) po	1.2	0.22	-
T _{max} (h) po	1.5	0.75	-
AUC _{0-inf} (μM*hr) po	3.8	0.59	-
F (%) po	79	9.5	-

Total brain:total plasma (K_p)	0.31	1.0	-
Unbound	0.37	0.84	-
brain:unboundplasma ($K_{p,uu}$)			

^aValues represent means from two to three animals.

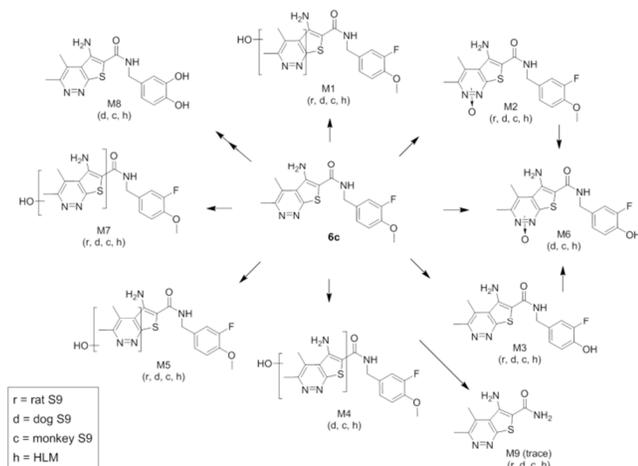


Figure 3. Metabolite identification studies for **6c** across species (rat, dog, cyno monkey and human).

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 CYP₄₅₀ phenotyping experiments revealed 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., clozapine;olanzapine) possess a 1A2/2D6/3A4 phenotype). Furthermore, in a functional hERG assay, **6c** was inactive when tested at 11 μ M, as well as against a larger cardiac ion channel panel (IC_{50} s > 33 μ M). 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 μ M) were noted, including both binding and functional assays for cardiac ion channels, with the exception of a 1.2 μ M IC_{50} (radioligand binding) at the rat GABA_A receptor.²¹

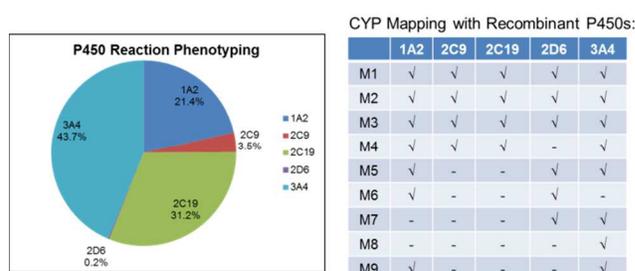


Figure 4. P450 phenotyping and CYP mapping for **6c**, indicating that multiple CYP₄₅₀s (3A4/2C19/1A2 and to a lesser extent, 2C9) catalyze biotransformation.

Evaluation in a rat amphetamine-induced hyperlocomotion (AHL) study, a traditional preclinical model of antipsychotic activity,^{10,12-14} revealed that **6c** (**Figure 3**) 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 hr) plasma and brain concentrations of 1.8 μ M (0.06 μ M unbound) and 0.56 μ M (0.02 μ M unbound), respectively, and showing greater efficacy than our previously reported rat tool M₄ PAM, VU0467154.^{13,14}

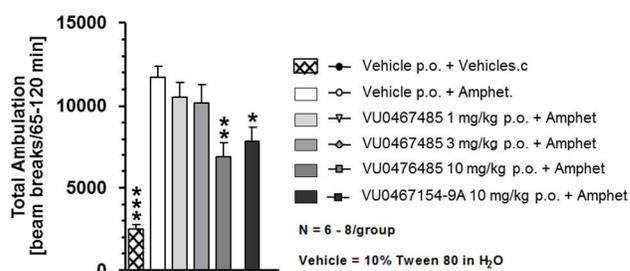


Figure 5. **6c** has antipsychotic-like activity in an AHL rat model. **6c** dose-dependently (1-10 mg/kg, po) reverses AHL (amphetamine, 0.75 mg/kg, s.c., *p < 0.05 vs. vehicle + amphetamine, ** p < 0.01 vs. vehicle + amphetamine, *** p < 0.001 vs. vehicle + amphetamine. N = 6-8rats/group. Vehicle = 10% Tween 80 in H₂O

Based on the fact that **6c** was the first M₄ PAM discovered in our program with similar M₄ PAM activity across all pre-clinical 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 six-hour 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-hour 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} (hr)	1.9 – 6.6
F (%) ^c	71
k _a (1/hr) ^d	0.55

^aPredicted by multi-species IVIVE; ^bpredicted by scaling of unbound V_{ss} from rat and dog; ^cpredicted 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}; ^dpredicted 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 0.5% methylcellulose in water.

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 M₄ PAM to overcome major species differences in potency while maintaining high selectivity versus M₂ (rat, dog, cyno and human EC_{50s} >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.

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Author Contributions

CWL, TMB, MRW, PJC, CMN, NJB, MED and CJK drafted/corrected the manuscript. BJM, KDN, MSP, MAH and DWE performed the chemical synthesis. CWL, MWR, TMB, PJC, CMN, CJK, MJN, NJB, MWW, MED and ALR oversaw the target selection and interpreted the biological data. MJN, ALR, AL, VBL and RLW performed the *in vitro* molecular pharmacology studies. TMB, ALB and SC performed the *in vitro* and *in vivo* DMPK studies. CKJ and MB performed the *in vivo* experiments. All authors have given approval to the final version of the manuscript.

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ASSOCIATED CONTENT

Supporting Information. General methods for the synthesis and characterization of all compounds, and methods for the *in vitro* and *in vivo* DMPK protocols and supplemental tables. This material is available free of charge via the Internet at <http://pubs.acs.org>.

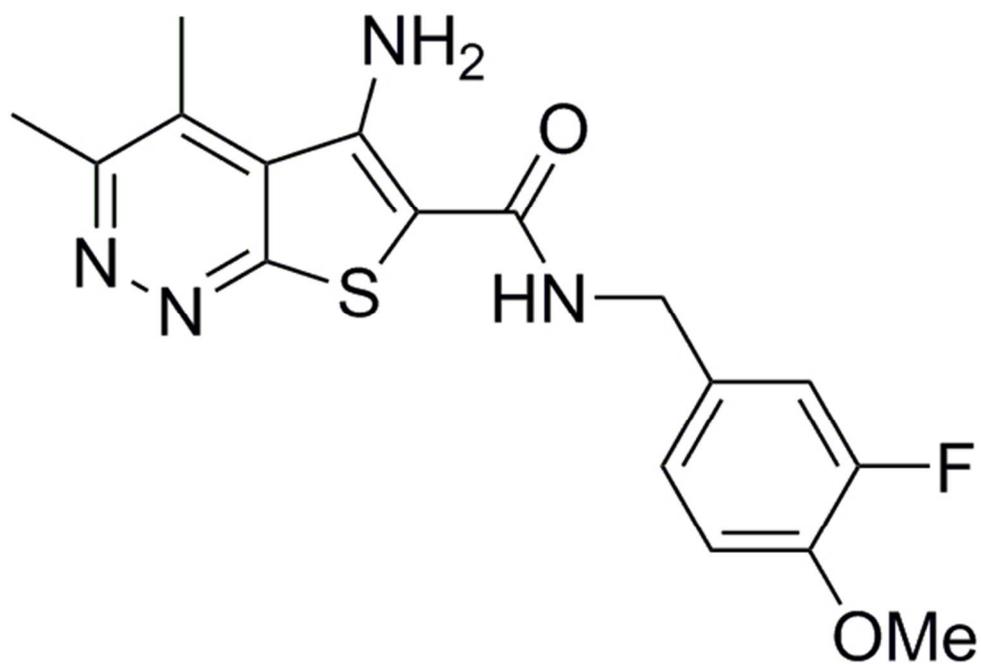
ABBREVIATIONS

DCM, dichloromethane; AHL, amphetamine-induced hyperlocomotion; MED, minimum effective dose; muscarinic acetylcholine receptor, (mAChR); 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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