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Structure-Activity Relationships of Pan-G $\alpha_{q/11}$ Coupled Muscarinic Acetylcholine Receptor Positive Allosteric Modulators

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Abstract

Recent years have seen a large increase in the discovery of allosteric ligands targeting muscarinic acetylcholine receptors (mAChRs). One of the challenges in screening such compounds is to understand their mechanisms of action and define appropriate parameter estimates for affinity, cooperativity and efficacy. Herein we describe the mechanisms of action and structure—activity relationships for a series of “pan-G_q-coupled” muscarinic acetylcholine (ACh) receptor (mAChR) positive allosteric modulators (PAMs). Using a combination of radioligand binding, functional inositol phosphate accumulation assays, receptor alkylation and operational data analysis, we show that most compounds in the series derive their variable potency and selectivity from differential cooperativity at the M₁, M₃ and M₅ mAChRs. None of the PAMs showed greater than 10-fold subtype selectivity for the agonist-free receptor, but VU6007705, VU6007678, and VU6008555 displayed markedly increased cooperativity compared to the parent molecule and M₅ mAChR-preferring PAM, ML380 ($\alpha\beta > 100$), in the presence of ACh. Most of the activity of these PAMs derives from their ability to potentiate ACh binding affinity at mAChRs, though VU6007678 was notable for also potentiating ACh signaling efficacy and robust allosteric agonist activity. These data provide key insights for the future design of more potent and subtype-selective mAChR PAMs.

Graphical abstract

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Author Contributions

A.E.B. performed the experimental in vitro pharmacology. A.M.B. performed synthetic/medicinal chemistry. A.E.B., A.C., and C.J.L. analyzed data and wrote the manuscript. C.W.L., P.J.C., P.M.S., C.J.L., and A.C. oversaw and designed the chemistry and molecular pharmacology.

The authors declare no competing financial interest.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscchemneuro.8b00136.

Graphical representation of global EC₅₀ shift analysis to ACh potencies; graphical representation of absolute sum-of-squares analyses for global fits; allosteric ternary complex parameters for PAMs and [³H]-NMS binding (PDF)

Keywords

Muscarinic acetylcholine receptor; positive allosteric modulator; cooperativity; affinity; efficacy; operational model

INTRODUCTION

Muscarinic acetylcholine receptors (mAChRs) are Class A GPCRs that consist of five subtypes, denoted M₁–M₅. The M₁, M₃, and M₅ mAChR subtypes primarily couple to G_{q/11} proteins, which leads to the activation of phospholipase C and facilitates the release of calcium, while the M₂ and M₄ mAChR subtypes couple primarily to G_{i/o} proteins and inhibit adenylyl cyclase activity.^{1,2} The mAChRs are widely expressed within both the central nervous system (CNS) and peripheral tissues and have a key role in many physiological processes.^{3,4} Indeed, mAChRs have been implicated in cognition, motor control, attention, reward and motivation, appetite, muscle contractility, regulation of heart rate, thermoregulation and other functions.^{3–9} As a consequence, these receptors have emerged as interesting drug targets for treating a wide-range of CNS and peripheral disorders including schizophrenia, Alzheimer's disease, drug addiction, asthma, and chronic obstructive pulmonary disease (COPD).^{2,4}

Efforts to develop subtype selective mAChR therapeutics have historically focused on ligands that engage the highly conserved orthosteric (ACh) binding sites on these proteins.^{2,3,10,11} However, the high degree of sequence conservation makes identifying truly subtype selective ligands for individual mAChRs very challenging. Furthermore, directly targeting mAChRs with orthosteric agonists or antagonists increases the likelihood of on-target side effects by disruption of the spatiotemporal aspect of endogenous receptor signaling.^{4,6} In order to address these limitations, research has focused on developing small subtype-selective ligands that target allosteric sites on mAChRs, which are topographically distinct from the orthosteric binding site and less well conserved, offering opportunities for increased selectivity.¹² Of note, the selectivity of an allosteric ligand for a given receptor subtype can be driven by two main factors. First, ligands can be developed to take advantage of structural differences at the level of the allosteric binding site to engender affinity-based selectivity between mAChRs.¹² Second, allosteric ligands may bind to relatively well-conserved allosteric sites with similar affinity across all subtypes, but still only exert a positive, negative or neutral effect on an orthosteric ligand depending on the receptor subtype as a consequence of selective cooperativity between orthosteric and allosteric sites on the cobound receptor. Indeed, cooperativity driven selectivity is emerging as an alternative means of developing or understanding selective allosteric ligands,^{12,13} as evidenced by the proposed mechanisms of action of M₄ mAChR-selective PAMs (such as thiochrome and LY2033298^{13–15}) and allosteric ligands targeting class C GPCRs.⁴⁴

Medicinal chemistry efforts applied to allosteric modulator drug discovery programs are often considered challenging, with descriptions of structure—activity relationships (SAR) described as “flat” or “shallow”.^{16,17} However, this often reflects a focus of allosteric SAR on overall modulator potency in the presence of orthosteric agonist, which itself is composed of, at a minimum, three key molecular properties: allosteric modulator affinity for its binding site, its cooperativity with the orthosteric agonist, and the potential for direct activation of the receptor by the modulator itself, i.e., allosteric agonism.¹⁸ The delineation of the differential impact that chemical manipulation can have on each of these properties can often overcome common challenges associated with allosteric ligand SAR that focuses predominantly on potency measures.¹²

As an interesting case in point, VU0119498 was originally identified from a functional, cell-based, high throughput screen (HTS) as a PAM at M₁, M₃ and M₅ mAChRs, while being devoid of appreciable activity at M₂ and M₄ mAChRs, thereby providing the first example of a “pan-G_q” mAChR PAM.¹⁹ Its discovery served as the impetus for subsequent studies focused on identifying more subtype-selective allosteric modulators for M₁ and M₅ mAChRs in particular,^{20–22} but despite improved mAChR subtype selectivity, next-generation allosteric modulators generated from these studies have limited rodent CNS penetration,^{20,21} thus hindering further translational studies.

Further medicinal chemistry efforts led to the identification of ML380 as a moderately selective M₅ mAChR PAM;²³ subsequent studies yielded a range of newer analogues with improved CNS penetration,^{23,35} and these modifications are summarized in Figure 1. While compounds from this series have been characterized for their potency (i.e., their capacity to potentiate ACh-mediated responses) and mAChR subtype selectivity, there has been no mechanistic characterization of these modulators to determine the molecular allosteric parameters that engender activity and selectivity. Accordingly, the aim of the current study was to characterize the *in vitro* pharmacology of a cohort of pan-G_q mAChR PAMs based on a novel chemotype and predicted to have varying degrees of activity at M₁, M₃, and M₅ mAChRs.^{23,35} Using both functional inositol phosphate (IP1) accumulation and [³H]-NMS binding studies we show that selectivity among these pan-G_q mAChR PAMs is driven by selective cooperativity, rather than binding affinity. Interestingly, in addition to a potentiation of ACh affinity, some of the PAMs also demonstrate modest efficacy modulation. An N-ethyl/propyl moiety appears to be important to the activity of all PAMs at the G_q-coupled mAChRs and the incorporation of a chiral indane motif yields higher cooperativity but relatively nonselective pan-G_q coupled PAM activity. Future studies with compounds of this series will be important to refine subtype selectivity while retaining increased activity.

RESULTS AND DISCUSSION

The discovery of a novel chemotype with M₅ mAChR preferring PAM activity led to a series of allosteric modulators with improved mAChR subtype selectivity, but also modulators with diverse pan-G_q coupled mAChR PAM activity (Figure 1).²³ Herein we have characterized the binding and functional properties of a selection of allosteric modulators at the M₁, M₃ and M₅ mAChRs to understand which pharmacological parameters drive modulator activity and selectivity at G_q coupled mAChRs. Radioligand binding and functional assays were

performed in whole CHO-hM₁, -hM₃, or -hM₅ cells with a maximal binding capacities of 21.8 ± 0.6 , 17.5 ± 0.3 or 7.3 ± 1.2 fmol/10⁵ cells, respectively. In order to reduce the receptor reserve and visualize potential PAM-mediated effects on maximal agonist response(s), a subset of functional assays was performed after receptor alkylation with phenoxybenz-amine (PBZ), as previously described.²⁴

ML380 (1-((1*H*-Indazol-5-yl)sulfonyl)-*N*-ethyl-*N*-(2-(trifluoromethyl)benzyl)piperidine-4-carboxamide).

For comparison, we built on existing pharmacological data available for the PAM ML380, which was noteworthy for its M₅ mAChR potency and selectivity. We confirmed previous reports that ML380 is among the most potent M₅ mAChR PAMs identified to date.^{23,24} In IP1 accumulation, ML380 demonstrated similar affinity for each G_q coupled mAChR and increased ACh potency at the M₁ and M₅ mAChR subtypes; the size of effect at the M₅ mAChR being similar to previous reports (Figure 2A-C; Table 1).²⁴ ML380 exhibited moderate positive cooperativity with ACh at the M₁ and M₅ mAChR subtypes and very weak positive cooperativity with ACh at the M₃ mAChR (Figure 2A-C). ML380 also demonstrated intrinsic efficacy at G_q coupled mAChRs, the magnitude of which appeared to track with the PAM's cooperativity at each receptor (Figure 2A-C). Indeed, ML380 appeared as almost a full agonist at the M₅ mAChR, with minimal partial agonism at the M₃ mAChR (Figure 2B, C). In [³H]-NMS equilibrium binding, the positive affinity cooperativity estimates were sufficient to explain the cooperativity estimates obtained from the functional data sets (Figure 2D-F, Tables 1 and 2), suggesting that ML380 exerts its effects mainly via changes in ACh affinity. This was confirmed with receptor alkylation studies, where ML380 (10 μ M) caused a left-shift in the ACh curve with no effect on the ACh maximal response (Figure 2G-I). Given that ML380 displayed similar affinities for each of the G_q coupled mAChRs (Table 1), this suggests that any subtype selectivity for this PAM is derived from cooperativity.

VU0488129 ((*R*)-1-((1*H*-Indazol-5-yl)sulfonyl)-*N*-(2,3-dihydro-1*H*-inden-1-yl)-*N*-ethylpiperidine-4-carboxamide).

The introduction of a chiral center by replacement of the (trifluoromethyl)benzene with the 2,3-dihydro-1*H*-indene moiety reduced the number of possible conformations the compound is able to exhibit as compared to the more flexible ML380.

Unlike ML380, which has modest functional M₅ mAChR selectivity, VU0488129 had very weak positive cooperativity with ACh at the M₅ mAChR in IP1 assays, but moderate positive cooperativity at the M₁ and M₃ mAChR subtypes. These effects tracked broadly with its intrinsic agonist activity at the three receptor subtypes, consistent with a two-state model of receptor activation (Figure 3; Table 1).^{25,26} However, VU0488129 had 100-fold *higher* affinity at the M₅ mAChR compared to ML380, suggesting that the reduced flexibility improved M₅ mAChR binding, but reduced its cooperativity and functional subtype selectivity.

Similar to the case of ML380, the affinity cooperativity estimates from [³H]-NMS binding assays tracked with (and were sufficient to explain) the combined cooperativity estimates for

the same interactions in IP1 accumulation assays (Figure 3D—F; Table 2), a finding that was reflected in the receptor alkylation experiments where VU0488129 modestly potentiated ACh potency but did not significantly change the maximal ACh response (Figure 3G—I). Interestingly, as with ML380, the mechanism underlying the ability of VU0488129 to differentially potentiate ACh activity at mAChRs was driven by selective cooperativity between the modulator and ACh at the receptor.

VU6007438 ((*R*)-1-((1*H*-Indazol-5-yl)sulfonyl)-*N*-(2,3-dihydro-1*H*-inden-1-yl)-*N*-(ethyl-1,1-*d*2)piperidine-4-carboxamide).

The deuterated analogue of VU0488129, VU6007438, was synthesized to address any metabolic liability associated with the *N*-ethyl moiety.²³ Unsurprisingly, given the very modest change, VU6007438 had very similar affinity and positive cooperativity with ACh at the M₁ and M₃ mAChRs compared to VU0488129 (Figure 4A, B; Table 1), and although it had >10-fold higher positive cooperativity at the M₅ mAChR, this came at the cost of >10-fold reduced affinity for this subtype compared to VU0488129 (Figure 4C; Table 1). Notably, VU6007438 displayed higher intrinsic agonism at the M₅ mAChR as compared to VU0488129. These observations imply that the incorporation of deuterium atoms into the *N*-ethyl moiety may subtly alter the binding affinity and transmission of positive cooperativity between VU6007438 and ACh at this mAChR subtype.

VU6007705 ((*R*)-1-((1*H*-Indazol-5-yl)sulfonyl)-*N*-propyl-*N*-(1,2,3,4-tetrahydronaphthalen-1-yl)piperidine-4-carboxamide).

VU6007705 was the product of two modifications made to VU0488129: a change in the left-hand side from a 2,3-dihydro-1*H*-indene to a 1,2,3,4-tetrahydronaphthalene moiety and the replacement of the *N*-ethyl moiety with an *N*-propyl chain (Figures 3 and 5). These changes resulted in a dramatic change in ligand pharmacology, whereby VU6007705 had very high cooperativity with ACh in both [³H]-NMS binding and IP1 accumulation assays at all receptor subtypes (Figure 5A—C; Table 1). With such high positive cooperativity, it was unsurprising that the ligand displayed robust intrinsic allosteric agonism at each subtype (the magnitude of which tracked with the cooperativity between the modulator and ACh; Table 1) that was preserved even after receptor alkylation with PBZ (Figure 5 G-I). Despite the high positive cooperativity and robust allosteric agonism VU6007705 had no effect on the maximum ACh responses at the mAChRs, suggesting that the primary driver of cooperativity is the increase in ACh affinity (Tables 1 and 2). It is notable that despite markedly increased cooperativity across the receptor subtypes compared with ML380 and VU6007438, VU6007705 had similar affinities to these ligands, suggesting that the reduced flexibility of the structure may lock the ligand into a position that promotes contacts with binding site residues important to the transmission of cooperativity between the allosteric and orthosteric binding sites.

VU6007678 (*R*)-1-((1*H*-Indazol-5-yl)sulfonyl)-*N*-(4-fluoro-2,3-dihydro-1*H*-inden-1-yl)-*N*-propylpiperidine-4-carboxamide.

Fluorination of the indanyl core at the 4-position of VU0488129, and extension of the *N*-ethyl chain to propyl, yielded VU6007678, which has similar affinity for all G_q coupled mAChRs and high positive cooperativity with respect to both the signaling and binding

affinity of ACh at all subtypes (Figure 6; Tables 1 and 2). These modifications seem to engender a broad increase in positive cooperativity with respect to ACh compared to that of VU0488129, with a modest (<10-fold) selectivity for the M₅ mAChR, similar to that of ML380 (Figure 6; Tables 1 and 2).

The affinity cooperativity estimates derived from the [³H]-NMS binding studies correlate with, but do not fully account for, the extent of the combined cooperativity estimates from the IP1 accumulation data. This suggests further effects on ACh signaling efficacy, a hypothesis validated after receptor alkylation, where VU6007678 increased maximal ACh responses and retained its allosteric agonism, even with reduced receptor reserve (Figure 6I—G). As with the other modulators, the degree of allosteric agonism tracked with the amount of cooperativity exhibited between ligands (Figure 6; Tables 1 and 2).

VU6008555 ((*R*)-1-((4-Acetamidophenyl)sulfonyl)-*N*-(2,3-dihydro-1*H*-inden-1-yl)-*N*-ethyl-4-fluoropiperidine-4-carboxamide).

Replacement of the indazole moiety of VU0488129 with the *N*-phenylacetamide moiety and the fluorination at the 4-position of the central piperidine ring yielded VU6008555. This change in the compound structure had little effect on the pharmacology of the ligand at the M₁ and M₃ mAChRs. Indeed, VU6008555 potentiated ACh signaling to a similar extent as VU0488129 and the affinity was largely unchanged at both the M₁ and M₃ mAChRs (Figures 3 and 7A, B, D, E; Tables 1 and 2). Interestingly, when compared to VU0488129, VU6008555 had >250-fold reduced affinity at the M₅ mAChR, an effect largely offset by an ~100-fold increased positive cooperativity (Figures 3 and 7C, F; Tables 1 and 2). As with VU0488129 and ML380, the affinity cooperativity estimates in the binding assays were sufficient to account for the overall cooperativity estimates in the functional assays. This, combined with the lack of effect on ACh maximal responses after receptor alkylation, suggests that affinity cooperativity alone drives the observed functional effects of the PAM (Tables 1 and 2).

VU6007976 ((*R*)-1-((4-Acetamidophenyl)sulfonyl)-*N*-(chroman-4-yl)-*N*-propylpiperidine-4-carboxamide).

Replacement of the left-hand side 2,3-dihydro-1*H*-indene moiety with a chromane (along with an *N*-ethyl chain to the *N*-propyl extension) yielded VU6007976. This compound has similar pharmacology to VU6007438, with relatively nonselective, moderately positive modulator activity in the IP1 assay across all the receptor subtypes, highest affinity for the M₅ mAChR, and affinity cooperativity estimates that correlated with the functional assays (Figure 8A—F; Table 1). VU6007976 was without marked effects on the maximal ACh responses after PBZ treatment, suggesting that the cooperativity is driven through changes in ACh binding (Figure 8I—G).

Broadly speaking, our findings suggest that the compounds in this pan-G_q modulator series are relatively nonselective across the G_q coupled mAChRs with regard to affinity for the allosteric pocket on the unoccupied receptor; none of the compounds tested displayed any more than ~10-fold selectivity for any individual subtype. While highly subtype selective PAMs of the M₁ mAChR have been extensively reported,^{18,27–33} identifying selective PAMs

for the M₃ and M₅ receptor subtypes has met with less success, though efforts in this direction are fairly nascent. Indeed, any limited selectivity seems to derive from cooperativity; ML380 has similar binding affinity for all G_q coupled mAChRs but only shows PAM and allosteric agonist activity at the M₁ and M₅ mAChR subtypes, and only weakly potentiates ACh affinity at the M₃ mAChR. This extends more generally to positive modulation across the series; VU6007678, VU6007705 and VU6008555 all have up to 60-fold higher cooperativity than ML380 at the G_q coupled mAChRs, though they have approximately the same affinity for the unoccupied receptor(s). With the exception of VU0488129 at the M₅ mAChR, none of the compounds displayed submicromolar affinity for any of the receptor subtypes in the absence of agonist.

Given that the SAR depends heavily on changes on cooperativity between the modulators and ACh, the complementary use of [³H]-NMS binding and receptor alkylation studies to dissect any effects on affinity and efficacy was vital in providing additional mechanistic insights, suggesting that these complementary approaches should be more routinely incorporated into modulator SAR studies on key tool compounds. In general, there was a high degree of correlation between the overall cooperativity in the functional IP1 assays (log $\alpha\beta$) and the log α value estimated by the binding assay (Figure 9A), suggesting that changes in ACh binding affinity, rather than changes in signaling efficacy, drive the overall cooperativity. However, receptor alkylation studies provided some additional texture to these data, suggesting that VU6007678 can also modestly increase the maximal ACh responses after treatment of the receptors with PBZ (Figure 6). This implies that some compounds in the series can potentially modulate signaling efficacy, as an increase in the maximal agonist response cannot be reconciled in a model whereby the modulator increases only the affinity of the orthosteric agonist.

Given the high degrees of positive cooperativity displayed by some of the modulators, it was unsurprising that these same compounds displayed robust allosteric agonist activity, some of which was even preserved even after reduction of receptor reserve with PBZ. There was a significant correlation between the degree of allosteric agonism (as quantified by the parameter log τ_B) and the degree of positive cooperativity in the functional IP1 assay (log β ; Figure 9B). This correlation implies that the modulators generally follow a two-state model of receptor activation and allostery, such that the binding of the PAMs shift the equilibrium toward a more active population of receptors, yielding both intrinsic allosteric agonism and increased sensitivity to ACh. Consistent with this mechanism, some of the PAMs also exerted weak effects to inhibit the binding of the inverse agonist radioligand, [³H]-NMS; however these effects were not always evident and most likely limited by the low affinity and/or solubility limit of the compounds under test.

Another general observation was that the incorporation of a chiral center into the structure correlated well with pan-G_q coupled PAM activity, and the N-substitution on the R₂ moiety and N-ethyl/propyl moiety appeared important to PAM activity. More specifically, the incorporation of deuterium into VU6007488 favored M₅ mAChR activity. Further investigation into analogues with these modifications may yield more selective M₅ mAChR PAMs. It would be of interest to compare the in vivo efficacy of compounds with this

mechanism of modulation as compared to pure affinity modulators, such as ML380, to determine which type of modulation, if any, is preferred for in vivo activity.

Since these PAMs exhibit different degrees of selectivity between G_q coupled mAChR subtypes, it would also be of interest to determine their binding sites on mAChRs. While structural studies have defined a prototypical allosteric modulator site in the extracellular vestibule of the M_1 - M_4 mAChRs, currently there is minimal understanding as to the location and nature of the allosteric binding site on the M_5 mAChR; it remains to be seen whether it is analogous to that described for other members of the receptor family.^{24,25,28,34}

In summary, this study has provided the first detailed pharmacological characterization for the mechanistic basis of activity of a selection of pan- G_q coupled mAChR PAMs. We have shown that cooperativity, rather than binding affinity, appears to be the major mechanism driving the PAM activity and/or selectivity of these ligands. Moreover, this study has identified key structural modifications in the chemotype that increase the PAM activity of G_q coupled mAChR PAMs. Ideally, future studies will couple this increased cooperativity with greater selectivity for the M_3 and M_5 mAChRs, for which potent and selective modulators are still generally lacking relative to other mAChR subtypes.

METHODS

Materials.

Flp-In-Chinese hamster ovary (CHO) cells were obtained from Life Technologies (Mulgrave, Australia). Dulbecco's modified Eagle's medium was purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum was purchased from ThermoTrace (Melbourne, Australia). [3 H]-*N*-Methylscopolamine ([3 H]-NMS; specific activity, 70 Ci/mmol) and MicroScint scintillation liquid were purchased from PerkinElmer Life Sciences. The IP1 assay kit was purchased from Cisbio (Codolet, France). ACh and phenoxybenzamine (PBZ) were purchased from Sigma-Aldrich (St. Louis, MO). ML380, VU0488129, VU6007705, VU6007678, VU6008555, VU6007438, and VU6007976 were a generous gift of Craig Lindsley (Vanderbilt University, Nashville, TN³). All other chemicals, reagents and kits were from Sigma-Aldrich. For all procedures, purified water (18.2 MV cm) from a Milli-Q PF Plus system was used.

Cell Culture.

Human mAChR (hM₁, hM₃, and hM₅ mAChR; Origene) constructs were isogenically integrated into Flp-In CHO cells (Invitrogen), and cells were selected in the presence of 600 mg/mL hygromycin B at 37 °C, 5% CO₂, as previously described for the hM₁ mAChR.²⁷ All cells were subcultured and seeded as previously described for the CHO-hM₅ cells.²⁴

[3 H]-NMS Equilibrium Binding.

[3 H]-NMS equilibrium binding assays were performed in CHO-hM₁-hM₃ and -hM₅ mAChR cells as previously described.²⁴

Inositol Phosphate Accumulation.

The IP1 assay kit (Cisbio) was used for direct quantitative measurement of inositol phosphate accumulation in CHO-hM₁, -hM₃, or -hM₅ mAChR cells, as previously described in ref ²⁴.

Receptor Alkylation Studies.

To determine functional affinity (K_A) and efficacy (τ_A) estimates for acetylcholine at hM₁, hM₃, and hM₅ mAChRs, in addition to determining the contributions made via affinity modulation (α) and efficacy modulation (β) to the overall PAM activity of allosteric ligands at each G_q coupled mAChR, additional IP1 accumulation assays were performed in CHO-hM₁, -hM₃, and -hM₅ cells pretreated with phenoxybenzamine (PBZ) as per described.²⁴

Data Analysis.

GraphPad Prism version 7.02 (San Diego, CA) was used for all curve fitting. For direct determination of the functional ACh equilibrium dissociation constants (K_A) from the alkylation experiments, ACh concentration-response curves (in the presence or absence of PBZ) were globally fitted to the operational model of agonism as previously described in.^{24,36}

For functional interaction studies between acetylcholine and PAMs in the IP1 accumulation assays, the following operational model of allosterism was applied:³⁷

$$Y = \text{basal} + \frac{\{ (E_M - \text{basal})(\tau_A[A](K_B + \alpha\beta[B]) + \tau_B[B]K_A)^n \}}{([A]K_B + K_A K_B + K_A[B] + \alpha[A][B])^n + (\tau_A[A](K_B + \alpha\beta[B]) + \tau_B[B]K_A)^n} \quad (1)$$

where basal is the response in the absence of ligand, E_m is the maximal response of the system and n is the slope of the transducer function linking occupancy to response. $[A]$ and $[B]$ represent the concentrations of the orthosteric agonist (ACh) and allosteric ligand, respectively, and K_A and K_B represent their respective equilibrium dissociation constants. τ_A and t_b denote the relative efficacies of the orthosteric and allosteric ligands, respectively. α denotes the affinity cooperativity between ACh and an allosteric modulator and β represents a scaling factor, which defines the magnitude and the direction of the effect of the allosteric modulator on ACh efficacy. For all data sets the K_A was constrained to the value derived from the alkylation experiments for the same mAChR (M₁ mAChR $pK_A = 4.97 \pm 0.21$; M₃ mAChR $pK_A = 5.44 \pm 0.21$; M₅ mAChR $pK_A = 5.84 \pm 0.15$; $n = 5-8$), $\log a$ was constrained to 0 such that the resulting $\log \beta$ value was actually representative of the combined affinity and efficacy cooperativity (i.e., $\log \alpha\beta$) between the interaction of a modulator with ACh for a particular mAChR. Derived cooperativity values greater than 1 indicate positive cooperativity; values < 1 (but > 0) indicate negative cooperativity, and values of unity indicate neutral cooperativity.

In instances where application of eq 1 to interaction studies did not yield a reliable fit of the model or the model did not converge, the following two-step process was applied. First, the

ACh concentration- response curves were fitted to the following four parameter logistic function:

$$Y = \text{basal} + \frac{(E_{\max} - \text{basal})}{1 + 10^{-(X - \log EC_{50})^n}} \quad (2)$$

where basal and $E_{\max}$ represent the shared minimum and maximal asymptotes, respectively, n is the shared Hill slope, and the $\log EC_{50}$ is the logarithm of the midpoint of the ACh stimulation curves. Second, agonist potencies for ACh in the presence and absence of modulator derived from eq 2 were then plotted against the corresponding concentration of the modulator, and eq 3 (below) was applied to the data to derive PAM affinity (K_B) and combined affinity/efficacy cooperativity ($\alpha\beta$) estimates.^{38,39} This analysis was applied to interactions of ACh with VU0488129 at the M_1 mAChR; VU6007678 at the M_1 , M_3 , and M_5 mAChRs; VU6008555 at the M_1 mAChR; and VU6007438 at the M_1 mAChR (Supporting Information Figure 1).

$$pEC_{50} = -\log([B] + 10^{-pK_B}) + \log(\alpha\beta[B] + 10^{-pK_B}) - \log d \quad (3)$$

where $[B]$ is as previously described and d is the estimate of the ACh EC_{50} in the absence of the modulator. Interaction studies were then refitted to eq 1, where $\log a = 0$, the $\log K_B$ and $\log \beta$ were fixed to the values derived from application of eq 3 to data sets so that an estimate for the remaining parameter (intrinsic efficacy (τ_B) of the allosteric modulator) not yet quantified could be determined.

Finally, where eq 3 was applied to IP1 accumulation data sets and the model did not converge, a scanning of parameter space based on reduction in global absolute sum of squares was performed.^{40,41} Specifically, $\log \alpha$ was constrained to 0 and the $\log K_B$ was constrained to values ranging from -7.5 to -1.0 , in 0.1 log unit increments while eq 1 was applied to each of the data sets under each of the aforementioned conditions until the global reduction in the sum of squares of the resulting fit approached an asymptotic value, suggesting that no further improvement in the fit could be obtained by changing the $\log K_B$. From this analysis, the reported functional $\log K_B$ values were -4.5 for VU0488129 at the M_3 mAChR, -4.0 for VU6008555 at the M_3 and M_5 mAChRs, -4.0 for VU6007438 at the M_3 mAChR, and -4.5 and -4.0 for VU6007976 at the M_1 and M_3 mAChRs, respectively (Supporting Information Figure 2; Table 1); these values were used to fit the interaction data sets according to eq 1 to obtain an estimate of the combined cooperativity ($\log \alpha\beta$) of the PAM with ACh and an estimate of the allosteric agonism ($\log \tau_B$) (Table 1).

For interaction [3H]-NMS equilibrium binding studies, data were analyzed according to an allosteric ternary complex model (ATCM) as previously described in refs 24 and 39.

$$Y = \frac{B_{\max}[A]}{[A] + \left(\frac{K_A K_B}{\alpha_A [B] + K_B} \right) \left(1 + \frac{[I]}{K_I} + \frac{[B]}{K_B} + \frac{\alpha_I [I][B]}{K_I K_B} \right)} \quad (4)$$

where $[A]$, $[B]$, and $[I]$ represent the concentrations of the radioligand ($[^3\text{H}]\text{-NMS}$), allosteric ligand, and orthosteric inhibitor, respectively, K_A , K_B , and K_I represent their respective equilibrium dissociation constants, and $B_{\max}$ is the total number of receptors in a sample of tissue. The K_A was fixed to 0.39 nM for interactions at the M_1 mAChR, 0.34 nM for interaction at the M_3 mAChR, or 0.3 nM for interactions at the M_5 mAChR, as determined in separate saturation binding studies (data not shown). α_A and α_I denote the cooperativity values between the allosteric ligand and the radioligand or orthosteric inhibitor, respectively; values greater than 1 indicate positive cooperativity; values < 1 (but > 0) indicate negative cooperativity, and values of unity indicate neutral cooperativity.

All estimates for potency, affinity, and cooperativity were reported as logarithms.⁴² Where appropriate, fitted parameters were compared by extra sum-of-squares F-test.⁴³ Data are expressed as either a percentage of the maximal ACh response or as a percentage of specific $[^3\text{H}]\text{-NMS}$ binding and represent the mean $\pm$ SEM of at least three independent experiments performed in duplicate.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

CHO	Chinese hamster ovary
mAChR	muscarinic acetylcholine receptor
NMS	N-methyl scopolamine
PAM	positive allosteric modulator

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Figure 1. Modifications made to the novel chemotype that yielded the PAMs described in this study.²³

Figure 2.

Functional and radioligand binding studies showing the effect of ML380 on ACh-stimulated IP1 accumulation in whole FlpIn-CHO cells stably expressing M₁, M₃, or M₅ mAChRs (A–C; G–I) and on ACh-mediated inhibition of [³H]-NMS binding for the same cell lines (D–F), respectively. IP1 accumulation assays were performed with (A–C) or without (G–I) a 30 min pretreatment with PBZ (3 μM) to reduce the receptor reserve. ML380 (10 μM) did not change the maximal ACh response after PBZ treatment (extra sum-of-squares F-test: M₁ mAChR: *P* = 0.14; M₃ mAChR: *P* = 0.50; M₅ mAChR: *P* = 0.35). Data are expressed as either a percentage of the maximal ACh response or as a percentage of specific [³H]-NMS binding and represent the mean ± SEM of at least three independent experiments performed in duplicate.

Figure 3.

Functional and radioligand binding studies showing the effect of VU0488129 on ACh-stimulated IP1 accumulation in whole FlpIn-CHO cells stably expressing M₁, M₃, or M₅ mAChRs (A-C; G—I) and on ACh-mediated inhibition of [3H]-NMS binding for the same cell lines (D-F) as in Figure 2. VU0488129 (10 μM) did not change the maximal ACh response after PBZ treatment (M₁ mAChR: $P = 0.28$; M₃ mAChR: $P = 0.12$; M₅ mAChR: $P = 0.77$; G—I). Data are expressed as either a percentage of specific [3H]-NMS binding or as a percentage of the maximal ACh response and represent the mean ± SEM of at least four independent experiments performed in duplicate.

Figure 4.

Functional and radioligand binding studies showing the effect of VU6007438 on ACh-stimulated IP1 accumulation in whole FlpIn-CHO cells stably expressing M_1 , M_3 , or M_5 mAChRs (A-C; G—I) and on ACh-mediated inhibition of [3 H]-NMS binding for the same cell lines (D-F) as in Figure 2. VU6007438 (10 μ M) modestly but significantly enhanced the maximal ACh response at all three G_q coupled mAChRs following PBZ treatment (M_1 mAChR: $P < 0.0001$; M_3 mAChR: $P = 0.0049$; M_5 mAChR: $P = 0.0052$; G—I). Data are expressed as either a percentage of specific [3 H]-NMS binding or as a percentage of the maximal ACh response, respectively, and represent the mean $\pm$ SEM of at least four independent experiments performed in duplicate.

Figure 5.

Functional and radioligand binding studies showing the effect of VU6007705 on ACh-stimulated IP1 accumulation in whole FlpIn-CHO cells stably expressing M₁, M₃, or M₅ mAChRs (A–C; G–I) and on ACh-mediated inhibition of [³H]-NMS binding for the same cell lines (D–F) as in Figure 2. Following PBZ treatment, VU6007705 (10 μM) did not change the maximal ACh response at M₁ mAChRs ($P = 0.39$) but had a modest, significant, enhancement of the maximal ACh response at M₃ ($P = 0.022$; 10 μM) and M₅ mAChRs ($P = 0.022$; 1 μM), respectively (G–I). Data are expressed as either a percentage of specific [³H]-NMS binding or as a percentage of the maximal ACh response, respectively, and represent the mean ± SEM of at least four independent experiments performed in duplicate.

Figure 6.

Functional and radioligand binding studies showing the effect of VU6007678 on ACh-stimulated IP1 accumulation in whole FlpIn-CHO cells stably expressing M_1 , M_3 , or M_5 mAChRs (A-C; G—I) and on ACh-mediated inhibition of $[^3\text{H}]\text{-NMS}$ binding for the same cell lines (D-F) as in Figure 2. VU6007678 increased the maximal ACh response at mAChRs (M_1 mAChR at $10\ \mu\text{M}$, $P = 0.024$; M_3 mAChR at $10\ \mu\text{M}$, $P = 0.0001$; M_5 mAChR at $300\ \text{nM}$, $P < 0.0001$). Data are expressed as either a percentage of specific $[^3\text{H}]\text{-NMS}$ binding or as a percentage of the maximal ACh response, respectively, and represent the mean $\pm$ SEM of at least three independent experiments performed in duplicate.

Figure 7.

Functional and radioligand binding studies showing the effect of VU6008555 on ACh-stimulated IP1 accumulation in whole FlpIn-CHO cells stably expressing M₁, M₃, or M₅ mAChRs (A—C; G—I) and on ACh-mediated inhibition of [³H]-NMS binding for the same cell lines (D—F) as in Figure 2. Following PBZ pretreatment, 10 μM VU6008555 did not change the maximal ACh response at (G—I). VU6008555 (10 μM) had no effect on maximal ACh responses after PBZ treatment at M₁ mAChRs ($P = 0.59$) or M₅ mAChRs ($P = 0.22$), but did cause a small, but significant, increase in the response at M₃ mAChRs ($P = 0.03$; H). Data are expressed as either a percentage of specific [³H]-NMS binding or as a percentage of the maximal ACh response, respectively, and represent the mean ± SEM of at least four independent experiments performed in duplicate.

Figure 8.

Functional and radioligand binding studies showing the effect of VU6007976 on ACh-stimulated IP1 accumulation in whole FlpIn-CHO cells stably expressing M₁, M₃, or M₅ mAChRs (A-C; G-I) and on ACh-mediated inhibition of [3H]-NMS binding for the same cell lines (D-F) as in Figure 2. Following PBZ pretreatment, VU6007976 (10 μM) increased the maximal ACh response at M₃ mAChRs ($P = 0.0022$) and M₅ mAChRs ($P < 0.0001$), but not at M₁ mAChRs ($P = 0.11$; G-I). Data are expressed as either a percentage of specific [3H]-NMS binding or as a percentage of the maximal ACh response, respectively, and represent the mean $\pm$ SEM of at least four independent experiments performed in duplicate.

Figure 9.

Positive correlation between the composite functional cooperativity estimates ($\alpha\beta$) and (A) affinity cooperativity (α) and (B) relative efficacy (τ_B) estimates for allosteric modulators with ACh at the G_q coupled mAChRs. Each data point represents a parameter estimate for a single compound at an individual mAChR as determined by whole-cell [^3H]-NMS competition binding and/or IP1 accumulation assays (Tables 1 and 2).

Table 1.

operational Model Estimates for the Interaction between ACh and the Indicated Pan-G_q Coupled PAMs at M₁, M₃, and M₅ mAChRs in IP1 accumulation Assays^d

	Structure		M ₁ mAChR				M ₃ mAChR				M ₅ mAChR			
	R	R ₂	X	R ₃	<i>a</i> _{pK_B}	<i>b</i> _{Logβ}	<i>c</i> _{Logτ_B}	<i>a</i> _{pK_B}	<i>b</i> _{Logβ}	<i>b</i> _{Logτ_B}	<i>a</i> _{pK_B}	<i>b</i> _{Logβ}	<i>c</i> _{Logτ_B}	
ML380			H		4.34 ± 0.37	0.95 ± 0.30	0.16 ± 0.26	4.65 ± 0.34	0.13 ± 0.13	-0.25 ± 0.21	4.43 ± 0.30	0.97 ± 0.35	0.91 ± 0.26	
U0488129			H		\$5.28 ± 0.07	\$1.28 ± 0.04	-0.14 ± 0.07	= \$4.5	1.51 ± 0.06	-0.33 ± 0.14	6.43 ± 0.32	0.27 ± 0.09	-0.74 ± 0.16	
U06007438			H		\$4.99 ± 0.19	\$1.59 ± 0.13	-0.04 ± 0.06	= \$4.0	1.62 ± 0.06	0.01 ± 0.12	5.07 ± 0.25	1.14 ± 0.22	0.08 ± 0.12	

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Structure		M ₁ mACHR				M ₃ mACHR				M ₅ mACHR			
R	R ₂	X	R ₃	<i>a</i> _{pK_B}	<i>b</i> _{Log_B}	<i>c</i> _{Log_B}	<i>a</i> _{pK_B}	<i>b</i> _{Log_B}	<i>c</i> _{Log_B}	<i>a</i> _{pK_B}	<i>b</i> _{Log_B}	<i>c</i> _{Log_B}	
		H		4.87 ± 0.21	2.12 ± 0.21	0.42 ± 0.15	4.54 ± 0.24	1.75 ± 0.23	0.59 ± 0.20	4.59 ± 0.47	2.61 ± 0.48	1.03 ± 0.41	
U6007705													
		H		\$4.94 ± 0.17	\$1.90 ± 0.13	0.34 ± 0.04	\$5.52 ± 0.04	\$1.28 ± 0.03	−0.03 ± 0.04	\$4.82 ± 0.14	\$2.77 ± 0.13	0.84 ± 0.04	
U6007678													
		F		\$4.74 ± 0.19	\$2.09 ± 0.15	0.42 ± 0.05	= \$4.0	1.91 ± 0.05	0.36 ± 0.07	= \$4.0	2.29 ± 0.08	0.95 ± 0.05	
U6008555													
		H		= \$4.5	1.46 ± 0.08	−0.003 ± 0.09	= \$4.0	1.39 ± 0.08	−0.49 ± 0.26	5.15 ± 0.24	1.09 ± 0.25	0.32 ± 0.13	
U6007976													

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^aNegative logarithm of the equilibrium dissociation constant for each pan G_q mAChR PAM for mAChRs. When the model did not converge, this parameter was constrained to a value derived from either an allosteric global pEC₅₀ analysis (eq 3), as denoted by superscript \$, or an absolute sum of squares analysis, as denoted by superscript &.

^bLogarithm of the efficacy scaling factor for the effect of the indicated pan G_q mAChR PAMs on ACh responses at the specified mAChRs; when the logarithm of the affinity cooperativity between ACh and the PAM is equal to zero ($\log a = 0$), it is equivalent to the combined functional cooperativity between ligands ($\text{Log } a\beta$). Combined cooperativity values with a corresponding \$ or & were derived from applying an allosteric global pEC₅₀ analysis (eq 3) or an absolute sum of squares analysis, respectively.

^cLogarithm of the operational efficacy parameter of the indicated pan G_q mAChR PAMs at the specified mAChRs.

^dEstimated parameters represent the mean $\pm$ SEM of at least three experiments performed in duplicate or triplicate. When the full operational model of allosterism (eq 1) was applied to data sets the negative logarithm of the equilibrium dissociation constant for ACh (pK_A) was constrained to a predetermined value by applying the operational model of agonism to the ACh IPI accumulation concentration—response curves (in the presence or absence of PBZ) from the alkylation experiments for the same mAChR. In the case of experiments at M1 mAChRs the $pK_A = 4.97 \pm 0.21$, at M3 mAChRs the $pK_A = 5.44 \pm 0.21$, and at M5 mAChRs the $pK_A = 5.84 \pm 0.15$.

Allosteric Ternary Complex Model Parameter Estimates for the Interaction between the Orthosteric Antagonist/Inverse Agonist [^3H]-NMS and Agonist ACh with the Indicated Pan-G_q mAChR PAMs at M₁, M₃, and M₅ mAChRs^c

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Structure		M ₁ mAChR				M ₃ mAChR				M ₅ mAChR			
R	R ₂	X	R ₃	a _{pK_B}	b _{Log_aCh}	a _{pK_B}	b _{Log_aCh}	a _{pK_B}	b _{Log_aCh}	a _{pK_B}	b _{Log_aCh}	a _{pK_B}	b _{Log_aCh}

VU6007705			H		= 4.87	1.39 ± 0.07	= 4.54	1.83 ± 0.04	= 4.59	2.37 ± 0.08			
VU6007678			H		= 4.94	1.25 ± 0.08	= 5.52	1.10 ± 0.07	= 4.82	1.89 ± 0.08			
VU6008555			F		1.56 ± 0.07	= 4.00	1.88 ± 0.06	= 4.00	2.12 ± 0.07				
VU6007976			H		= 4.50	0.75 ± 0.11	= 4.00	1.48 ± 0.09	= 5.15	0.85 ± 0.10			

^aNegative logarithm of the equilibrium dissociation constant of each pan G_q mAChR PAM (constrained to the estimate from the functional IP1 assays).

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q Logarithm of the affinity cooperativity between ACh and the indicated pan G_q mAChR PAM.

ζ Estimated parameters represent the mean $\pm$ SEM of at least three experiments performed in duplicate.