Published in final edited form as:

ACS Chem Neurosci. 2018 July 18; 9(7): 1572–1581. doi:10.1021/acscchemneuro.8b00126.

Discovery and Optimization of Potent and CNS Penetrant M₅-Preferring Positive Allosteric Modulators Derived from a Novel, Chiral N-(Indanyl)piperidine Amide Scaffold

Aaron M. Bender^{#†,||}, Hyekyung P. Cho^{#†,||}, Kellie D. Nance^{‡,||}, Kaelyn S. Lingenfelter^{||}, Vincent B. Luscombe^{||}, Patrick R. Gentry^{||}, Karl Voigtritter^{†,||}, Alice E. Berizzi[#], Patrick M. Sexton[#], Christopher J. Langmead[#], Arthur Christopoulos[#], Charles W. Locuson^{†,||}, Thomas M. Bridges^{†,||}, Sichen Chang^{†,||}, Jordan C. O'Neill^{†,||}, Xiaoyan Zhan^{†,||}, Colleen M. Niswender^{†,||,⊥}, Carrie K. Jones^{†,||}, P. Jeffrey Conn^{†,||,⊥}, and Craig W. Lindsley^{*†,‡,§,||}

[†]Department of Pharmacology, Vanderbilt University, Nashville, Tennessee 37232-6600, United States

[‡]Department of Chemistry, Vanderbilt University, Nashville, Tennessee 37232-6600, United States

[§]Department of Biochemistry, Vanderbilt University, Nashville, Tennessee 37232-6600, United States

^{||}Vanderbilt Center for Neuroscience Drug Discovery, Vanderbilt University, Nashville, Tennessee 37232-6600, United States

[⊥]Vanderbilt Kennedy Center, Vanderbilt University, Nashville, Tennessee 37232-6600, United States

[#]Drug Discovery Biology, Monash Institute of Pharmaceutical Sciences, Monash University, Parkville, Victoria 3052, Australia

[#] These authors contributed equally to this work.

Abstract

***Corresponding Author:** craig.lindsley@vanderbilt.edu. Phone: 615-322-8700. Fax: 615-343-6532. .

Author Contributions

C.W.Lindsley, P.J.Conn, C.M.Niswender, and P.M.Sexton, C.J.Langmead and A.Christopoulou, and C.W.Locuson and T.M.Bridges oversaw and designed the chemistry, molecular pharmacology, and DMPK, respectively. A.M.Bender, P.R.Gentry, and K.Voigtritter performed synthetic and medicinal chemistry and scaled-up key compounds for *in vivo* studies. H.P.Cho designed and executed advanced molecular pharmacology assays. K.D.Nance, P.R.Gentry, A.E.Berizzi, and K.S.Lingenfelter performed molecular pharmacology assays. C.W.Locuson, T.M.Bridges, X.Zhan, J.C.O'Neill, and S.Chang conducted the DMPK assays and studies. C.W.Lindsley and P.J.Conn wrote the manuscript.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscchemneuro.8b00126. Compound characterization and additional methods, rat M₁–M₅ selectivity for three pan-G_q PAMs, and [³H]NMS binding at human M₅ and M₃ (PDF)

The authors declare no competing financial interest.

The pharmacology of the M_5 muscarinic acetylcholine receptor (mAChR) is the least understood of the five mAChR subtypes due to a historic lack of selective small molecule tools. To address this shortcoming, we have continued the optimization effort around the prototypical M_5 positive allosteric modulator (PAM) ML380 and have discovered and optimized a new series of M_5 PAMs based on a chiral *N*-(indanyl)piperidine amide core with robust SAR, human and rat M_5 PAM EC_{50} values <100 nM and rat brain/plasma K_p values of ~0.40. Interestingly, unlike M_1 and M_4 PAMs with unprecedented mAChR subtype selectivity, this series of M_5 PAMs displayed varying degrees of PAM activity at the other two natively G_q -coupled mAChRs, M_1 and M_3 , yet were inactive at M_2 and M_4 .

Keywords

M_5 ; muscarinic acetylcholine receptor; positive allosteric modulator (PAM); SAR; selectivity

INTRODUCTION

Of the five muscarinic acetylcholine receptors (M_1 – M_5), M_5 has the lowest expression level and distribution as well as the fewest chemical tools to probe the receptor's biology.^{1–10} Until recently, M_5 knockout (KO) mice and resultant phenotypic observations afforded the only insight into the physiological role of and therapeutic potential for M_5 modulation.^{11–14} From these studies, M_5 emerged as a potentially ideal target for drug addiction,^{15–17} and recent work with the M_5 negative allosteric modulator (NAM) ML375 (**1**)¹⁸ recapitulated the M_5 KO mouse data, displaying robust efficacy in models of cocaine use disorder,¹⁹ ethanol seeking,²⁰ and opiate abuse.²¹ Beyond ML375, several other highly selective M_5 inhibitors, such as the short half-life NAM **2**²² and the highly selective orthosteric M_5 antagonist **3**²³ (Figure 1) have been reported. Observations from the M_5 KO mice also suggest that selective activation of M_5 would be beneficial for cognition, Alzheimer's disease, schizophrenia, and ischemic stroke, by enhancing dilation of CNS vasculature and increasing cerebral blood flow.^{11–14} However, to validate this hypothesis, potent, selective, and CNS penetrant M_5 positive allosteric modulators (PAMs) are required. Efforts with M_5 PAMs have been more limited (Figure 1). We reported the first M_5 PAM, ML129 (**4**),²⁴ derived from a pan- $M_{1,3,5}$ PAM,²⁵ which then led to the discovery of a more potent congener, ML326 (**5**).²⁶ However, neither of these isatin-based PAMs possessed acceptable CNS penetration.^{24–26} A new high-throughput screen (HTS) then afforded a new M_5 PAM-preferring chemotype, exemplified by ML380 (**6**),²⁷ yet M_5 PAM potency and pharmacokinetics precluded this PAM from serving as an *in vivo* tool. Here, we report of the further optimization of ML380 leading to the discovery of a new chiral indanyl core with enantiospecific activity that provided highly potent M_5 PAMs (at both rat and human M_5) and new insights into the origins of muscarinic acetylcholine receptor (mAChR) PAM subtype selectivity.²⁸

RESULTS AND DISCUSSION

Toward the Next Generation of M₅ PAMs.

Initially, optimization of ML380 (hM₅ EC₅₀ = 190 nM) used a calcium mobilization assay with a 96 well-format FlexStation II microplate reader (Molecular Devices, LLC). When the recent optimization campaign initiated, we had developed a rat M₅ cell line and were routinely assessing functional activity at both human and rat M₅ on an industry-standard 384-well-format Hamamatsu FDSS7000 screening system.²² As a benchmark, we re-evaluated ML380 under these new optimal screening conditions and noted an ~5-fold rightward shift in M₅ PAM potency (hM₅ EC₅₀ = 1.1 μ M); thus for a robust *in vivo* tool, we also needed to increase M₅ PAM activity ~10-fold. As all of our M₅ NAMs displayed enantiospecific activity,^{18,22,23} we were driven to identify locations within the ML380 scaffold where we could introduce a chiral center and hopefully engender a significant increase in functional potency that might also afford new avenues for productive, tractable SAR. As ML380 possessed a lipophilic CF₃ moiety in the 2 position and in close proximity to the benzylic position,²⁷ we envisioned the introduction of a cyclic constraint in the form of an indanyl ring system providing racemic **7** (Figure 2), which proved to be an equipotent M₅ PAM (hM₅ EC₅₀ = 1.06 μ M, pEC₅₀ = 5.98 $\pm$ 0.03, 89% $\pm$ 1% ACh Max). Synthesis of the two single enantiomers of **7** did afford enantiospecific M₅ PAM activity, with the (*R*)-enantiomer, VU0488129 (**8**), displaying improved potency (hM₅ EC₅₀ = 481 nM, pEC₅₀ = 6.34 $\pm$ 0.08, 71% $\pm$ 4% ACh Max; rM₅ EC₅₀ = 409 nM, pEC₅₀ = 6.42 $\pm$ 0.06, 63% $\pm$ 3% ACh Max) relative to ML380, while the (*S*)-enantiomer (**9**) was inactive at both hM₅ and rM₅. To further evaluate **8** as a putative new lead, we determined several *in vitro* and *in vivo* DMPK properties. PAM **8** displayed attractive free fraction in rat and human plasma (*f*_{u,p} = 0.043 and 0.019, respectively) as well as rat brain (*f*_{u,br} = 0.017). However, predicted hepatic clearance in both rat and human was high (CL_{hep} = 69.3 and 20.8 mL·min⁻¹·kg⁻¹, respectively, based on microsomal intrinsic clearance (CL_{int})), but with CNS penetration (rat brain/plasma *K*_p = 0.17, *K*_{p,uu} = 0.07) rivaling centrally active M₁ PAMs. Thus, PAM **8** became the lead for further optimization for which we would survey functionalized indanyl systems, ring-expanded congeners, piperidine mimetics, and alternate sulfonamides. In parallel (*vide infra*), we initiated *in vitro* metabolite experiments to understand the high predicted hepatic clearance.

Chemistry and Limited Structure–Activity Relationships (SARs).

The chemistry to access this new series of M₅ PAMs was straightforward (Scheme 1).²⁹ From commercial **10**, a standard HATU coupling with (*R*)-indanyl amine (or the tetrahydronaphthyl amine) provides secondary amides **11** in yields ranging from 82% to 89%. *N*-Alkylation of **11** to form the tertiary amides **12** proceeds in moderate to good yields (33–97%), followed by a Boc removal to deliver the free piperidine, which was then converted directly to the sulfonamides **13** (17–74% from the HCl salt). Piperidine mimetics (such as azetidine or [3.3.0] and [3.1.0] ring systems) utilized starting materials analogous to **10**, but all were devoid of M₅ PAM activity.

Initial SAR was robust, and we were pleased to see that this series did not fall into “flat” SAR (Table 1).⁶ The indazole moiety of **8** could be replaced with either a piperonyl group

(**13b**), a cyclic carbamate (**13c**), or a 4-acetamide derivative (**13f**) without any significant loss in M₅ PAM potency. This was unanticipated, and there appears to be remarkable tolerance for a wide range of hydrogen bond donors and acceptors. A quinoline congener (**13g**) lost about 2.5-fold in potency relative to **8**, but a naphthalene analog (**13h**) surprisingly lost all activity. Finally, ring-expanded analogs **13k** and **13l** proved more potent than **8**, with EC₅₀ values of 256 nM and 255 nM, respectively. Overall, remarkable tolerability of changes to both the eastern and western portions of this new M₅ PAM scaffold and a 4-fold improvement in potency over the prototypical M₅ PAM **6** (ML380)²⁷ were observed.

Based on the high predicted hepatic clearance of **8**, we evaluated several of the new analogs **13** (**13a**, **13b**, **13c**, **13f**, **13k**, and **13l**) and found that all were predicted to be cleared at hepatic blood flow rates in both rat and human (CL_{hep} >68 and >20 mL·min⁻¹·kg⁻¹, respectively) despite possessing unique structural motifs. In rat iv PK cassette studies, there was a good *in vitro/in vivo* correlation (IVIVC) with all analogs showing high clearance (CL_p > 65 mL·min⁻¹·kg⁻¹) and short half-lives (*t*_{1/2} < 15 min). In parallel, we conducted metabolite identification (MetID) studies in both rat and human hepatic microsomes with PAM **8** in an attempt to discern metabolic “hot spots” that could then be addressed via chemistry to provide PAMs with suitable PK for *in vivo* studies. The MetID studies quickly rationalized the high *in vitro* and *in vivo* clearance values, as PAM **8** was subject to extensive oxidative metabolism (Figure 3). M1 was the major metabolite in rat, resulting from *N*-dealkylation of the tertiary amide and oxidation of the indane ring. In human, there were three major metabolites: M2 (oxidation of the indane ring), M3 (presumed further oxidation of M2 to a ketone), and M5 (*N*-dealkylation of the indane ring).

Thus, we elected to chemically modify analogs **13** based on the rat microsomal MetID data, and we first directed attention to the tertiary amide moiety. Analogs in this series were prepared using similar alkylation chemistry as shown in Scheme 1, from additional secondary amides **11** (see Supporting Information for details). As shown in Table 2, the secondary amide **14a** (also metabolite M6) was inactive, while the truncated methyl congener lost ~5-fold PAM activity. Homologation increased M₅ PAM potency, with an *n*-propyl derivative, **14c**, being the most potent M₅ PAM thus far (hM₅ EC₅₀ = 112 nM). Finally, a deuterated version of **8**, **14e**, was of comparable potency to **8**, but the reliance on the kinetic isotope effect in this instance had no impact on reducing clearance.³⁰ As pretreatment with a pan-CYP₄₅₀ inhibitor, such as 1-ABT, has previously increased *C*_{max} and AUC for other PAMs,^{27,31} we explored this option with a representative compound to potentially enable target validation studies. However, this approach was not successful as 1-ABT pretreatment had negligible impact on *C*_{max} and only afforded <10% increase in AUC following a single 10 mg/kg intraperitoneal (ip) administration to male Sprague–Dawley rats (data not shown). In this case, dosing via the ip route to partially avoid the hepatic first pass metabolism provided plasma *C*_{max} levels approaching 2 μM (total), but estimated unbound concentrations were below the compound's *in vitro* EC₅₀.

As modifications to the tertiary amide were not productive, we turned our attention to the indane ring. Here, we developed a synthetic route to enable a “fluorine walk”⁶ around the indane ring system, as well as allowing for fluorine incorporation in the 4-position of the piperidine ring (Scheme 2). In cases when the chiral amine starting materials were not

commercially available, Ellman sulfinimine chemistry³² on substituted indanones **15** provided (*R*)-**16** after NaBH₄-mediated sulfinimine reduction, which were then hydrolyzed to key fluorinated (*R*)-indanes **17**. Following the sequence described for Scheme 1 (HATU coupling, alkylation, Boc deprotection, and sulfonylation), analogs **18** were prepared. In cases where direct alkylation of the secondary amide proved difficult, commercially available primary chiral amines could also first be alkylated under reductive amination conditions to give secondary amine intermediates **19** (see Supporting Information for details) prior to amide coupling.²⁹ As shown in Table 3, fluorine incorporation on the indane ring had a major impact on M₅ PAM activity, with only the 4-F congener **18a** retaining activity (EC₅₀ = 500 nM); all other isomers decreased 5–10-fold in M₅ PAM activity, which once again highlights the value of the fluorine walk in allosteric modulator optimization.⁶ Fluorine incorporation at the 4-position of the piperidine, as in **18e** and **18f**, led to only a slight increase in potency, but CNS penetration improved (**18f**, rat brain/plasma *K_p* = 0.40, *K_{p,uu}* = 0.13).

However, homologation of the ethyl side in **18a** to an *N*-propyl analog (**18g**) afforded >10-fold increase in M₅ PAM potency (EC₅₀ = 41 nM), and this held true for alternate sulfonamides as well (**18h**, EC₅₀ = 58 nM). Ring expansion (**18j**) provided comparable potency (EC₅₀ = 161 nM) to **14c**, yet expansion to a seven-membered ring further increased M₅ PAM activity (EC₅₀ = 76 nM). Incorporation of an oxygen atom into **18j** led to a slight loss in potency (EC₅₀ = 298 nM). Despite the significant structural changes across analogs **13**, **14**, and **18**, both predicted hepatic clearance and *in vivo* clearance in rat were high (CL_{hep} > 65 mL·min⁻¹·kg⁻¹ and CL_p > 60 mL·min⁻¹·kg⁻¹) with rat brain/plasma *K_p* values in the 0.15 to 0.40 range (and favorable CNS MPO scores³³). Thus, this new series appears to be relegated for use as *in vitro* tools (or electrophysiology tools); however, more extensive PK studies employing alternative routes of administration may enable *in vivo* utility. Prior to these decisions, we elected to take a more detailed examination of the molecular pharmacology and ensure comparable activity at rat M₅ and overall mAChR selectivity.²⁹

Molecular Pharmacology Studies.

We were pleased to find that there were no major species disconnects in potency for this series of M₅ PAMs, especially for the more potent analogs **18** (Table 4) between human and rat M₅. However, when we assessed selectivity at human and rat M₁–M₄, we were surprised to find that all of these analogs showed varying degrees of PAM activity at the G_q-coupled mAChRs M₁ and M₃ yet were very weak to inactive at M₂ and M₄. As an example, the most potent M₅ PAM in this set, **18g** (VU6007678) proved to be M₅-preferring, but was only 10–20-fold selective versus M₃ and M₁, respectively (Figure 4). We had encountered pan-G_q M_{1,3,5} PAMs previously²⁴ but were able to develop highly selective M₁ PAMs³⁴ and M₅ PAMs²⁵ from that lead; however, selective M₃ PAMs remained elusive.

Within this new series of M₅ PAMs, we were unable to completely dial-out activity at human and rat M₁ and M₃. In radioligand competition binding assays with [³H]NMS, there is no binding interaction with the orthosteric site at either M₅ (or M₃), though there is a hint of positive cooperativity with the antagonist at higher concentrations (see Supporting Information).²⁹ What could be the cause for the lack of subtype selectivity for this series of

M₅ PAMs? In contrast to the historical dogma of allosteric modulators, are there conserved allosteric sites across the G_q-PAMs? Or, could there be varying degrees of cooperativity that give rise to varying selectivity profiles at M₁, M₃, and M₅? Emerging data suggest the lack of mAChR selectivity may arise from differential cooperativity via a “common” allosteric site, as opposed to pure differences in allosteric site sequence divergence; our current findings are in support of this hypothesis.²⁸

CONCLUSIONS

We have reported a novel series of M₅ PAMs, based on a chiral *N*-(indanyl)piperidine amide core with robust SAR, human and rat M₅ PAM EC₅₀ values <100 nM, rat brain/plasma *K_p* values of ~0.40 and enantiospecific activity. Once again, the fluorine walk proved essential in the optimization effort to develop the most potent M₅ PAMs to date, for example, **18g** (VU6007678), EC₅₀ = 41 nM. However, the series was plagued with significant metabolic liabilities that could not be abrogated by either pretreatment with a pan-CYP inhibitor or utilization of the kinetic isotope effect. Interestingly, unlike M₁ and M₄ PAMs with unprecedented mAChR subtype selectivity, this series of M₅ PAMs displayed varying degrees of PAM activity at the other two G_q-coupled mAChRs, M₁ and M₃, yet were inactive at M₂ and M₄; moreover, molecular pharmacology studies suggest the lack of selectivity is due to differential cooperativity. Thus, while compounds from this series will not likely become useful as *in vivo* tools, they may prove highly valuable in facilitating understanding of mechanisms underlying mAChR allosteric modulator selectivity.

METHODS

Chemical Synthesis and Purification.

All reactions were carried out employing standard chemical techniques under inert atmosphere. Solvents used for extraction, washing, and chromatography were HPLC grade. All reagents were purchased from commercial sources and were used without further purification. Analytical HPLC was performed on an Agilent 1200 LCMS with UV detection at 215 and 254 nm along with ELSD detection and electrospray ionization (ESI), with all final compounds showing 95% purity and a parent mass ion consistent with the desired structure. All NMR spectra were recorded on a 400 MHz Bruker AV-400 instrument. ¹H chemical shifts are reported as δ values in ppm relative to the residual solvent peak (MeOD = 3.31, CDCl₃ = 7.26). Data are reported as follows: chemical shift, multiplicity (br = broad, s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet), coupling constant (Hz), and integration. ¹³C chemical shifts are reported as δ values in ppm relative to the residual solvent peak (MeOD = 49.0, CDCl₃ = 77.16). When visible, minor rotamer peaks are denoted with an * in ¹H NMR spectra. Low resolution mass spectra were obtained on an Agilent 1200 LCMS with electrospray ionization, with a gradient of 5–95% MeCN in 0.1% TFA water solution over 1.5 min. High resolution mass spectra were obtained on an Agilent 6540 Ultra High Definition (UHD) Q-TOF with ESI source. Automated flash column chromatography was performed on an Isolera One by Biotage. Preparative purification of library compounds was performed on a Gilson 215 preparative LC system. Optical rotations were acquired on a Jasco P-2000 polarimeter at 23 °C and 589 nm. The specific rotations

were calculated according to the equation $[\alpha]_D^{23} = (100\alpha)/(l \times c)$ where l is path length in decimeters and c is the concentration in g/100 mL. For full experimental procedures, please see the Supporting Information.

Synthesis of Compound 8 (VU0488129).

tert-Butyl 4-[[[(1R)-Indan-1-yl]carbamoyl]piperidine-1-carboxylate (11a).—To a solution of **10** (2.5 g, 10.9 mmol) and (*R*)-(–)-1-aminoindane hydrochloride (2.77 g, 16.4 mmol, 1.5 equiv) in DCM (45 mL) was added DIPEA (4.75 mL, 27.3 mmol, 2.5 equiv). After 5 min stirring, HATU (8.29 g, 21.8 mmol, 2 equiv) was added. The resulting mixture was stirred at rt overnight, after which time sat. NaHCO₃ was added. Aqueous layer was extracted with DCM. Combined organic extracts were washed with brine and dried with MgSO₄. Solvents were filtered and removed, and crude residue was purified by column chromatography (12–100% EtOAc in hexanes) to give product as a white solid (3.08 g, 82%). ¹H NMR (400 MHz, CDCl₃) δ 7.25–7.22 (m, 4H), 5.83 (d, J = 8.2 Hz, 1H), 5.49 (q, J = 7.8 Hz, 1H), 4.15 (br, 2H), 3.02–2.95 (m, 1H), 2.92–2.84 (m, 1H), 2.74 (br, 2H), 2.64–2.56 (m, 1H), 1.85–1.64 (m, 6H), 1.47 (s, 9H). ¹³C NMR (101 MHz, CDCl₃) δ 174.17, 154.66, 143.40, 143.16, 128.02, 126.82, 124.86, 123.87, 79.62, 54.44, 43.40, 34.09, 30.21, 28.85, 28.61, 28.44. LCMS (215 nm) RT = 0.978 min (>98%); m/z 289.4 [M + H]⁺, minus *t*-butyl. HRMS (TOF, ES+) C₂₀H₂₉N₂O₃ [M + H]⁺ calcd mass 345.2178, found 345.2173. Specific rotation $[\alpha]_D^{23} = +67.7^\circ$ (c = 0.91, MeOH).

tert-Butyl 4-[Ethyl-[(1R)-indan-1-yl]carbamoyl]piperidine-1-carboxylate (12a).—To a dry flask was added NaH (45.3 mg, 1.13 mmol, 2 equiv, 60% dispersion in mineral oil). DMF (5 mL) was then added, and the flask was cooled to 0 °C under an inert atmosphere. Compound **11a** (195 mg, 0.57 mmol) in DMF (3 mL) was then added dropwise. The flask was warmed to rt and stirred for 30 min, after which time it was cooled back to 0 °C, and iodoethane (0.18 mL, 2.26 mmol, 4 equiv) was added dropwise. The resulting solution was warmed to rt and stirred for 2.5 h, after which time the reaction was quenched with the addition of sat. NH₄Cl and extracted with EtOAc. Combined organic extracts were washed with H₂O (3×) and brine (1×) and dried with MgSO₄. Solvents were filtered and removed to give product as a white solid, which was pure by LCMS and used without further purification (202 mg, 96%). Note: substitutions with longer alkyl halides do not proceed to completion under these conditions and require purification by column chromatography (hex/EtOAc). ¹H NMR (1.2:1 rotamer ratio, asterisk denotes minor rotamer peaks where separable 400 MHz, CDCl₃) δ 7.30–7.17 (m, 3H), 7.12, 7.06* (d, J = 7.2 Hz, 1H), 6.20*, 5.45 (t, J = 7.9 Hz, 1H), 4.18 (br, 2H), 3.42–3.31, 3.28–3.20* (m, 1H), 3.13–2.86 (m, 3H), 2.85–2.63 (m, 3H), 2.50–2.42 (m, 1H), 2.17–1.70 (m, 5H), 1.49*, 1.48 (s, 9H), 1.14*, 1.10 (t, J = 7.0 Hz, 3H). ¹³C NMR (101 MHz, CDCl₃) δ 175.50, 174.53, 154.72, 143.71, 142.90, 142.10, 141.32, 128.21, 127.69, 126.85, 126.55, 125.29, 124.91, 124.05, 123.64, 79.58, 79.55, 62.84, 59.18, 43.47, 39.79, 38.54, 31.34, 30.46, 30.32, 29.97, 29.34, 29.05, 28.82, 28.65, 28.45, 17.37, 14.58. LCMS (215 nm) RT = 1.135 min (>98%); m/z 317.4 [M + H]⁺, minus *t*-butyl. HRMS (TOF, ES+) C₂₂H₃₃N₂O₃ [M + H]⁺ calcd mass 373.2491, found 373.2484. Specific rotation $[\alpha]_D^{23} = +57.9^\circ$ (c = 0.74, MeOH).

N-Ethyl-N-[(1R)-indan-1-yl]-1-(1H-indazol-5-ylsulfonyl)-piperidine-4-

carboxamide (8, VU0488129).—Compound **12a** (109 mg, 0.29 mmol) was dissolved in 4 M HCl in 1,4-dioxane solution (10 mL) and stirred at rt for 1 h, after which time solvents were removed under reduced pressure, and the resulting amine was used directly as the HCl salt (white solid) (87 mg, 96%); m/z 273.4 $[M + H]^+$. To a solution of the HCl salt (31.2 mg, 0.101 mmol) in DCM (1 mL) was added DIPEA (0.035 mL, 0.202 mmol, 2 equiv), followed by 1H-indazole-5-sulfonyl chloride (32.8 mg, 0.152 mmol, 1.5 equiv). The resulting solution was stirred at rt for 1 h, after which time solvents were concentrated. Crude residue was purified by RP-HPLC, and fractions containing product were basified with sat. NaHCO_3 . Aqueous layer was extracted with 3:1 chloroform/IPA. Solvents were filtered through a phase separator and concentrated to give product as a colorless oily solid (11.9 mg, 26%). ^1H NMR (1.4:1 rotamer ratio, asterisk denotes minor rotamer peaks where separable, 400 MHz, CDCl_3) δ 8.27*, 8.24 (s, 1H), 8.19*, 8.16 (s, 1H), 7.75–7.69 (m, 1H), 7.59 (t, $J = 10.1$ Hz, 1H), 7.19–7.11 (m, 3H), 7.00–6.97 (m, 1H), 6.09*, 5.25 (t, $J = 7.8$ Hz, 1H), 3.90–3.80 (m, 2H), 3.33–2.74 (m, 4H), 2.51–2.31 (m, 4H), 2.07–1.68 (m, 5H), 1.02, 0.98* (t, $J = 7.2$ Hz, 3H). ^{13}C NMR (101 MHz, CDCl_3) δ 175.03, 174.01, 143.80, 142.90, 141.85, 141.39, 141.00, 135.94, 129.03, 128.81, 128.35, 127.87, 126.93, 126.65, 125.37, 125.24, 125.03, 124.11, 123.54, 122.76, 110.75, 110.69, 62.90, 59.39, 45.78, 45.66, 38.68, 38.47, 31.32, 30.53, 30.36, 29.96, 28.92, 28.67, 28.42, 28.35, 17.34, 14.59. LCMS (215 nm) RT = 0.999 min (>98%); m/z 453.4 $[M + H]^+$. HRMS (TOF, ES+) $\text{C}_{24}\text{H}_{29}\text{N}_4\text{O}_3\text{S}$ $[M + H]^+$ calcd mass 453.1960, found 453.1950. Specific rotation $[\alpha]_{\text{D}}^{23} = +51.0^\circ$ ($c = 0.32$, MeOH).

Cell Lines and Calcium Mobilization Assay.

Chinese hamster ovary (CHO) cells stably expressing human and rat M_5 were maintained in Ham's F-12 growth medium containing 10% FBS, 20 mM HEPES, antibiotic/antimycotic, and 500 $\mu\text{g/mL}$ G418 in the presence of 5% CO_2 at 37 °C. To determine the potency and efficacy of M_5 PAMs, calcium flux was measured using the Functional Drug Screening System (FDSS7000, Hamamatsu, Japan). Briefly, the M_5 -CHO cells were plated in black-walled, clear-bottomed 384 well plates (Greiner Bio-One, Monroe, NC) at 20 000 cells/well in 20 μL of growth medium without G418 the day before assay. The following day, cells were washed with assay buffer (Hank's balanced salt solution, 20 mM HEPES, and 2.5 mM probenecid) and immediately incubated with 20 μL of 1.15 μM Fluo-4-acetomethoxyester (Fluo-4 AM) dye solution prepared in assay buffer for 45 min at 37 °C. During the incubation time, all compounds were serially diluted (1:3) in DMSO for 10-point concentration–response curves (CRCs) and further diluted in assay buffer at starting final concentration 30 μM using Echo liquid handler (Labcyte, Sunnyvale CA). Dye was removed and replaced with assay buffer. Immediately, calcium flux was measured using the FDSS7000 as previously described.^{24,27} The compounds or vehicle was added to cells for 2.5 min, and then an EC_{20} concentration of acetylcholine (ACh) was added and incubated for 1 min. EC_{max} concentration was also added to cells that were incubated with DMSO vehicle to ensure the EC_{20} calcium response. Using a four-point logistical equation in GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA), the concentration–response curves were generated for determination of the potency and efficacy of the PAM.

Radioligand Binding Assay.

Competition binding assay was performed using [³H]-*N*-methylscopolamine ([³H]NMS, PerkinElmer, Boston, MA) as previously described.¹⁸ Compounds were serially diluted 1:3 in DMSO for 11-point CRC, then further diluted at starting final concentration of 30 μ M in binding buffer (20 mM HEPES, 10 mM MgCl₂, and 100 mM NaCl, pH 7.4). Membranes from either human M₅-CHO cells or M₃-CHO cells (10 μ g) were incubated with the serially diluted compounds in the presence of a *K_d* concentration of [³H]NMS, 0.376 nM, at room temperature for 3 h with constant shaking. Nonspecific binding was determined in the presence of 10 μ M atropine. Binding was terminated by rapid filtration through GF/B Unifilter plates (PerkinElmer) using a Brandel 96-well plate Harvester (Brandel Inc., Gaithersburg, MD), followed by three washes with ice-cold harvesting buffer (25 mM Tris-HCl, pH 7.4, 150 mM NaCl). Plates were air-dried overnight, 50 μ L of Microscint20 was added to the plate, and radioactivity was counted using a TopCount Scintillation Counter (PerkinElmer Life and Analytical Sciences).

Drug Metabolism Methods.

In Vitro.—Plasma protein binding, brain homogenate binding, and hepatic microsomal intrinsic clearance assays with the M₅ PAMs were conducted using the same methods described previously.^{30,31} Metabolite identification experiments were also performed essentially as described previously using rat and human hepatic microsomes.^{23,31}

In Vivo.—Select M₅ PAMs were administered to male Sprague–Dawley rats via single IV or IP administrations of cassette doses (0.20–0.25 mg/kg; *n* = 1 animal per cassette) formulated in ethanol/PEG400/DMSO vehicles (varying concentrations of excipients to afford solutions, dependent upon the compounds' solubilities; 0.5 mL/kg iv or 3 mL/kg ip dose volumes). For determination of pharmacokinetics and brain distribution, serial sampling of plasma and nonserial sampling of plasma and brain with subsequent quantitation of analytes via LC-MS/MS were conducted essentially as described previously.^{31,35} All animal studies were approved by the Vanderbilt University Institutional Animal Care and Use Committee. The animal care and use program is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International.

Liquid Chromatography/Mass Spectrometry Analysis.—Samples from *in vitro* and *in vivo* assays or studies with M₅ PAMs were analyzed via electrospray ionization (ESI) on an AB Sciex API-4000 or Q-Trap 5500 (Foster City, CA) triple-quadrupole mass spectrometer instrument that was coupled with Shimadzu LC-10AD or LC-20AD pumps (Columbia, MD) and a Leap Technologies CTC PAL autosampler (Carrboro, NC). Analytes were separated by reverse-phase gradient elution and monitored by analyte-specific multiple reaction monitoring (MRM) utilizing a Turbo-Ionspray source in positive ionization mode (5.0 kV spray voltage), essentially as described previously. All raw data were analyzed using AB Sciex Analyst (v. 1.4.2 or later) software, and pharmacokinetic noncompartmental analysis (NCA) of time–concentration data was performed using Pharsight Phoenix WinNonLin (v. 6.0 or later; Certara L.P., Princeton, NJ).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding

This work was funded by the NIH, NIMH and NIDA (Grants MH082867, MH106839, and DA037207). We thank William K. Warren, Jr., and the William K. Warren Foundation who funded the William K. Warren, Jr., Chair in Medicine (to C.W.L.).

ABBREVIATIONS:

ip	intraperitoneal
iv	intravenous
ABT	aminobenzotriazole
GPCR	G-protein-coupled receptor
M₅	muscarinic acetylcholine receptor subtype 5
PAM	positive allosteric modulator
ESI	electrospray ionization
NCA	noncompartmental analysis
MRM	multiple reaction monitoring
NMS	<i>N</i> -methylscopolamine
CHO	Chinese hamster ovary
CL_{hep}	predicted hepatic clearance
CL_{int}	intrinsic clearance
<i>f_{u,p}</i>	fraction unbound in plasma
<i>f_{u,br}</i>	fraction unbound in brain
HATU	1-[bis-(dimethylamino)methylene]-1 <i>H</i> -1,2,3-triazolo[4,5- <i>b</i>]-pyridinium 3-oxide hexafluorophosphate
DIPEA	<i>N,N</i> -diisopropylethylamine
DCM	dichloromethane
DMF	dimethylformamide
THF	tetrahydrofuran
ELSD	evaporative light scattering detector

RT	retention time
rt	room temperature
DMPK	drug metabolism and pharmacokinetics

REFERENCES

- (1). Dencker D, Thomsen M, Wortwein G, Weikop P, Cui Y, Jeon J, Wess J, and Fink-Jensen A (2012) Muscarinic acetylcholine receptor subtypes as potential drug targets for the treatment of schizophrenia, drug abuse, and Parkinson's disease. *ACS Chem. Neurosci* 3, 80–89. [PubMed: 22389751]
- (2). Conn PJ, Jones C, and Lindsley CW (2009) Subtype selective allosteric modulators of muscarinic receptors for the treatment of CNS disorders'. *Trends Pharmacol. Sci* 30, 148–156. [PubMed: 19201489]
- (3). Bridges TM, LeBois EP, Hopkins CR, Wood MR, Jones JK, Conn PJ, and Lindsley CW (2010) Antipsychotic potential of muscarinic allosteric modulation. *Drug News Perspect.* 23, 229–240. [PubMed: 20520852]
- (4). Levey AI, Kitt CA, Simonds WF, Price DL, and Brann MR (1991) Identification and localization of muscarinic acetylcholine receptor proteins in brain with subtype-specific antibodies. *J. Neurosci* 11, 3218–3226. [PubMed: 1941081]
- (5). Levey AI, Edmunds SM, Koliatsos V, Wiley RG, and Heilman CJ (1995) Expression of M1-M4 muscarinic acetylcholine receptor proteins in rat hippocampus and regulation by cholinergic innervations. *J. Neurosci* 15, 4077–4092. [PubMed: 7751967]
- (6). Melancon BJ, Hopkins CR, Wood MR, Emmitte KA, Niswender CM, Christopoulos A, Conn PJ, and Lindsley CW (2012) Allosteric modulation of 7 transmembrane spanning receptors: theory, practice and opportunities for CNS drug discovery. *J. Med. Chem* 55, 1445–1464. [PubMed: 22148748]
- (7). Conn PJ, Lindsley CW, Meiler J, and Niswender CM (2014) Opportunities and challenges in the discovery of allosteric modulators of GPCRs for the treatment of CNS disorders'. *Nat. Rev. Drug Discovery* 13, 692–708. [PubMed: 25176435]
- (8). Conn PJ, Christopoulos A, and Lindsley CW (2009) Allosteric Modulators of GPCRs as a Novel Approach to Treatment of CNS Disorders. *Nat. Rev. Drug Discovery* 8, 41–54. [PubMed: 19116626]
- (9). Vilaro MT, Palacios JM, and Mengod G (1990) Localization of M5 muscarinic receptor mRNA in rat brain examined by in situ hybridization histochemistry. *Neurosci. Lett* 114, 154–159. [PubMed: 2395528]
- (10). Foster DJ, Gentry PR, Lizardi-Ortiz JE, Bridges TM, Wood MR, Niswender CM, Sulzer D, Lindsley CW, Xiang Z, and Conn PJ (2014) M5 receptor activation produces opposing physiological outcomes in dopamine neurons depending on the receptor's location. *J. Neurosci* 34, 3253–3262. [PubMed: 24573284]
- (11). Wess J, Eglen RM, and Gautam D (2007) Muscarinic acetylcholine receptors: mutant mice provide new insights for drug development. *Nat. Rev. Drug Discovery* 6, 721–733. [PubMed: 17762886]
- (12). Thomsen M, Wortwein G, Fink-Jensen A, Woldbye DPD, Wess J, and Caine SB (2007) Decreased prepulse inhibition and increased sensitivity to muscarinic, but not dopaminergic drugs in M5 muscarinic acetylcholine receptor knockout mice. *Psychopharmacology* 192, 97–110. [PubMed: 17310388]
- (13). Araya R, Noguchi T, Yuhki M, Kitamura N, Higuchi M, Saido TC, Seki K, Itohara S, Kawano M, Tanemura K, Takashima A, Yamada K, Kondoh Y, Kanno I, Wess J, and Yamada M (2006) Loss of M5 muscarinic acetylcholine receptors leads to cerebrovascular and neuronal abnormalities and cognitive deficits in mice. *Neurobiol. Dis* 24, 334–344. [PubMed: 16956767]
- (14). Yamada M, Lamping KG, Duttaroy A, Zhang W, Cui Y, Bymaster FP, McKinzie DL, Felder CC, Deng C-X, Faraci FM, and Wess J (2001) Cholinergic dilation of cerebral blood vessels is

abolished in M5 muscarinic acetylcholine receptor knockout mice. *Proc. Natl. Acad. Sci. U. S. A* 98, 14096–14101. [PubMed: 11707605]

- (15). Thomsen M, Woldbye D, Wörtwein G, Fink-Jensen A, Wess J, and Caine SB (2005) Reduced cocaine self-administration in muscarinic M5 acetylcholine receptor-deficient mice. *J. Neurosci* 25, 8141–8149. [PubMed: 16148222]
- (16). Basile AS, Fedorova I, Zapata A, Liu X, Shippenberg T, Duttaroy A, Yamada M, and Wess J (2002) Deletion of the M5 muscarinic acetylcholine receptor attenuates morphine reinforcement and withdrawal but not morphine analgesia. *Proc. Natl. Acad. Sci. U. S. A* 99, 11452–11455. [PubMed: 12154229]
- (17). Fink-Jensen A, Fedorova I, Wortwein G, Woldbye DP, Rasmussen T, Thomsen M, Bolwig TG, Knitowski KM, McKinzie DL, Yamada M, Wess J, and Basile A (2003) Role for M5 muscarinic acetylcholine receptors in cocaine addiction. *J. Neurosci. Res* 74, 91–96. [PubMed: 13130510]
- (18). Gentry PR, Kokubo M, Bridges TM, Kett NR, Harp JM, Cho HP, Smith E, Chase P, Hodder PS, Niswender CM, Daniels SJ, Conn PJ, Wood MR, and Lindsley CW (2013) Discovery of the first M₅-selective and CNS Penetrant negative allosteric modulator (NAM) of a muscarinic acetylcholine receptor: (*S*)-9b-(4-chlorophenyl)-1-(3,4-difluorobenzoyl)-2,3-dihydro-1*H*-imidazo[2,1-*a*]isoindol-5(9*bH*)-one (ML375), an M₅ Selective NAM'. *J. Med. Chem* 56, 9351–9355. [PubMed: 24164599]
- (19). Gunter B, Gould R, Bubser M, McGowan KM, Lindsley CW, and Jones CK (2017) Selective inhibition of M₅ muscarinic acetylcholine receptors attenuates cocaine self-administration in rats'. *Addiction Biology*, 10.1111/adb.12567.
- (20). Berizzi AE, Perry CJ, Shackelford D, Lindsley CW, Jones CK, Chen NA, Sexton PM, Langmead CJ, Christopoulos A, and Lawrence AJ (2018) Muscarinic M5 receptors modulate ethanol seeking in rats. *Neuropsychopharmacology*, 10.1038/s41386-017-0007-3.
- (21). Manuscript in preparation.
- (22). McGowan KM, Nance KD, Cho HP, Bridges TM, Conn PJ, Jones CK, and Lindsley CW (2017) Continued optimization of the M₅ NAM ML375: Discovery of VU6008667, an M₅ NAM with high CNS penetration and a desired short half-life in rat for addiction studies. *Bioorg. Med. Chem. Lett* 27, 1356–1359. [PubMed: 28237763]
- (23). Gentry PR, Kokubo M, Bridges TM, Cho HP, Smith E, Chase P, Hodder PS, Utley TJ, Rajapakse A, Byers F, Niswender CM, Morrison RD, Daniels JS, Wood MR, Conn PJ, and Lindsley CW (2014) Discovery, synthesis and characterization of a highly muscarinic acetylcholine receptor (mAChR)-selective M₅-orthosteric antagonist, VU0488130 (ML381): A novel molecular probe. *ChemMedChem* 9, 1677–1682. [PubMed: 24692176]
- (24). Bridges TM, Marlo JE, Niswender CM, Jones JK, Jadhav SB, Gentry PR, Weaver CD, Conn PJ, Lindsley CW, and Plumley HC (2009) Discovery of the first highly M₅-preferring muscarinic acetylcholine receptor ligand, an M₅ positive allosteric modulator derived from a series of 5-trifluoromethoxy *N*-benzyl isatins. *J. Med. Chem* 52, 3445–3448. [PubMed: 19438238]
- (25). Bridges TM, Kennedy JP, Cho HP, Conn PJ, Lindsley CW, et al. (2010) Chemical optimization of an M₁, M₃, M₅ positive allosteric modulator (PAM) lead. Part I. Development of a highly selective M₅ PAM. *Bioorg. Med. Chem. Lett* 20, 558–562. [PubMed: 20004578]
- (26). Gentry PR, Bridges TM, Lamsal A, Vinson PN, Smith E, Chase P, Hodder PS, Engers JL, Niswender CM, Daniels JS, Conn PJ, Wood MR, and Lindsley CW (2013) Discovery of ML326: The first sub-micromolar, selective M₅ PAM'. *Bioorg. Med. Chem. Lett* 23, 2996–3000. [PubMed: 23562060]
- (27). Gentry PR, Kokubo M, Bridges TM, Cho HP, Smith E, Hodder PS, Niswender CM, Daniels JS, Conn PJ, Lindsley CW, Wood MR, et al. (2014) Development of a Highly Potent, Novel M₅ Positive Allosteric Modulator (PAM) Demonstrating CNS Exposure: 1-((1*H*-indazol-5-yl)sulfonyl)-*N*-ethyl-*N*-(2-(trifluoromethyl)benzyl)piperidine-4-carboxamide (ML380). *J. Med. Chem* 57, 7804–7810. [PubMed: 25147929]
- (28). Berizzi AE, Bender AM, Conn PJ, Lindsley CW, Sexton PM, Langmead CJ, and Christopoulos A (2018) Structure-activity relationships of pan-Gαq/11 coupled muscarinic acetylcholine receptor positive allosteric modulators. *ACS Chem. Neurosci*, submitted for publication.
- (29). See Supporting Information for full experimental details.

- (30). Wenthur CJ, Morrison R, Daniels JS, Conn PJ, Lindsley CW, et al. (2013) Discovery of (*R*)-(2-fluoro-4-((4-methoxyphenyl)ethynyl)phenyl)(3-hydroxypiperidin-1-yl)methanone (ML337), an mGlu₃ selective and CNS penetrant negative allosteric modulator (NAM). *J. Med. Chem* 56, 5208. [PubMed: 23718281]
- (31). Bridges TM, Rook JM, Noetzel MJ, Morrison RD, Zhou Y, Gogliotti RD, Vinson PN, Jones CK, Niswender CM, Lindsley CW, Stauffer SR, Conn PJ, Daniels JS, and Xiang Z (2013) Biotransformation of a novel positive allosteric modulator of metabotropic glutamate receptor subtype 5 contributes to seizures in rats involving a receptor agonism-dependent mechanism. *Drug Metab. Dispos* 41, 1703–1714. [PubMed: 23821185]
- (32). Ellman JA, Owens TD, and Tang TP (2002) *N-tert*-butylsulfinyl imines: versatile intermediates for the asymmetric synthesis of amines. *Acc. Chem. Res* 35, 984–995. [PubMed: 12437323]
- (33). Wager TT, Hou X, Verhoest PR, and Villalobos A (2016) Central Nervous System Multiparameter Optimization Desirability: Application in Drug Discovery. *ACS Chem. Neurosci* 7, 767–775. [PubMed: 26991242]
- (34). Bridges TM, Kennedy JP, Conn PJ, Lindsley CW, et al. (2010) Chemical optimization of a pan Gq mAChR M₁, M₃, M₅ positive allosteric modulator (PAM) lead. Part II. Development of a highly selective M₁ PAM. *Bioorg. Med. Chem. Lett* 20, 1972–1975. [PubMed: 20156687]
- (35). Bridges TM, Morrison RD, Byers FW, Daniels SJ, and Luo S (2014) Use of a novel rapid and resource-efficient cassette dosing approach to determine the pharmacokinetics and CNS distribution of small molecule 7-transmembrane receptor allosteric modulators in rat. *Pharmacol. Res. Perspect* 2, e00077. [PubMed: 25505618]

Figure 1.
Structures of reported M₅ NAMs **1** and **2**, and the orthosteric M₅ antagonist **3**, along with the disclosed M₅ PAMs (**4–6**).

Figure 2.

Optimization plan to introduce a cyclic constraint and a chiral center into the ML380 scaffold to afford racemic indanyl analog **7**. Enantiospecific activity was noted, with the (*R*)-enantiomer **8** displaying an improved EC₅₀ of 481 nM, while the (*S*)-enantiomer **9** was inactive.

Figure 3. Metabolite identification studies with **8** in rat and human hepatic microsomes identified six discrete metabolites with different major (based on relative peak areas) metabolites in rat and human (proposed structures and major pathways based on LC-UV/MSⁿ data).

Figure 4.

Muscarinic M₁–M₅ selectivity for **18g** (VU6007678). (A) PAM activity for **18g** at human M₁ (EC₅₀ = 1034 nM, 67% ACh Max), M₂ (inactive), M₃ (EC₅₀ = 466 nM, 71% ACh Max), M₄ (inactive), and M₅ (EC₅₀ = 41 nM, 74% ACh Max). (B) PAM activity for **18g** at rat M₁ (EC₅₀ = 904 nM, 69% ACh Max), M₂ (inactive), M₃ (EC₅₀ = 772 nM, 51% ACh Max), M₄ (EC₅₀ = 3913 nM, 49% ACh Max), and M₅ (EC₅₀ = 74 nM, 76% ACh Max).

Scheme 1. Synthesis of Indane and Tetrahydronaphthalene Analogs 13^a

^aReagents and conditions: (a) chiral indane or tetrahydronaphthyl amine, HATU, DIPEA, DCM, rt, 82–89%; (b) iodoethane, NaH, DMF, rt, 33–97%; (c) HCl, dioxanes, rt; (d) sulfonyl chloride, DIPEA, DCM, rt, 17–74%.

Scheme 2. Synthesis of Indane and Tetrahydronaphthalene Analogs 18^a

^aReagents and conditions: (a) (*R*)-(+)-2-methyl-2-propanesulfinamide, $\text{Ti}(\text{OEt})_4$, THF, 0–75 °C; (b) NaBH_4 , THF, –78 °C to rt; 19–32% (c) HCl, 1,4-dioxanes, MeOH, rt; 80–100% (d) **10** or 1-Boc-4-fluoro-4-piperidinecarboxylic acid, HATU, DIPEA, DCM, rt, 65–93%; (e) iodoalkane, NaH, DMF, rt, 20–87%; (f) HCl, dioxanes, rt; (g) sulfonyl chloride, DIPEA, DCM, rt, 27–82%.

Table 1.
Structure and Activities of Analogs 13^a

Compound	Ar	n	hM ₅ EC ₅₀ (nM) (pEC ₅₀ ±SEM)	hM ₅ % ACh Max
8		1	481 (6.34±0.08)	71±4
13a		1	976 (6.05±0.13)	80±5
13b		1	672 (6.18±0.06)	75±6
13c		1	889 (6.07±0.10)	79±6
13d		1	2,216 (5.67±0.09)	80±4
13e		1	2,823 (5.56±0.06)	68±3
13f		1	606 (6.25±0.09)	77±6

Compound	Ar	n	hM ₅ EC ₅₀ (nM) (pEC ₅₀ ±SEM)	hM ₅ % ACh Max
13g		1	1,005 (6.00±0.02)	73±5
13h		1	> 10,000 (<5)	<25
13i		1	1,713 (5.80±0.12)	74±4
13j		1	5,102 (5.31±0.09)	71±4
13k		2	256 (6.59±0.03)	74±3
13l		2	255 (6.61±0.12)	76±0

^ahM₅ pEC₅₀, and ACh Max data reported as averages ± SEM from our functional calcium assay; *n* = 3 determinations.

Table 2.
Structure and Activities of Analogs 14^a

compd	R	hM ₅ EC ₅₀ (nM) (pEC ₅₀ ± SEM)	hM ₅ % ACh Max
14a	H	>10000 (<5)	<25
14b	Me	2535 (5.60 ± 0.03)	70 ± 5
14c	<i>n</i> -Pr	112 (7.08 ± 0.27)	73 ± 4
14d	<i>n</i> -Bu	349 (6.46 ± 0.02)	77 ± 3
14e	CD ₂ CH ₃	456 (6.36 ± 0.07)	74 ± 3

^ahM₅ pEC₅₀, and ACh Max data reported as averages ± SEM from our functional calcium assay; *n* = 3 determinations.

Table 3.
Structure and Activities of Analogs 18^a

Compound	Structure	hM ₅ EC ₅₀ (nM) (pEC ₅₀ ±SEM)	hM ₅ % ACh Max
18a		500 (6.42±0.26)	83±5
18b		5,413 (5.27±0.05)	64±4
18c		2,612 (5.60±0.08)	74±4
18d		2,515 (5.61±0.07)	68±2
18e		631 (6.23±0.08)	77±5
18f		314 (6.53±0.08)	70±6
18g		41 (7.46±0.15)	74±6
18h		58 (7.35±0.13)	72±7
18i		76 (7.20±0.12)	79±6
18j		161 (6.86±0.11)	76±6

Compound	Structure	hM ₅ EC ₅₀ (nM) (pEC ₅₀ ±SEM)	hM ₅ % ACh Max
18k		298 (6.55±0.09)	69±4

^a hM₅ pEC₅₀, and ACh Max data reported as averages ± SEM from our functional calcium assay; *n* = 3 determinations.

Table 4.Human and Rat M₅ PAM Activities of Select Analogs 18^a

compd	hM ₅ EC ₅₀ (nM) (pEC ₅₀ ± SEM)	hM ₅ % ACh Max	rM ₅ EC ₅₀ (nM) (pEC ₅₀ ± SEM)	rM ₅ % ACh Max
18a	500 (6.42 ± 0.26)	83 ± 5	418 (6.40 ± 0.10)	82 ± 4
18g	41 (7.46 ± 0.15)	74 ± 6	74 (7.13 ± 0.04)	76 ± 7
18h	58 (7.35 ± 0.13)	72 ± 7	79 (7.12 ± 0.07)	84 ± 2
18i	76 (7.20 ± 0.12)	79 ± 6	71 (7.19 ± 0.14)	75 ± 8
18j	161 (6.86 ± 0.11)	76 ± 6	196 (6.71 ± 0.02)	81 ± 3

^ahM₅ and rM₅ pEC₅₀ and ACh Max data reported as averages ± SEM from our functional calcium assay; *n* = 3 determinations.