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Diverse Effects on M₁ Signaling and Adverse Effect Liability within a Series of M₁ Ago-PAMs

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Abstract

Both historical clinical and recent preclinical data suggest that the M₁ muscarinic acetylcholine receptor is an exciting target for the treatment of Alzheimer's disease and the cognitive and negative symptom clusters in schizophrenia; however, early drug discovery efforts targeting the orthosteric binding site have failed to afford selective M₁ activation. Efforts then shifted to focus on selective activation of M₁ via either allosteric agonists or positive allosteric modulators (PAMs). While M₁ PAMs have robust efficacy in rodent models, some chemotypes can induce cholinergic adverse effects (AEs) that could limit their clinical utility. Here, we report studies

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J.M.R., M.A., and H.P.C. contributed equally to this work. C.W.L., P.J.C., and C.M.N., and J.M.R. and C.K.J., and A.L.B. oversaw and designed the chemistry, molecular pharmacology, behavioral pharmacology and DMPK, respectively. M.A. and D.W.E., and J.L.E. performed synthetic/medicinal chemistry and scaled-up key compounds for in vivo studies. H.P.C. designed and executed advanced molecular pharmacology assays. K.D.N. and P.M.G.-B. performed molecular pharmacology assays. S.C. and J.J.F. performed DMPK and bioanalysis. C.W.L., P.J.C., and J.M.R. wrote the manuscript.

Notes

The authors are developing M₁ PAMs for the treatment of schizophrenia and AD, and have an open-IND for the same as well as a patent portfolio of M₁ PAMs.

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Supporting Information

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Compound characterization, additional methods and supplemental pharmacology and drug metabolism data (PDF)

aimed at understanding the subtle structural and pharmacological nuances that differentiate efficacy from adverse effect liability within an indole-based series of M₁ ago-PAMs. Our data demonstrate that closely related M₁ PAMs can display striking differences in their in vivo activities, especially their propensities to induce adverse effects. We report the discovery of a novel PAM in this series that is devoid of observable adverse effect liability. Interestingly, the molecular pharmacology profile of this novel PAM is similar to that of a representative M₁ PAM that induces severe AEs. For instance, both compounds are potent ago-PAMs that demonstrate significant interaction with the orthosteric site (either bitopic or negative cooperativity). However, there are subtle differences in efficacies of the compounds at potentiating M₁ responses, agonist potencies, and abilities to induce receptor internalization. While these differences may contribute to the differential in vivo profiles of these compounds, the in vitro differences are relatively subtle and highlight the complexities of allosteric modulators and the need to focus on in vivo phenotypic screening to identify safe and effective M₁ PAMs.

Graphical abstract

Keywords

M₁; muscarinic acetylcholine receptor; agonist; positive allosteric modulator (PAM); ago-PAM; seizure

INTRODUCTION

Both Alzheimer's disease (AD) and schizophrenia represent significant unmet medical needs for which treatment options are limited and offer, at best, only palliative treatment of symptoms and often with intolerable side-effects.^{1,2} Dysregulation and reduction in cholinergic projections to cortical and forebrain regions is a hallmark of AD,³⁻⁹ and specific reductions in densities of M₁ subtype of muscarinic acetylcholine receptor (mAChR) are present in postmortem schizophrenic brain.^{10,11} In addition, M₁ functionally interacts with the *N*-methyl-D-aspartate subtype of the glutamate receptor (NMDAR)¹² and has been shown to reverse behavioral pharmacological (phencyclidine and MK-801) and genetic (NR1 KD mice) NMDA receptor deficits (i.e., the NMDA receptor hypofunction hypothesis of schizophrenia).¹³⁻²³ Over the last 30 years, the M₁ muscarinic acetylcholine receptor has emerged as an attractive molecular target for both of these devastating CNS disorders; however, while early efforts with orthosteric muscarinic agonists (activating M₁-M₅ at varying degrees) showed a trend toward cognitive enhancing efficacy in patients, they failed to meet significant end points for cognition enhancement, a result attributed to dose-limiting nonselective cholinergic agonist adverse effects mediated by the concomitant activation of peripheral M₂ and M₃ receptors.¹³⁻²⁵ Similarly, functionally M₁ selective allosteric agonists garnered interest early on. However, many of these compounds proved to interact with both

the orthosteric and allosteric binding sites of the receptor and bound bitopically to the receptor.^{26–35} This resulted in a lack of true M₁ selectivity as potency was increased,^{33–35} as well as receptor reserve-dependent efficacy (i.e., brain region dependent pharmacology).³⁶ Therefore, with the exception of a recent advancement of an M₁ partial agonist, HTL9936, into clinical trials by Heptares Therapeutics, strategies of targeting the orthosteric binding site were largely abandoned.³⁷

Based on these previous efforts, it has become increasingly clear that targeting a less-conserved, allosteric site on the M₁ receptor, may represent the best path forward for this promising target.^{38–40} Fortunately, this approach proved fruitful with the discovery of BQCA (**1**), the first and prototypical M₁ positive allosteric modulator (PAM).^{41,42} Within the BQCA series, structure–activity relationships (SARs) were steep, with little modifications tolerated in the context of the β -keto acid moiety, as in **1** and **2** (Figure 1).⁴³ Subsequent optimization identified the (*S,S*)-hydroxycyclohexyl amide moiety in **3** as a replacement for the β -ketoacid,⁴⁴ which was then parlayed into the key nonhuman primate tool compound PQCA (**4**)^{45,46} and the advanced M₁ PAM (**5**).⁴⁷ Recently, Scammells and co-workers⁴⁸ further optimized **5** into a novel fused arylpyrimidone core with improved physiochemical properties.

In parallel, we conducted a high-throughput screening (HTS) campaign and identified a novel isatin hit **6** (VU0119498) as an M_{1,3,5} PAM (Figure 1)⁴⁹ from which we were able to develop highly selective M₁ PAMs **7** and **8**,^{50,51} as well as the first selective M₅ PAMs.⁵² Further optimization afforded **9** and, finally, **10** (VU0453595),^{22,53} an important in vivo proof-of-concept tool within this series that is structurally divergent from **5**. A second HTS hit, VU0108370 (**11**), represented a third unique chemotype based on an indole core. Chemical optimization around **11** afforded ML169 (**12**) and by surveying azaindoles, diverse amides and sulfones, as well as significant heterobiaryl development, VU0456940 (**14**) was discovered.^{53–56} Overall, this series had significant liabilities that precluded it from further development as a clinical candidate, including modest to low brain penetration ($K_p/K_{p,uu}$), interaction with the orthosteric site, and varying degrees of allosteric agonist activity. Allosteric agonists are referred to as ago-PAMs or PAM-agonists and defined as an allosteric modulator with PAM pharmacology, but that also activates the receptor in the absence of the orthosteric endogenous ligand similar to an agonist. Typically, the allosteric agonist activity demonstrates weaker potency than the PAM activity and is a feature to be avoided with pro-convulsant CNS targets.^{57,58}

Shortly thereafter, multiple M₁ PAM programs were launched by generating chimeras **15** between the (*S,S*)-hydroxycyclohexyl amide and the indole/azaindole cores (Figure 2).⁵⁹ Davoren et al. then published their series, highlighted by PF-06764427 (**16**), a potent M₁ ago-PAM with reported efficacy in reversing amphetamine-induced hyperlocomotion (AHL) in mice.⁶⁰ This report in particular caught our attention, as we, and others, have not reported efficacy in reversing hyperlocomotion induced by psychostimulants with M₁ PAMs, and instead use cognition models to drive the optimization effort.^{33–45} Moreover, a 2009 study found that the attenuation of amphetamine-induced activity by xanomeline, an M₁/M₄ preferring agonist, was absent in M₄ knockout mice, indicating that the efficacy of xanomeline in amphetamine-induced hyperlocomotion is driven by M₄ activation.^{24,61}

These results were further validated by reports of in vivo efficacy in reversing AHL with M_4 -selective PAMs.^{62,63}

Previous studies provide strong evidence that overactivation of M_1 can induce seizures and fully generalized convulsions in rodent models.^{58,64,65} These findings, coupled with the robust ago-PAM activity of some M_1 -selective PAMs, raises the possibility that direct agonist activity of some M_1 PAMs could contribute to the ability of these compounds to induce seizure activity and other adverse effects.⁵⁷ Furthermore, a propensity of some M_1 PAMs to induce seizure activity, particularly at doses lower than those required to produce generalized behavioral convulsions, could potentially contribute to the reduction in AHL rather than the reflection of potential antipsychotic-like effects. Since the initial report of BQCA, all subsequent reports of M_1 PAMs have been generated in cognition assays (e.g., reversing scopolamine-induced deficits in contextual fear conditioning). Moreover, there is building literature with structurally conserved M_1 ago-PAMs, possessing M_1 agonism-imparting (*S,S*)-hydroxycyclohexyl amide, regarding cholinergic toxicity and other adverse events.^{44,65} It is important to recognize that this class of M_1 ago-PAMs, as well as the BQCA-derived ago-PAMs, possesses low K_p 's (<0.3) and $K_{p,uu}$'s (<0.1), requiring high peripheral exposure to achieve meaningful CNS levels.^{40–46,59} Thus, we performed a series of studies evaluating effects of these previously reported compounds, along with novel difluoro indole congeners **17**, to gain a further understanding of the subtle structural and pharmacological nuances that differentiate efficacy from adverse effect liability within these indole-based M_1 ago-PAMs.

RESULTS AND DISCUSSION

Chemistry and Structure–Activity Relationships (SARs)

The chemistry to access difluoro indole analogues **17** has deviated from that previously published for the sulfone congeners (Scheme 1).^{54,55} Here, commercial 4,6-difluoroindole **18** is a linchpin to access diversely functionalized derivatives **21**, **24**, and **27**. Analogues **21** are readily prepared in four steps by first deprotonation of **18** and alkylation with the desired benzyl bromide to provide **19**. Installation of the trifluoromethyl ketone was achieved in moderate to excellent yields by treatment with TFAA to deliver **20**. Hydrolysis of the trifluoromethyl ketone to the corresponding carboxylic acid and BOP-mediated coupling affords analogues **21** in variable yields. Heterocyclic congeners **24** and **27** required four or five steps. In this case, indole **18** is alkylated with 2-bromo-5-(bromomethyl)pyridine to deliver **22** in 84% yield. A Suzuki coupling (exact conditions depend on the nature of the boronic acid or ester) then gives **23**. Installation of the trifluoromethyl ketone, hydrolysis to the acid and BOP-mediated coupling generates analogues **24**. Alternatively, the trifluoromethyl ketone can first be installed onto indole **18** to yield **25**, followed by repetition of the aforementioned sequences delivering analogues **27**.

We elected to drive the SAR utilizing human M_1 coupled with CNS penetration in a plasma/brain level (PBL) cassette approach.^{55,62} Table 1 highlights select SARs from analogues of **24** and **27**, defined here as **28** in which the (*S,S*)-hydroxycyclohexyl amide is held constant. A diverse array of heterobiaryl motifs were surveyed and found to be well tolerated in terms

of human M₁ PAM potency (hM₁ EC₅₀s 210 nM to 3 μ M), but K_p (0.11 to 0.18) and $K_{p,uu}$ (0.01 to 0.027) were uniformly low (as expected). Deletion of the 2-hydroxy moiety in **28a** led to a 50-fold loss in potency, but the K_p increased to 0.75, strongly suggesting that the critical (*S,S*)-hydroxycyclohexyl amide is responsible for the low CNS penetration of the class. In terms of SAR, both regioisomeric *N*-methyl pyrazoles, **28a** and **28c**, were equipotent and equi-CNS penetrant. All other fluorinated aryl congeners, **28e–g**, were uniformly 4- to 8-fold less potent, and 6-membered heterocycles such as pyridine (**28h**), pyrimidine (**28i**), and pyridazine (**28k**) either lost M₁ PAM potency or offered no advantage in terms of CNS penetration. From this first generation of analogs, **28a** emerged as the most attractive compound.

Next, we held the southern heterobiaryl tail of **28a** constant and surveyed other amide moieties (Table 2) evaluating both hM₁ PAM potency and CNS penetration for analogues **29**. Replacing the cyclohexyl moiety with regioisomeric pyrans, such as **29a** and **29b**, retained or improved hM₁ PAM potency, but both K_p (0.04) and $K_{p,uu}$ (0.01 to 0.02) diminished. Constriction to the cyclobutyl congener **29c** resulted in the loss of ~10-fold PAM activity, and the cyclopentyl derivative **29d** lost ~4-fold; moreover, both displayed reduced CNS penetration relative to **28a**. Interestingly, expansion to the cycloheptyl ring (**29e**) retained potency and doubled the K_p (0.24) and $K_{p,uu}$ (0.08); however, the predicted in vitro hepatic clearance (CL_{hep}) was at rat hepatic blood flow. All other amides surveyed, such as **29f–h**, were inactive.

These data led us to one final library of analogues based on **28a**, where we surveyed additional diversity in the southern tail with analogues **30** (Table 3; Figure 3). Replacing the central pyridine core of the heterobiaryl with a 2-fluorophenyl moiety (**30a**) resulted in a 4-fold loss in PAM potency, and replacement with a 2,6-difluorophenyl ring (**30b**) reduced potency ~10-fold. Moving the pyridine nitrogen, as in **30c**, resulted in a ~5-fold reduction in potency and replacement of the *N*-methyl pyrazole with a methyl group (**30d**) reduced potency by ~8-fold. Interestingly, replacement of the heterobiaryl moiety with a simple 4-*N*-methylacetamide phenyl group (**30e**) retained, and slightly improved, hM₁ PAM potency, but at the expense of CNS penetration. Replacement of the acetamide with a methyl sulfone (**30f**) resulted in a considerable loss of PAM activity. Therefore, **28a** emerged as the key 2,6-difluoroindole analogue for further profiling and to delve into the pharmacodynamic (PD) data reported by Davoren et al.⁵⁹ with **16**. However, to ensure against structural subtleties underlying the PD phenomenon, we also prepared the regioisomeric *N*-methyl pyrazole congener of **28a** (VU6004256) that matches **16** (PF-06764427), **28c** (VU6006251). In addition, we also prepared **31**, the *trans*-racemic congener of **16**, and the regioisomeric *N*-methyl pyrazole congener of **16** that matches **28a**, as both a *trans*-racemate (**32**) as well as the single *trans*-(1*S*,2*S*) analogue **33**. Could a 3-versus 4-*N*-methyl pyrazole result in divergent pharmacology?

Assessment of Adverse Effects

From pioneering pharmacology in the 1990s, studies with the muscarinic agonist pilocarpine and M₁–M₅ knockout (KO) mice have clearly demonstrated the pro-convulsive/seizure liabilities of excessive M₁ activation.^{58,64,65} It has also been shown that potent allosteric

agonist activity must be avoided to minimize adverse effect liability with another pro-convulsant GPCR, mGlu₅, suggesting that a similar strategy might also provide a higher safety window for ligands targeting M₁.⁵⁷ One strategy to allow for early detection of adverse events (AEs) is to administer M₁ PAMs/ago-PAMs at 100 mg/kg intraperitoneally (i.p.) in mice (a species with high susceptibility to cholinergic seizure activity)⁵⁸ within compound workflow to eliminate compounds or chemotypes that may engender AEs in development. Previous unpublished work identified key AEs (pro-convulsive activity) with BQCA and related M₁ ago-PAM congeners in mice. Therefore, we administered 100 mg/kg i.p. of **1**, **16**, **28a**, **28c**, **31**, **32**, and **33** and behavioral convulsions were assessed for 3 h (Figure 4) using the modified Racine scale (0–5).⁵⁷ As anticipated, BQCA (**1**) induced robust seizure activity, reaching stage 4–5 behavioral convulsions on the Racine scale. Likewise, multiple other M₁ PAMs in this class induced fully generalized behavioral convulsions (Figure 4).

Interestingly, neither difluoro indole **28a** nor **28c** (both regioisomeric *N*-Me pyrazoles) induced behavioral convulsions during the 3 h assessment period, nor did the *trans*-racemic congener **31** (VU6005263) or **16** (PF-06764427) (Figure 4). The *trans*-racemic analogue of **31** with the 4-versus 3-*N*-Me pyrazole, **32**, induced robust seizure activity in contrast to both **31** and **28a**. The single *trans*-(1*S*,2*S*)-isomer **33** of racemic **32** also induced seizure activity in mice. Importantly, **16** (PF-06764427) also induced pronounced behavioral convulsions between 30 min and 1 h post-administration, and resulted in Racine scale 5 seizures by 3 h. As shown in Figure 4B, the seizure activity was dose-dependent, with no measurable behavioral convulsions at 10 mg/kg, but becoming significant at 30 mg/kg and 60 mg/kg. However, mice given the 10 mg/kg dose were already displaying significant decreases in spontaneous locomotor activity (Figure 7), raising the possibility that this dose may induce behavioral alterations impacting the interpretation of the AHL data previously published.^{59,65} Additionally, we observed seizure activity with PF-06764427 at a dose as low as 30 mg/kg, i.p., which correlates to total and unbound brain concentrations of 1.95 μ M and 195 nM, respectively, 2 h following administration. This correlates to unbound brain levels of 4-fold and 1.9-fold higher than the M₁ PAM and M₁ allosteric agonist potency (mEC₅₀ = 100 nM, 91% ACh max), respectively. However, a 100 mg/kg, i.p. dose of VU6004256 results in total and unbound maximum brain concentrations of 49.9 μ M and 649 nM, respectively. The free brain levels also correlate to a 4-fold and 1.4-fold increase in PAM and allosteric agonist potency (mEC₅₀ = 450 nM, 86% ACh max), respectively. These data suggest that the brain levels achieved relative to the potency at M₁ does not appear to contribute to the adverse effect liability observed with some M₁ PAMs.

Overall, these data highlight how subtle structural variations in this series of M₁ PAMs can lead to dramatic differences in adverse effect liability.⁶⁶ When comparing **31** to **32**, the regioisomeric *N*-Me pyrazole is critical, as is the aza-versus the difluoro indole core. However, these structural elements alone do not complete the picture, and detailed molecular and behavioral pharmacology studies, as well as assessments in exposure, are required to further elucidate the origins of the AE versus safe profiles.

Molecular and Drug Metabolism Studies

Based on the SARs for the proconvulsant activity, we elected to evaluate the molecular pharmacological profiles of **16**, **28a**, **28c**, **31**, **32**, and **33** across human, rat, and mouse M₁ in both PAM and agonist modes (Tables 4–6) to determine if these M₁ PAMs can be differentiated based on their effects on M₁ signaling.⁶⁰ As shown in Table 4, the difluoro indoles **28a** and **28c** were potent M₁ PAMs on both human (EC₅₀ = 210 nM and EC₅₀ = 224 nM, respectively) and rat (EC₅₀ = 141 nM and EC₅₀ = 191 nM, respectively) receptors with comparable efficacies (86–92% ACh Max) and rat CNS penetration (K_p 's = 0.11, $K_{p,uu}$'s = 0.02); however, they were both 5- to 14-fold less potent than **16** (hEC₅₀ = 31 nM, rEC₅₀ = 30 nM), **31** (hEC₅₀ = 59 nM, rEC₅₀ = 39 nM), **32** (hEC₅₀ = 48 nM, rEC₅₀ = 24 nM), and **33** (hEC₅₀ = 17 nM, rEC₅₀ = 18 nM) but with similar efficacies and K_p 's/ $K_{p,uu}$'s. Thus, absolute M₁ PAM potency was a differentiating point; however, **31** was similarly potent to the M₁ ago-PAMs (**16**, **32**, and **33**) that induced seizures. Therefore, M₁ PAM potency alone or in combination with CNS exposure could not account for the AE liability. In addition, all six ago-PAMs were completely selective for rat and human M₁ over rat and human M₂–M₅ (see Supporting Information Figures 1 and 2); thus, it appears unlikely that AEs are derived from off-target mAChR activity.

Interestingly, differences between the allosteric agonist activity of these compounds at M₁ may provide insight regarding adverse effect liability as opposed to differences in PAM potencies (Table 5), and the raw calcium traces highlight the robust agonist activity of these ago-PAMs (see Supporting Information Figures 3 and 4). Both **28a** and **28c** display modest M₁ agonist activity at both human (EC₅₀ = 4.8 μ M and EC₅₀ = 5.9 μ M, respectively) and rat (EC₅₀ = 4.0 μ M and EC₅₀ = 4.6 μ M, respectively) with comparable efficacies (48–68% ACh Max, depending on the species). The other four ago-PAMs all displayed sub-micromolar agonist activity in our human and rat M₁ cell lines with high agonist efficacy (47–81% ACh Max, depending on the species). Importantly, **16** was an ~600 nM agonist (65% and 77% ACh Max, respectively) at human and rat M₁; in contrast, **28a** was 7-fold weaker M₁ agonist.

Since the AEs were noted in mouse, we also profiled the six ago-PAMs on a murine M₁ cell line (Table 6). Once again, both **28a** and **28c** were potent mouse M₁ PAMs (EC₅₀s of 155 nM and 263 nM, respectively) with robust PAM efficacy (77–79% ACh Max), but 3- to 5-fold less potent than **16** (EC₅₀ = 46 nM, 79% ACh Max) and **31–33**. Mouse CNS penetration was consistent for **16** (K_p = 0.21, $K_{p,uu}$ = 0.16), but was significantly, and unexpectedly, enhanced for **28a** (K_p = 4.84, $K_{p,uu}$ = 2.61). Quickly, our efforts centered on a direct head-to-head comparison of **16** to **28a** as these two ago-PAMs began to clearly diverge in vitro and in vivo. Next, we assessed mouse agonist activity and found both **16** (EC₅₀ = 100 nM, 91% ACh Max) and **28a** (EC₅₀ = 450 nM, 86% ACh Max) had agonist activity at mouse M₁ at sub-micromolar concentrations, yet **16** remained 4.5-fold more potent. To confirm, we also assessed mouse M₁ agonist in a low expressing mouse M₁ cell line, and found similar data, but with diminished ACh Max (15–18%). Therefore, these compounds are both potent M₁ mouse agonists and PAMs.

Previous studies suggest that structurally related M₁ PAMs can also display differences with regards to stimulus bias, and that this can contribute to different in vivo profiles.⁴⁹ Interestingly, in a Discover X bias panel,⁶⁷ both **16** and **28a** displayed comparable M₁ PAM potency and efficacy in a β -arrestin recruitment assay (see Supporting Information Table 1), suggesting no signaling bias in this pathway. However, **28a** had no effect on receptor internalization (EC₅₀ > 10 μ M), whereas **16** induced M₁ internalization with an EC₅₀ of 2.1 μ M. Another question concerned the mechanism of the M₁ agonism and whether this was due to orthosteric or allosteric agonism. To assess the interactions of these M₁ PAMs with antagonist binding to the orthosteric site, we performed the radioligand binding assay with [³H]-NMS in human M₁ membranes (Figure 5). Interestingly, both ago-PAMs induced slight inhibition of binding of the orthosteric radioligand at high concentrations.

Mechanistically, we found that both **16** and **28a** act as PAMs by significantly increasing the affinity of ACh for the M₁ receptor (Figure 6). Here as well, **16** was a more efficacious M₁ PAM, shifting the ACh K_i a maximum of 430-fold to the left (at 10 μ M) versus only 178-fold for **28a**. Even at micromolar concentrations of the ago-PAMs, **16** increased ACh affinity to a 3-fold greater extent than **28a**; however, both showed a significant impact on ACh affinity.

To evaluate other potential differences between **16** and **28a**, we performed a battery of in vitro and in vivo DMPK assays (see Supporting Information Table 2) and found no major differences in terms of plasma protein binding across species, CYP450 profiles or predicted hepatic clearance. Soft-spot analysis in mouse, rat, and human S9 liver microsomes also showed very similar stability and conserved metabolism to the amide headgroup and dealkylation of the pyrazole moiety (see Supporting Information Figures 5 and 6). These metabolites are considerably more polar and thus unlikely to cross the blood-brain barrier. In addition, both ago-PAMs were profiled in a Eurofin Lead Profiling radioligand binding panel (see Supporting Information Tables 3 and 4), against 68 GPCRs, ion channels, and transporters, and found to have no significant ancillary pharmacology (no percent inhibition > 50% at 10 μ M).^{68,69} Thus, we evaluated **16** and **28a** in rodent AHL and other pharmacodynamic assays for which M₁ PAMs have been reported to be efficacious.

Behavioral Pharmacology Assessment

In their recent paper,⁵⁹ Davoren et al. reported pharmacodynamic efficacy of **16** in mouse AHL at dose of 3 and 10 mg/kg, but discussed that there was no dose–response despite increased drug exposure. It is possible that the effect noted may not represent a pharmacological reversal of a hyperdopaminergic state, but may be mediated by an overall decreased basal locomotor activity, thereby not affording a dose–response. Therefore, (Figure 7), we repeated the design of the published study⁵⁹ and found that **16** (PF-06764427) has pronounced effects on spontaneous locomotor activity (Figure 7A) that correlate with reversal of AHL in mice (Figure 7C). In contrast, **28a**, a weaker ago-PAM shows significantly less effect on spontaneous locomotor activity (Figure 7B), and did not reverse AHL in mice (Figure 7D). The effect of **16** is mediated by M₁, as the effect is lost in M₁ knockout (KO) mice (Figure 7E). While these studies do not provide definitive insights into the mechanism by which **16** reduces AHL, it is possible that doses of this compound

that are below doses required for inducing fully generalized convulsions result in seizure activity below the threshold required for behavioral convulsions but that could result in disruption of locomotor activity. The finding that **28a** is devoid of measurable pro-convulsant activity yet has no activity in the AHL assay is consistent with that possibility, and suggests that the ability to decrease AHL is not a common feature of M₁ PAMs.

As mice are more sensitive to cholinergic activation of seizure activity, we next evaluated the two ago-PAMs in rat AHL. As shown in Figure 8, neither M₁ ago-PAM significantly reversed AHL in rats at a dose of 30 mg/kg, which is consistent with the possibility that the proconvulsant activity of **16** in mice may play a role in the reduced locomotor activity in AHL.

As we and others have demonstrated, selective activation of M₁ is well validated for cognitive enhancement in wild-type animals, upon pharmacological challenge, or in genetic models of either AD or NMDA hypofunction.^{22,23,40–44,54} To ensure sufficient potentiation of M₁ was present in the aforementioned rat AHL studies (dosed at 30 mg/kg), we evaluated **16** and **28a** in the novel object recognition paradigm in rats (Figure 9). Here, at doses of 1, 3, and 10 mg/kg, **16** did not enhance cognitive performance in this task ($p = 0.0529$), but did trend toward significance. In contrast, **28a** dose-dependently enhanced recognition memory in rats. Thus, at doses of **28a** that are fully efficacious in rat models of cognitive performance, there is no effect on AHL.

Finally, we performed a Modified Irwin Toxicology Battery test (Figure 10) in mice to assess any potential adverse effects of the ago-PAMs **16** and **28a** on the CNS and GI effects to compare with the seizure data in mice (Figure 4) shown previously. Here, **28a** displayed no pronounced cholinergic effects. In contrast, **16** immediately produced effects on the corneal and pinnae reflex loss, salivation and motor activity. Additionally, the best battery was terminated due to significant behavioral convulsions. These data, coupled with the proconvulsant effects of **16**, suggest that these two M₁ PAMs can be clearly differentiated in terms of their adverse effect liabilities, despite highly conserved chemotypes and only subtle variations in pharmacological profiles.

CONCLUSIONS

We have reported a novel series of M₁ ago-PAMs, represented by **28a**, that have robust activity in potentiating M₁ responses to acetylcholine and in enhancing recognition memory. However, this compound is devoid of the severe adverse effect liability, including behavioral convulsions and peripheral cholinergic AEs that are observed with previously reported M₁ ago-PAMs, such as **16**. Furthermore, our studies suggest that the reported efficacy of M₁ ago-PAMs, such as **16**, in reversing AHL in mice is not observed with other M₁ PAMs and could be related to actions that are not shared by other PAMs, such as proconvulsant activity. Unfortunately, the current data do not provide a clear mechanistic basis for the fundamental differences between these closely related M₁ ago-PAMs. These compounds do display subtle differences in their effects on M₁ signaling that could be relevant for these differences in *in vivo* activities. For instance, **16** has slightly higher *in vitro* efficacy as a PAM than does **28a**. Furthermore, **16** has more potent agonist activity when compared with **28a**. Thus, it is

possible that **16** has a tendency to induce more excessive activation of M₁ than does **28a** and that this contributes to the striking differences in the AE liabilities of these two compounds. However, the differences between these compounds in the assays included here are relatively subtle and are not likely to fully explain the differences of these compounds in terms of adverse effect liability. Recent advances make it increasingly clear that structurally related allosteric modulators can have diverse effects on different aspects of GPCR signaling that can influence their in vivo activity. In addition to differences in agonist activity reported here, this can include differential effects on coupling of GPCRs to different signaling cascades, or stimulus bias, different effects on homodimeric versus heterodimeric forms of receptors, different effects on receptor desensitization, and/or different effects on receptor signaling that are impacted by differences in cell type specific receptor reserve. For instance, we have found that we can avoid adverse effect liability of mGlu₅ PAMs by optimizing compounds that are fully devoid of allosteric agonist activity and display stimulus bias so that they do not potentiate coupling of mGlu₅ to potentiation of NMDA receptor currents. Based on these complexities and data such as those reported in this paper, it is prudent to incorporate in vivo assays of AE liability as an early screening tier when optimizing M₁ PAMs. Furthermore, the potential liabilities of excessive activation of M₁ encourage optimization of compounds that are completely devoid of allosteric agonist activity, as we have done for optimization of mGlu₅ PAMs. Based on the strong data suggesting that M₁ PAMs may have robust therapeutic effects in improving cognitive function in AD and schizophrenia and reducing negative symptoms in schizophrenia, it will be important to optimize drug candidates that are devoid of major adverse effect liabilities and are suitable for advancing through clinical development for treatment of these devastating brain disorders. Further studies to elucidate more definitive underpinnings that give rise to AEs with M₁ ago-PAMs are underway and will be reported in due course.

METHODS

Chemical Synthesis and Purification

All ¹H and ¹³C NMR spectra were recorded on Bruker AV-400 (400 MHz) or Bruker AV-NMR (600 MHz) instrument. Chemical shifts are reported in ppm relative to residual solvent peaks as an internal standard set to δ H 7.26 or δ C 77.0 (CDCl₃) and δ H 3.31 or δ C 49.0 (CD₃OD). Data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, br = broad, m = multiplet), integration, coupling constant (Hz). IR spectra were recorded as thin films and are reported in wavenumbers (cm⁻¹). Low resolution mass spectra were obtained on an Agilent 1200 LCMS with electrospray ionization. High resolution mass spectra were recorded on a Waters Qtof-API-US plus Acquity system. The value Δ is the error in the measurement (in ppm) given by the equation $\Delta = [(ME - MT)/MT] \times 106$, where ME is the experimental mass and MT is the theoretical mass. The HRMS results were obtained with ES as the ion source and leucine enkephalin as the reference. Optical rotations were measured on a PerkinElmer-341 polarimeter. Analytical thin layer chromatography was performed on 250 μ M silica gel 60 F254 plates. Visualization was accomplished with UV light, and/or the use of ninhydrin, anisaldehyde and ceric ammonium molybdate solutions followed by charring on a hot-plate. Chromatography on silica gel was performed using Silica Gel 60 (230–400 mesh) from Sorbent Technologies.

Analytical HPLC was performed on an Agilent 1200 analytical LCMS with UV detection at 214 and 254 nm along with ELSD detection. Solvents for extraction, washing, and chromatography were HPLC grade. All reagents were purchased from Aldrich Chemical Co. and were used without purification. All polymer-supported reagents were purchased from Biotage, Inc. Flame-dried (under vacuum) glassware was used for all reactions. All reagents and solvents were commercial grade and purified prior to use when necessary. High-resolution mass spectrometry (HRMS) data were obtained using a Micromass Q-T of API-US mass spectrometer. For full experimental procedures, please see the Supporting Information.

4,6-Difluoro-N-(1S,2S)-2-hydroxycyclohexyl-1-((6-(1-methyl-1H-pyrazol-4-yl)pyridine-3-yl)methyl)-1H-indole-3-carboxamide (VU6004256, 28a). **1-((6-Bromopyridin)-3-yl)methyl)-4,6-difluoro-1H-indole (22)**—To a round-bottom flask was added at room temperature 4,6-difluoroindole **18** (750 mg, 4.90 mmol) dissolved in DMF (9.8 mL). To the solution was added sodium hydride (392 mg, 9.80 mmol, 60% oil dispersion) at 0 °C. Then 2-bromo-5-(bromomethyl)pyridine (1.48 g, 5.88 mmol) was added to the reaction mixture, followed by stirring at room temperature for 18 h. The reaction was monitored by TLC analysis and upon completion, an aqueous NH₄Cl solution (10 mL) and EtOAc (20 mL) were added to the reaction mixture. The reaction mixture was extracted with EtOAc (20 mL × 2). The organic layers were combined, dried over MgSO₄, filtered, and concentrated in vacuo. The crude material was purified on SiO₂ gel by flash column chromatography eluting with 0 to 100% hexane/EtOAc to afford 1.52 g (96% yield) of 1-[(6-bromo-3-pyridyl)methyl]-4,6-difluoro-1H-indole, **22**. LCMS: R_t = 1.128 min; >93% pure at 254 nm, *m/z* = 323 (M + H). ¹H NMR (400 MHz, CDCl₃) δ ppm): 8.25 (d, *J* = 2.1 Hz, 1H), 7.42 (d, *J* = 8.2 Hz, 1H), 7.16 (dd, *J* = 8.2 Hz, 2.1 Hz, 1H), 7.05 (d, *J* = 3.2 Hz, 1H), 6.70 (d, *J* = 9.1 Hz, 1H), 6.65–6.58 (m, 2H), 5.23 (s, 2H).

4,6-Difluoro-1-((6-Methyl-1H-pyrazol-4-yl)pyridine-3-yl)methyl)-1H-indole (23)—In a microwave vial, 1-[(6-bromo-3-pyridyl)methyl]-4,6-difluoro-1H-indole **22** (1.50 g, 4.64 mmol), 1-methylpyrazole-4-boronic acid pinacol ester (1.16 g, 5.52 mmol), potassium phosphate tribasic monohydrate (1.07 g, 4.46 mmol), tricyclohexylphosphine (32 mg, 0.116 mmol), and Pd₂(dba)₃ (42 mg, 0.0464 mmol) were added. The vessel was charged with a stir bar and dioxane/H₂O (2:1, 15.5 mL), and then sealed with a crimped-cap. The reaction was heated to 120 °C for 30 min in the microwave. After the reaction had cooled to ambient temperature, the reaction mixture was diluted with 1:1 CH₂Cl₂/water (20 mL). The layers were separated, and the aqueous layer was extracted with CH₂Cl₂ (10 mL × 2). The combined organic layers were dried over MgSO₄, filtered, and concentrated in vacuo. The crude material was purified on SiO₂ gel by flash column chromatography eluting with 0 to 100% hexane/EtOAc to afford 1.39 g (93% yield) of 4,6-difluoro-1-((6-methyl-1H-pyrazol-4-yl)pyridine-3-yl)methyl)-1H-indole, **23**. LCMS: R_t = 0.826 min; >98% pure at 254 nm, *m/z* = 325 (M + H). ¹H NMR (400 MHz, CDCl₃) δ ppm): 8.45 (s, 1H), 8.28 (br, 1H), 7.95 (s, 1H), 7.54 (d, *J* = 8.0 Hz, 1H), 7.47 (d, *J* = 8.0 Hz, 1H), 7.08 (d, *J* = 3.2 Hz, 1H), 6.74 (d, *J* = 8.9 Hz, 1H), 6.68–6.60 (m, 2H), 5.30 (s, 2H), 3.97 (s, 3H).

1-(4,6-Difluoro-1-((6-(1-methyl-1H-pyrazol-4-yl)pyridin-3-yl)-methyl)-1H-indol-3-yl)-2,2,2-trifluoroethan-1-one—To a round-bottom flask was added at room temperature 4,6-difluoro-1-((6-(1-methyl-1H-pyrazol-4-yl)pyridine-3-yl)methyl)-1H-indole, **23** (1.39 g, 4.29 mmol) dissolved in DMF (4.3 mL) and trifluoroacetic anhydride (894 μ L, 6.43 mmol). The resultant mixture was stirring at room temperature for 48 h. Upon completion of the reaction by LC/MS, a NaHCO₃ (saturated aqueous solution, 20 mL) and CH₂Cl₂ (20 mL) were added to the reaction mixture. The layers were separated, and the aqueous layer was extracted with CH₂Cl₂ (10 mL $\times$ 2). The combined organic layers were dried over MgSO₄, filtered, and concentrated in vacuo to afford 1.48 g (82% yield) of 1-(4,6-difluoro-1-((6-(1-methyl-1H-pyrazol-4-yl)pyridine-3-yl)methyl)-1H-indol-3-yl)-2,2,2-trifluoroethan-1-one. The residue was used in subsequent reactions without further purification. LCMS: R_t = 0.888 min; >98% pure at 254 nm, m/z = 421 (M + H). ¹H NMR (400 MHz, CDCl₃) δ ppm): 8.53 (s, 1H), 8.09 (br, 1H), 8.00 (s, 1H), 7.95 (s, 1H), 7.50 (d, J = 8.2 Hz, 1H), 7.45 (d, J = 8.2 Hz, 1H), 6.89–6.82 (m, 2H), 5.38 (s, 2H), 3.96 (s, 3H).

4,6-Difluoro-1-((6-(1-methyl-1H-pyrazol-4-yl)pyridin-3-yl)-methyl)-1H-indol-3-carboxylic acid—To a microwave vial was added 1-(4,6-difluoro-1-((6-(1-methyl-1H-pyrazol-4-yl)pyridine-3-yl)methyl)-1H-indol-3-yl)-2,2,2-trifluoroethan-1-one (1.28 g, 3.06 mmol). The vessel was charged with a stir bar and water (7.6 mL) was added, followed by sodium hydroxide (134 mg, 3.36 mmol) and then sealed with a crimped-cap. The reaction was heated to 150 °C for 30 min in the microwave. The reaction was cooled to ambient temperature. Upon completion of the reaction by LC/MS, the reaction mixture was concentrated and the residue was taken up in water (10 mL). Then 12N HCl was added dropwise to adjust the pH to 2. The resultant solid was filtered, washed with water, and dried in vacuo to provide 833 mg (74% yield) of 4,6-difluoro-1-((6-(1-methyl-1H-pyrazol-4-yl)pyridin-3-yl)-methyl)-1H-indol-3-carboxylic acid. LCMS: R_t = 0.636 min; >95% pure at 254 nm, m/z = 369 (M + H). ¹H NMR (400 MHz, (CD₃)₂SO) δ ppm): 8.60 (s, 1H), 8.36 (s, 1H), 8.27 (s, 1H), 7.98 (s, 1H), 7.70 (d, J = 8.3 Hz, 1H), 7.63 (d, J = 8.3 Hz, 1H), 7.52 (d, J = 9.1 Hz, 1H), 7.01 (t, J = 9.1 Hz, 1H), 5.51 (s, 2H), 3.90 (s, 3H).

4,6-Difluoro-N-(1S,2S)-2-hydroxycyclohexyl-1-((6-(1-methyl-1H-pyrazol-4-yl)pyridine-3-yl)methyl)-1H-indole-3-carboxamide (VU6004256, 28a)—To a vial at room temperature were added 4,6-difluoro-1-((6-(1-methyl-1H-pyrazol-4-yl)pyridin-3-yl)methyl)-1H-indol-3-carboxylic acid (250 mg, 0.679 mmol), (1S, 2S)-2-aminocyclohexanol (94 mg, 0.814 mmol), BOP reagent (benzotriazole-1-yl-oxy-tris(dimethylamino)-phosphonium hexafluorophosphate) (390 mg, 0.882 mmol), and triethylamine (378 μ L, 2.72 mmol), which were then dissolved in DMF (2.5 mL). This mixture was stirred for 20 min. The reaction was monitored by LC/MS and upon completion, the reaction mixture was diluted with 1:1 CH₂Cl₂/water (10 mL). The layers were separated, and the aqueous layer was extracted with CH₂Cl₂ (5 mL $\times$ 2). The organic layers were combined, passed through a phase separator, and concentrated in vacuo. The crude material was purified on SiO₂ gel by flash column chromatography eluting with 0 to 10% CH₂Cl₂/MeOH and then via reverse-phase preparative HPLC eluting with 24 to 79% H₂O/acetonitrile with 0.1% TFA. The obtained residue was stirred for 1 h in hexane/Et₂O (1:1). Then, the deposited solid was collected by filtration and dried under reduced pressure

to afford 219 mg (69% yield) of 4,6-difluoro-*N*-(1*S*,2*S*)-2-hydroxycyclohexyl-1-((6-(1-methyl-1*H*-pyrazol-4-yl)pyridine-3-yl)methyl)-1*H*-indole-3-carboxamide (VU6004256, **28a**). Specific rotation $[\alpha]_D^{23} = -18.8^\circ$ ($c = 1.0$, CHCl_3); LCMS: $R_t = 0.814$ min; ^1H NMR (400 MHz, CD_3OD) δ ppm): 8.46 (s, 1H), 8.15 (s, 1H), 8.01 (s, 2H), 7.64 (s, 2H), 7.21 (dd, $J = 10.8$ Hz, 1.6 Hz, 1H), 6.86 (dt, $J = 1.6$ Hz, 10.8 Hz, 1H), 5.49 (s, 2H), 3.96 (s, 3H), 3.86–3.77 (m, 1H), 3.54–3.46 (m, 1H), 2.20–2.02 (m, 2H), 1.85–1.71 (m, 2H), 1.50–1.23 (m, 4H); ^{13}C NMR (100.6 MHz, $(\text{CD}_3)_2\text{SO}$) δ ppm): 163.5, 159.4 (dd, $J_{\text{C-F}} = 237$ Hz, 12 Hz), 155.9 (dd, $J_{\text{C-F}} = 249$ Hz, 15 Hz), 152.3, 149.6, 138.9 (t, $J_{\text{C-F}} = 15$ Hz), 138.0, 137.0, 134.1, 130.5, 130.4, 123.3, 120.1, 112.4, 111.4 (d, $J_{\text{C-F}} = 19$ Hz), 97.6 (t, $J_{\text{C-F}} = 28$ Hz), 95.0 (d, $J_{\text{C-F}} = 31$ Hz), 72.3, 55.7, 47.9, 39.7, 35.0, 31.9, 25.1, 24.8 HRMS: calcd for $\text{C}_{25}\text{H}_{25}\text{N}_5\text{O}_2\text{F}_2$, 465.1976; found, 465.1979.

Cell Lines

Chinese hamster ovary (CHO) cells stably expressing human and rat M_1 were maintained in Ham's F-12 growth medium containing 10% FBS, 20 mM HEPES, antibiotic/antimycotic, and 500 $\mu\text{g}/\text{mL}$ G418 in the presence of 5% CO_2 at 37 °C as previously described.⁴⁹ To determine the functional activity at mouse M_1 , the mouse M_1 full-length open reading frame (ORF) was amplified from a mouse brain cDNA library (Clontech Laboratories, Mountain View, CA) and subcloned into the BamH1 and EcoR1 sites of pcDNA3.1 (+) vector (Life Technologies, Carlsbad, CA). Sequencing of the mouse M_1 -pcDNA3.1 (+) plasmid confirmed the presence of mouse M_1 ORF (NM_007698). CHO cells were transfected with mouse M_1 expression plasmid using Fugene 6 (Promega, Madison, WI), and the transfected cells were used for calcium mobilization to assess the functional activity at mouse M_1 . Also, the transfected cells were incubated with the selection medium containing 1 mg/mL G418 for 2 weeks, and the resulting polyclones were then plated in a 96-well plate for monoclonal generation. The positive monoclonal clones of mouse M_1 expression were identified in the calcium mobilization assay described below.

Calcium Mobilization Assay

To determine the potency and efficacy of M_1 ago-PAMs, calcium flux was measured using the Functional Drug Screening System (FDSS7000, Hamamatsu, Japan) as previously described.⁶² Briefly, the M_1 -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 (CRC), 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.⁴⁹ The CRC of 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 agonist and PAM.

Radioligand Binding Assay

Competition binding assay was performed using [^3H]-*N*-methylscopolamine ([^3H]-NMS, PerkinElmer, Boston, MA) as previously described.⁴⁸ Briefly, compounds were serially diluted 1:3 in DMSO for 11 point CRC, then further diluted at starting final concentration at 10 μM in binding buffer (20 mM HEPES, 10 mM MgCl_2 , and 100 mM NaCl, pH 7.4). During the dilution of the concentrated compound stocks in the binding buffer, stock solutions more than 20 μM were sonicated due to insolubility issue before adding to the binding assay plate. Membranes from human M_1 -CHO cells (10 μg) were incubated with the serially diluted compounds in the presence of K_d concentration of [^3H]-NMS, 0.075 nM, at room temperature for 1 h with constant shaking. Nonspecific binding was determined in the presence of 10 μM 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 μL of Microscint20 was added to the plate, and radioactivity was counted using a TopCount Scintillation Counter (PerkinElmer Life and Analytical Sciences).

For the measurement of the acetylcholine affinity at hM_1 , all binding conditions were the same as those for competition binding with the exception of addition of varying concentration of acetylcholine (serially diluted at 11 point CRC) in the presence of vehicle or the M_1 ago-PAMs (10, 1, and 0.3 μM).

Drug Metabolism Methods

In Vitro. Protein binding of M_1 PAMs were determined in rat plasma via equilibrium dialysis employing Single-Use RED Plates with inserts (ThermoFisher Scientific, Rochester, NY). Briefly, plasma (220 μL) was added to the 96-well plate containing test article (5 μL) and mixed thoroughly. Subsequently, 200 μL of the plasma-test article mixture was transferred to the *cis* chamber (red) of the RED plate, with an accompanying 350 μL of phosphate buffer (25 mM, pH 7.4) in the *trans* chamber. The RED plate was sealed and incubated 4 h at 37 $^\circ\text{C}$ with shaking. At completion, 50 μL aliquots from each chamber were diluted 1:1 (50 μL) with either plasma (*cis*) or buffer (*trans*) and transferred to a new 96-well plate, at which time ice-cold acetonitrile (2 volumes) was added to extract the matrices. The plate was centrifuged (3000 rpm, 10 min), and supernatants transferred to a new 96-well plate. The sealed plate was stored at -20°C until LC/MS/MS analysis. The metabolism of M_1 PAMs was investigated in multispecies hepatic microsomes (BD Biosciences, Billerica, MA) using substrate depletion methodology (percent of test article remaining). A potassium phosphate-buffered reaction mixture (0.1 M, pH 7.4) of test article (1 μM) and microsomes (0.5 mg/mL) was preincubated (5 min) at 37 $^\circ\text{C}$ prior to the addition of NADPH (1 mM). The incubations, performed in 96-well plates, were continued at 37 $^\circ\text{C}$ under ambient oxygenation and aliquots (80 μL) were removed at selected time intervals (0, 3, 7, 15, 25, and 45 min). Protein was precipitated by the addition of chilled acetonitrile (160 μL), containing glyburide as an internal standard (50 ng/mL), and centrifuged at 3000 rpm (4 $^\circ\text{C}$).

for 10 min. Resulting supernatants were transferred to new 96-well plates in preparation for LC/MS/MS analysis. The in vitro half-life ($t_{1/2}$, min, eq 1), intrinsic clearance (CL_{int} , mL/min/kg, eq 2), and subsequent predicted hepatic clearance (CL_{hep} , mL/min/kg, eq 3) were determined employing the following equations:

$$t_{1/2} = \ln(2)/k \quad (1)$$

where k represents the slope from linear regression analysis (percent of test article remaining).

$$CL_{int} = (0.693/t_{1/2}) \times (\text{rxn volume/mg of microsomes}) \\ \times (45 \text{ mg of microsomes/g of liver}) \\ \times (20^a \text{ g of liver/kg of body weight}) \quad (2)$$

where the a notes scale-up factors of 20 (human) and 45 (rat) and Q is hepatic perfusion.

$$CL_{hep} = \frac{Q \times CL_{int}}{Q + CL_{int}} \quad (3)$$

In Vivo—Male Sprague–Dawley rats ($n = 2$) weighing around 300 g were purchased from Harlon laboratories (Indianapolis, IN) and implanted with catheters in the carotid artery and jugular vein. The cannulated animals were acclimated to their surroundings for approximately 1 week before dosing and were provided food and water ad libitum. M_1 PAMs were administered intravenously (i.v.) to rats in cassette format via the jugular vein catheter in EtOH/PEG400/Saline at a total dose of 1 mg/kg (0.20 or 0.25 mg/kg per compound) and a dose volume of 1 mL/kg. Brain dissection and blood collections via the carotid artery were performed at 0.25 h post dose. Samples were collected into chilled, EDTA-fortified tubes, centrifuged for 10 min at 3000 rpm (4 °C), and resulting plasma aliquoted into 96-well plates for LC/MS/MS analysis. The brain samples were rinsed in PBS, snap frozen and stored at –80 °C. Prior to LC/MS/MS analysis, brain samples were thawed to room temperature and subjected to mechanical homogenation employing a Mini-Beadbeater and 1.0 mm Zirconia/Silica Beads (BioSpec Products). All animal studies were approved by the Vanderbilt University Medical Center 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. In Vivo Experiments—

M_1 PAMs were analyzed via electrospray ionization (ESI) on an AB Sciex API-4000 (Foster City, CA) triple-quadrupole instrument that was coupled with Shimadzu LC-10AD pumps (Columbia, MD) and a Leap Technologies CTC PAL autosampler (Carrboro, NC). Analytes were separated by gradient elution using a Fortis C18 2.1 × 50 mm, 3.5 μm column (Fortis Technologies Ltd., Cheshire, U.K.) thermostated at 40 °C. HPLC mobile phase A was 0.1%

NH₄OH (pH unadjusted), and mobile phase B was acetonitrile. The gradient started at 30% B after a 0.2 min hold and was linearly increased to 90% B over 0.8 min; held at 90% B for 0.5 min and returned to 30% B in 0.1 min followed by a re-equilibration (0.9 min). The total run time was 2.5 min, and the HPLC flow rate was 0.5 mL/min. The source temperature was set at 500 °C and mass spectral analyses were performed using multiple reaction monitoring (MRM) utilizing a Turbo-Ionspray source in positive ionization mode (5.0 kV spray voltage). All data were analyzed using AB Sciex Analyst 1.4.2 software.

In Vitro Experiments—M₁ PAMs were analyzed similarly to that described above (in vivo) with the following exceptions: LC/MS/MS analysis was performed employing a TSQ Quantum^{ULTRA} instrument that was coupled to a ThermoSurveyor LC system (Thermoelectron Corp., San Jose, CA) and a Leap Technologies CTC PAL autosampler (Carrboro, NC). Chromatographic separation of analytes was achieved with an Acquity BEH C18 2.1 × 50 mm, 1.7 μm column (Waters, Taunton, MA).

Mouse Plasma-Brain Exposure

M₁ PAMs were dissolved in 10% Tween 80 at the concentration of 1–10 mg/mL (base form) and administered intraperitoneally to male C57BL/6J (Jackson Laboratory, Sacramento, CA) mice aged 7–9 weeks at a volume of 10 mL/kg. The blood and brain were collected at 0.25 h. Animals were euthanized and decapitated, and the brains were removed, thoroughly washed with cold saline and immediately frozen on dry ice. Trunk blood was collected in EDTA coated Eppendorf tubes, and plasma was separated by centrifugation and stored at –80 °C until processed for LC/MS/MS analysis as previously described. Three animals were used for each time point.

In Vivo Studies

Animals—All present studies used 7–8 week old male C57BL/6 mice (Jackson Laboratory, Sacramento, CA) or male Sprague–Dawley rats weighing 275–300 g (Envigo, Indianapolis, IN), which were cared for in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All experimental procedures were approved by the Vanderbilt University Animal Care and Use Committee.

Spontaneous Locomotor Activity—The effects of M₁ PAMs on basal locomotor activity were assessed. Mice were administered vehicle or M₁ PAM (1–10 mg/kg, intraperitoneal (i.p.), 10% Tween 80 in water, 10 mL/kg, *n* = 12) and placed into an open field arena (MedAssociates, St. Albans, VT) following a 30 min pretreatment. Locomotor activity was then measured for 90 min. Changes in locomotor activity were measured as the total distance traveled (cm). Data are expressed as mean ± SEM and analyzed using one-way analysis of variance (ANOVA) with a Dunnett's post hoc test comparing all dosing groups to vehicle + amphetamine-treated controls. Statistical significance was determined as *p* < 0.05.

Amphetamine-Induced Hyperlocomotion—The effects of M₁ PAMs on amphetamine-induced hyperlocomotion were determined as previously described.⁶² Discovery of novel allosteric modulators of metabotropic glutamate receptor subtype 5 reveals chemical and functional diversity and in vivo activity in rat behavioral models of anxiolytic and

antipsychotic activity. *Mol. Pharmacol.* 78, 1105–1123). For studies in mice, animals were placed in an open field arena (MedAssociates, St. Albans, VT) for 90 min. At $t = 90$, mice were administered vehicle or M₁ PAM (1–10 mg/kg, i.p., 10% Tween 80 in water, 10 mL/kg, $n = 12$) or vehicle followed by amphetamine (3 mg/kg, subcutaneous (s.c.), saline, 10 mL/kg). Locomotor activity was then measured for an additional 60 min. Changes in locomotor activity were measured as the total distance traveled (cm) per 5 min bins. For locomotor activity assessment in rats, rats were placed in the activity chambers (KinderScientific, San Diego, CA) to habituate. At $t = 30$, rats were administered M₁ PAM (30 mg/kg i.p., 10% Tween 80 in water, 5 mL/kg) or vehicle and placed back in the activity chambers. Five minutes later the animals were injected with amphetamine (1 mg/kg s.c.; 1 mL/kg) or saline and placed back in the activity chambers. Locomotor activity was then measured for an additional 60 min. Changes in locomotor activity were measured as the total number of beam breaks per 5 min bins. Data are expressed as mean $\pm$ SEM and analyzed using one-way ANOVA (last 60 min) with a Dunnett's post hoc test comparing all dosing groups to vehicle + amphetamine-treated controls. Statistical significance was determined as $p < 0.05$.

Behavioral Manifestations of Seizure Activity—To evaluate induction of behavioral manifestation of seizure activity, C57Bl/6 mice received administration of vehicle or 100 mg/kg M₁ PAM. Compounds were formulated in 10% Tween 80 (pH 7.0) at a concentration of 10 mg/mL and injected i.p. ($n = 3$). Animals were monitored continuously and scored for behavioral manifestations of seizure activity at 5, 10, 15, and 30 min, and 1 and 3 h. Behavioral manifestations of seizures were scored using a modified Racine scoring system.⁷⁰ Modification of seizure activity by electrical stimulation. II. Motor seizure. *Electroencephalogr. Clin. Neurophysiol.* 32, 281). Briefly, a score of 0 represents no behavior alterations; score 1, immobility, mouth and facial movements, or facial clonus; score 2, head nodding, tail extension; score 3, forelimb clonus, repetitive movements; score 4, rearing and tonic clonic seizure; and score 5, continuous rearing and falling, severe generalized tonic clonic seizure.

Modified Irwin Toxicology Battery—The potential CNS adverse effects of M₁ PAMs were evaluated using the Modified Irwin Toxicology Battery. Baseline assessments were conducted prior to administration of compounds. Mice were then administered vehicle or M₁ PAM (100 mg/kg, 10% Tween 80, 10 mL/kg, i.p., $n = 3$), placed back into their home cages and evaluated by an observed blinded to dosing conditions at 15, 30, 60, 180, and 360 min for autonomic nervous and somatomotor system parameters outlined in Figure 10. Mice were assigned a score for each parameter of 0 (normal), 1 (slight/light), 2 (moderate), or 3 (marked) relative to vehicle-treated controls.

Novel Object Recognition Task—Rats were habituated for 10 min for 2 consecutive days in an empty novel object recognition (NOR) arena consisting of dark-colored plexiglass box (40 $\times$ 64 $\times$ 33 cm³). On day 3, rats were administered vehicle (10% tween 80) or M₁ PAM (1–10 mg/kg, i.p., 3 mL/kg, $n = 12$) and returned to their home cage for 30 min. Rats were then placed in the NOR arena containing two identical objects for 10 min. Following the exposure period, rats were placed back into their home cages for 24 h. The rats were then returned to the arena in which one of the previously exposed (familiar) objects was replaced

by a novel object and were video recorded for 5 min while they explored the two objects. Time spent exploring each object was scored by an observer blinded to the experimental conditions and the recognition index was calculated as [(time spent exploring novel object) – (time spent exploring familiar object)]/total time exploring objects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

i.p	intraperitoneal
p.o	oral dosing
LTS, GPCR	G protein-coupled receptor
M₁	muscarinic acetylcholine receptor subtype 1
NOR	novel object recognition
AHL	amphetamine-induced hyperlocomotion

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Figure 1.
Conserved structural motifs present in M₁ PAMs from Merck (1–5) and the Vanderbilt Center for Neuroscience Drug Discovery (VCNDD) (6–14).

Figure 2.

Recent scaffold hopping exercises, employing **3** and **13**, resulted in the patent applications listed above surrounding **15**, and an intriguing publication focused on **16**. Here, we disclose analogues **17**, derived from **13**, and insight into how to differentiate efficacy from adverse effect liability.

Figure 3. Structures of the six M₁ PAMs, both aza-indoles (**16**, **31–33**) and 4,6-difluoro indoles (**28a** and **28c**) that will be the subject of in-depth in vitro and in vivo characterization to understand subtle nuances that differentiate efficacy from adverse effects.

Figure 4.

Induction of behavioral convulsions by structurally distinct M₁ ago-PAMs. (A) Mice were administered 100 mg/kg compound, and seizure activity was measured for 3 h using the modified Racine scale (0–5). Compounds were formulated in 10% Tween 80 (10 mg/mL, pH 7.0) and injected intraperitoneally. BQCA (**1**), PF-06764427 (**16**), VU6004877 (**32**), and VU6006270 (**33**) induced robust behavioral convulsions, while VU6004256 (**28a**), VU6006251 (**28c**), and VU6005263 (**31**) did not display observable behavioral seizure activity. Data represent mean $\pm$ SEM ($n = 3$). (B) Dose-dependent induction of behavioral convulsions by M₁ PAM PF-06764427 (**16**). Mice were administered 10, 30, or 60 mg/kg compound, and seizure activity was measured for 2 h using the modified Racine scale (0–5). PF-06764427 (**16**) induced dose-dependent behavioral convulsions. Data represent mean $\pm$ SEM ($n = 4$).

Figure 5.

Inhibition of orthosteric radioligand binding with [³H]-NMS by **16** and **28c**. Both **16** and **28a** weakly displaced the orthosteric radioligand, [³H]-NMS (0.075 nM) in competition binding studies. As a positive control, the orthosteric antagonist, atropine, potently inhibited [³H]-NMS binding with a K_i of 0.9 nM (in agreement with the literature). These data suggest that both PAMs have some interaction with the orthosteric site at higher concentrations, suggesting, perhaps, a bitopic mode of activity. Data represent the mean $\pm$ SEM of three independent experiments.

Figure 6.

Evaluating the ability of M₁ PAMs **16** and **28a** to increase ACh affinity at the M₁ receptor. (A) In the presence of increasing fixed concentrations (0.3–10.0 μ M) of **16** (PF-06764427), the potency of ACh to displace [³H]-NMS binding is shifted leftward, yielding K_i values of 4847 ± 1230 (DMSO), 149 ± 49 nM (0.3 μ M **16**), 35 ± 4 nM (1.0 μ M **16**), and 11 ± 1 nM (10 μ M **16**), which represent 32-fold, 139-fold, and 430-fold shifts in ACh potency, respectively, when compared to the DMSO control. (B) In the presence of increasing fixed concentrations (0.3–10.0 μ M) of **28a** (VU6004256), the potency of ACh to displace [³H]-NMS binding is shifted leftward, yielding K_i values of 4599 ± 720 (DMSO), 426 ± 45 nM (0.3 μ M **28a**), 80 ± 10 nM (1.0 μ M **28a**), and 26 ± 1 nM (10 μ M **28a**), which represent 5-fold, 11-fold, 58-fold, and 178-fold shifts in ACh potency, respectively, when compared to the DMSO control. Data represent the mean $\pm$ SEM of three independent experiments, performed in duplicate.

Figure 7.

Effects of M₁ PAMs on spontaneous locomotor activity and amphetamine-induced hyperlocomotion. (A) PF-06764427 caused a dose-dependent reduction in basal locomotor activity with significant decreases observed beginning at the lowest dose of 1 mg/kg. (B) VU6004256 decreased locomotor activity at the highest dose of 10 mg/kg. Data represent mean distance traveled (cm) per 5 min intervals $\pm$ SEM ($n = 12$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (C, D) PF-06764427 dose-dependently reversed amphetamine-induced hyperlocomotion (AHL) in mice. Data are expressed as the mean total distance traveled (cm) per 5 min intervals $\pm$ SEM ($n = 12$). (E) Reversal of AHL by PF-06764427 is absent in M₁ KO mice.

Figure 8.

Reversal of amphetamine-induced hyperlocomotion in rats. PF-06764427 (**16**) slightly reversed AHL in SD rats, whereas VU6004256 (**28a**) has no effect. Data are represented as the mean ambulations $\pm$ SEM.

Figure 9.

Novel object recognition. VU6004256 (**28a**) dose-dependently enhanced recognition memory in rats. Pretreatment with 3 and 10 mg/kg VU6004256 (i.p., 10% Tween 80 in water, 30 min) prior to exposure to identical objects significantly enhanced recognition memory assessed 24 h later. PF-06764427 (**16**) did not enhance cognitive performance in this task. Data are expressed as mean $\pm$ SEM ($n = 12$). Statistical analysis was completed using a one-way analysis of variance. If significant ($p < 0.05$), comparison of group effects relative to the vehicle group was completed using a Dunnett's test, * $p < 0.05$.

	Time (min)	VU6004256					PF-06764427	
		15	30	60	180	360	15	30
Autonomic Nervous System		-	-	-	-	-		
Ptois		-	-	-	-	-	-	-
Exophthalmus		-	-	-	-	-	-	-
Miosis		-	-	-	-	-	-	-
Mydriasis		++	++	-	-	-	-	-
Corneal reflex loss		-	-	-	-	-	++	++
Pinna reflex loss		-	-	-	-	-	++	++
Piloerection			++	-	-	-	-	-
Respiratory rate		-	-	-	-	-	-	-
Writhing		-	-	-	-	-	-	-
Tail erection		-	-	-	-	-	-	-
Lacrimation		-	-	-	-	-	-	-
Salivation		-	-	-	-	-	+	++
Vasodilatation		-	-	-	-	-	-	-
Skin color		-	-	-	-	-	-	-
Irritability		-	-	-	-	-	-	-
Somatomotor systems		-	-	-	-	-	-	-
Motor activity		-	-	-	-	-	++	++
Convulsions		-	-	-	-	-	-	-
Arch/roll		-	-	-	-	-	-	-
Tremors		-	-	-	-	-	-	-
Leg weakness		-	-	-	-	-	-	-
Rigid stance		-	-	-	-	-	-	-
Spraddle		-	-	-	-	-	-	-
Placing loss		-	-	-	-	-	-	-
Grasping loss		-	++	-	-	-	-	-
Righting loss		-	-	-	-	-	-	-
Catalepsy		-	-	-	-	-	-	-
Tail pinch		-	-	-	-	-	-	-
Escape loss		++	++	-	-	-	-	-
The mean scores of eight animals per treatment are classified as follows:								
- no effect		(+) 0.01 – 0.25	+ 0.251 – 0.50		(++) 0.51 – 1.0			
++ 1.01 - 1.50		(+++)	1.51 - 2.0		+++ 2.01 - 2.50		(++++)	
						2.51 - 3.0		

Figure 10.

Modified Irwin Toxicology Battery test in rodents was used to assess any potential adverse effects of novel compounds on the CNS. To assess CNS and GI effects, animals were assessed prior to treatment to obtain baseline measurements. After administration of vehicle or compound, animals were evaluated at 15, 30, 50, 180, and 360 min for a number of behavioral and physiological changes from pretreatment baselines. An observer blinded to the compound treatment evaluated a series of autonomic nervous system, somatomotor system assessments and scored them on a scale from 0 (normal) to 3 (severe). The mean Modified Irwin Toxicology Battery test scores are classified as follows: – no effect, + 0.01–0.25, +0.251–0.50, (++) 0.51–1.0, ++ 1.01–1.50, (++++) 1.51–2.0, +++ 2.01–2.5, (+++++) 2.51–3.0. Pretreatment of VU6004256 (100 mg/kg, i.p.) did not cause significant observable adverse effects in normal, healthy mice as assessed by the modified Irwin Toxicology Battery test. However, the previously published M₁ PAM, PF-06764427, caused significant prolonged behavioral convulsions in mice beginning at 60 min, preventing continuation of the CNS assessment.

Scheme 1. Synthesis of VU0456940 Hybrid Amide M₁ PAMs^a

^aReagents and conditions: (a) NaH, ArCH₂Br, DMF, rt, 96–99%; (b) TFAA, DMF, rt, 38–82%; (c) NaOH, H₂O/MeOH, 90–150 °C, then HCl, 14–83%; (d) NEt₃, RNH₂, BOP, DMF, rt, 7–77%; (e) Cs₂CO₃, 2-bromo-5-(bromomethyl)pyridine, DMF, rt, 84%; (f) Pd₂(dba)₃, Cy₃P, Het-B(OH)₂ or Het-B(OR)₂, K₃PO₄, dioxane/H₂O, 120 °C, 61–99%.

Structures and Activities of Representative VU0456940 Hybrid Amide Analogues 28 Surveying Diverse Heterobiaryl Motifs^a**Table 1**

28							
Compound	Ar (Het)	hM ₁ EC ₅₀ (μM)	hM ₁ pEC ₅₀	hACh Max (%)	Rat K _p (K _{p_{muu}}) ^b		
28a		0.21	6.67±0.05	86±1	0.113 (0.025)		
28b		3.0	5.53±0.07	77±8	ND		
28c		0.22	6.65±0.05	86±1	0.115 (0.027)		
28d		0.85	6.07±0.05	78±5	ND

<p style="text-align: center;">28</p>					
Compound	Ar (Het)	hM ₁ EC ₅₀ (μM)	hM ₁ pEC ₅₀	hACh Max (%)	Rat K _p (K _{pnuu}) ^b
28e		2.2	5.65±0.01	55±5	ND
28f		1.3	5.90±0.02	36±4	ND
28g		0.77	6.11±0.15	44±3	ND
28h		0.52	6.28±0.13	83±6	0.18 (0.04)
28i		1.5	5.82±0.10	77±6	ND

28						
Compound	Ar (Het)	hM ₁ EC ₅₀ (μM)	hM ₁ pEC ₅₀	hACh Max (%)	Rat K _p (K _{p,unl}) ^b	
28k		0.87	6.06±0.18	80±9	ND	
28l		0.48	6.32±0.07	78±7	0.05 (0.01)	
28m		0.65	6.19±0.12	76±8	ND	

^a Calcium mobilization assays with hM₁-CHO cells performed in the presence of an EC₂₀ fixed concentration of acetylcholine. Values are the average of at least three independent determinations performed in triplicate ± SEM.

^b Total and calculated unbound brain/plasma partition coefficients determined at 0.25 h post-administration of an IV cassette dose (0.20–0.25 mg/kg) to male, SD rat (*n* = 1), in conjunction with in vitro rat plasma protein and brain homogenate binding assay data.

ND = not determined.

Structures and Activities of Representative VU0456940 Hybrid Amide Analogues 29 Surveying Diverse Amide Moieties

Table 2

Compound	R	hM ₁ EC ₅₀ (μM) ^a	hM ₁ pEC ₅₀	hACh Max (%) ^d	RatKp (K _{panu}) ^b
29					
28a		0.21	6.67±0.05	86±1	0.113 (0.025)
29a		0.30	6.53±0.12	81±10	0.04 (0.02)
29b		0.13	6.88±0.08	87±2	0.04 (0.01)
29c		2.8	5.56±0.12	84±4	0.051 (0.051)

29					
Compound	R	hM ₁ EC ₅₀ (μM) ^a	hM ₁ pEC ₅₀	hACh Max (%) ^a	RatKp (K _{pau}) ^b
29d		0.98	6.01±0.04	87±2	0.08 (0.05)
29e		0.34	6.47±0.16	81±7	0.24 (0.08)
29f		>10	>5	28	ND
29g		>10	>5	34	ND
29h		>10	>5	29	ND

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Calcium mobilization assays with hM1-CHO cells performed in the presence of a fixed EC20 concentration of acetylcholine. Values are the average of at least three independent determinations performed in triplicate $\pm$ SEM.

Total and calculated unbound brain/plasma partition coefficients determined at 0.25 h post-administration of an IV cassette dose (0.20–0.25 mg/kg) to male, SD rat ($n = 1$), in conjunction with in vitro rat plasma protein and brain homogenate binding assay data.

ND = not determined.

Table 3

Structures and Activities of Representative VU0456940 Hybrid Amide Analogues 30 Surveying Alternate Southern Tails^a

<p style="text-align: center;">30</p>				
Compound	R	hM ₁ EC ₅₀ (μM)	hM ₁ pEC ₅₀	hACh Max (%)
30a		0.95	6.02±0.03	82±6
30b		3.0	5.51±0.04	83±4
30c		1.0	5.98±0.06	82±7
30d		1.66	5.78±0.06	83±6
30e		0.13	6.90±0.13	73±8
30f		3.47	5.46±0.06	77±5

^aCalcium mobilization assays with hM₁-CHO cells performed in the presence of a fixed EC₂₀ concentration of acetylcholine. Values are the average of at least three independent determinations performed in triplicate ± SEM.

Table 4Human and Rat M₁ PAM Potency Coupled with Rat CNS Penetration

compd	hM ₁ EC ₅₀ (nM) ^a	hM ₁ pEC ₅₀	hACh Max (%) ^a	rM ₁ EC ₅₀ (nM) ^b	rM ₁ pEC ₅₀	rACh Max (%) ^b	rat K _p (K _{pau}) ^c
28a	210	6.67 ± 0.05	86 ± 1	141	6.86 ± 0.05	92 ± 4	0.113 (0.025)
28c	224	6.65 ± 0.05	86 ± 1	191	6.72 ± 0.01	92 ± 4	0.115 (0.027)
16	30	7.52 ± 0.05	82 ± 3	30	7.53 ± 0.01	90 ± 5	0.21 (0.05)
31	59	7.23 ± 0.03	90 ± 2	39	7.41 ± 0.03	91 ± 4	0.26 (0.21)
32	48	7.33 ± 0.05	88 ± 3	24	7.61 ± 0.02	94 ± 4	0.20 (0.06)
33	16	7.79 ± 0.07	83 ± 1	18	7.76 ± 0.07	89 ± 4	0.13 (0.05)

^a Calcium mobilization assays with hM₁-CHO cells performed in the presence of a fixed EC₂₀ concentration of acetylcholine. Values are the average of at least three independent determinations performed in triplicate ± SEM.

^b Calcium mobilization assays with rM₁-CHO cells performed in the presence of a fixed EC₂₀ concentration of acetylcholine. Values are the average of at least three independent determinations performed in triplicate ± SEM.

^c Total and calculated unbound brain/plasma partition coefficients determined at 0.25 h post-administration of an IV cassette dose (0.20–0.25 mg/kg) to male, SD rat (*n* = 1), in conjunction with in vitro rat plasma protein and brain homogenate binding assay data.

Table 5Human and Rat M₁ Agonist Potency

compd	hM ₁ EC ₅₀ (μM) ^a	hM ₁ pEC ₅₀	hACh Max (%) ^a	rM ₁ EC ₅₀ (μM) ^b	rM ₁ pEC ₅₀	rACh Max (%) ^b
28a	4.8	5.33 ± 0.15	56 ± 14	4.0	5.40 ± 0.05	68 ± 9
28c	5.9	5.23 ± 0.09	48 ± 12	4.6	5.34 ± 0.08	65 ± 9
16	0.59	6.23 ± 0.19	65 ± 10	0.58	6.24 ± 0.12	77 ± 2
31	0.65	6.19 ± 0.10	50 ± 13	0.40	6.40 ± 0.14	71 ± 8
32	0.44	6.36 ± 0.25	47 ± 11	0.44	6.36 ± 0.14	71 ± 11
33	0.63	6.20 ± 0.10	66 ± 7	0.33	6.48 ± 0.24	81 ± 4

^a Calcium mobilization assays with hM₁-CHO cells performed in agonist mode. Values are the average of at least three independent determinations performed in triplicate ± SEM.^b Calcium mobilization assays with rM₁-CHO cells performed in agonist mode. Values are the average of at least three independent determinations performed in triplicate ± SEM.

Table 6Mouse M₁ PAM Potency Coupled with Mouse CNS Penetration

compd	mM ₁ EC ₅₀ (nM) ^a	mM ₁ pEC ₅₀	mACh Max (%) ^a	Mouse K _p (K _{p,uu}) ^b
28a	155	6.81 ± 0.03	79 ± 1	4.84 (2.61)
28c	263	6.58 ± 0.02	77 ± 1	ND
16	46	7.34 ± 0.02	79 ± 1	0.21 (0.16)
31	83	7.08 ± 0.02	73 ± 1	ND
32	46	7.34 ± 0.02	75 ± 2	0.21 (0.16)
33	23	7.63 ± 0.02	78 ± 1	ND

^aCalcium mobilization assays with mM₁-CHO cells performed in the presence of a fixed EC₂₀ fixed concentration of acetylcholine. Values are the average of at least three independent determinations performed in triplicate ± SEM.

^bTotal and calculated unbound brain:plasma partition coefficients determined at 0.25 h post-administration of a discrete dose of compound (10 mg/kg, i.p.) to male mice (*n* = 3 or 4), in conjunction with in vitro mouse plasma protein and brain homogenate binding assay data.