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Chemical Modification of the M_1 Agonist VU0364572 Reveals Molecular Switches in Pharmacology and a Bitopic Binding Mode

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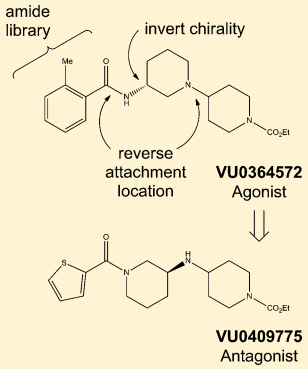
ABSTRACT: We previously reported the discovery of VU0364572 and VU0357017 as M_1-selective agonists that appear to activate M_1 through actions at an allosteric site. Previous studies have revealed that chemical scaffolds for many allosteric modulators contain molecular switches that allow discovery of allosteric antagonists and allosteric agonists or positive allosteric modulators (PAMs) based on a single chemical scaffold. Based on this, we initiated a series of studies to develop selective M_1 allosteric antagonists based on the VU0364572 scaffold. Interestingly, two lead antagonists identified in this series, VU0409774 and VU0409775, inhibited ACh-induced Ca^{2+} responses at rat M_{1-5} receptor subtypes, suggesting they are nonselective muscarinic antagonists. VU0409774 and VU0409775 also completely displaced binding of the nonselective radioligand [^{3}H]-NMS at M_1 and M_3 mAChRs with affinities similar to their functional IC_{50} values. Finally, Schild analysis revealed that these compounds inhibit M_1 responses through a fully competitive interaction at the orthosteric binding site. This surprising finding prompted further studies to determine whether agonist activity of VU0364572 and VU0357017 may also engage in previously unappreciated actions at the orthosteric site on M_1. Surprisingly, both VU0364572 and VU0357017 completely displaced [^{3}H]-NMS binding to the orthosteric site of M_1–M_5 receptors at high concentrations. Furthermore, evaluation of agonist activity in systems with varying levels of receptor reserve and Furchgott analysis using a cell line expressing M_1 under control of an inducible promotor was consistent with an action of these compounds as weak orthosteric partial agonists of M_1. However, consistent with previous studies suggesting actions at a site that is distinct from the orthosteric binding site, VU0364572 or VU0357017 slowed the rate of [^{3}H]-NMS dissociation from CHO-rM_1 membranes. Together, these results suggest that VU0364572 and VU0357017 act as bitopic ligands and that novel antagonists in this series act as competitive orthosteric site antagonists.



KEYWORDS: *Acetylcholine, GPCR, allosteric, orthosteric, agonist, antagonist*

Acetylcholine (ACh) is a major neurotransmitter that is important for multiple central nervous system (CNS) and peripheral functions through activation of nicotinic acetylcholine receptors (nAChRs) and muscarinic acetylcholine receptors (mAChRs).[1] The five mAChR subtypes are family A G protein-coupled receptors (GPCRs) and are divided into two groups based on their coupling preference to specific signal transduction pathways. M_1, M_3, and M_5 receptors induce the release of calcium from intracellular stores by activating $G\alpha_q$ subtype G proteins, whereas M_2 and M_4 act through $G\alpha_i$ to regulate adenylyl cyclase and ion channels, including G protein gated ion channels (GIRKs).[2] mAChRs are widely expressed throughout the body with varying degrees of expression at a particular target organ or synapse.[2] Consequently, mAChRs regulate an extensive array of signaling pathways and biological responses including cognition, attention, cardiovascular function, secretory, and GI function.[3]

The M_1 receptor has been viewed as an attractive therapeutic target and modulation of this subtype may provide a novel treatment strategy for a variety of disorders of the CNS.[4] M_1 receptors play multiple roles in different domains of cognitive function,[4,5] and activation of M_1 receptors has cognition-enhancing effects in a wide range of animal models.[6−8] Inhibition of M_1-mediated effects with highly selective antagonists may have potential utility in the treatment of epileptic and movement disorders.[9−11] Given such a broad influence, multiple groups have attempted to develop M_1-selective agonists and antagonists.[12] Unfortunately, previous attempts to develop highly selective ligands acting at the orthosteric ACh site of individual mAChR subtypes have failed,

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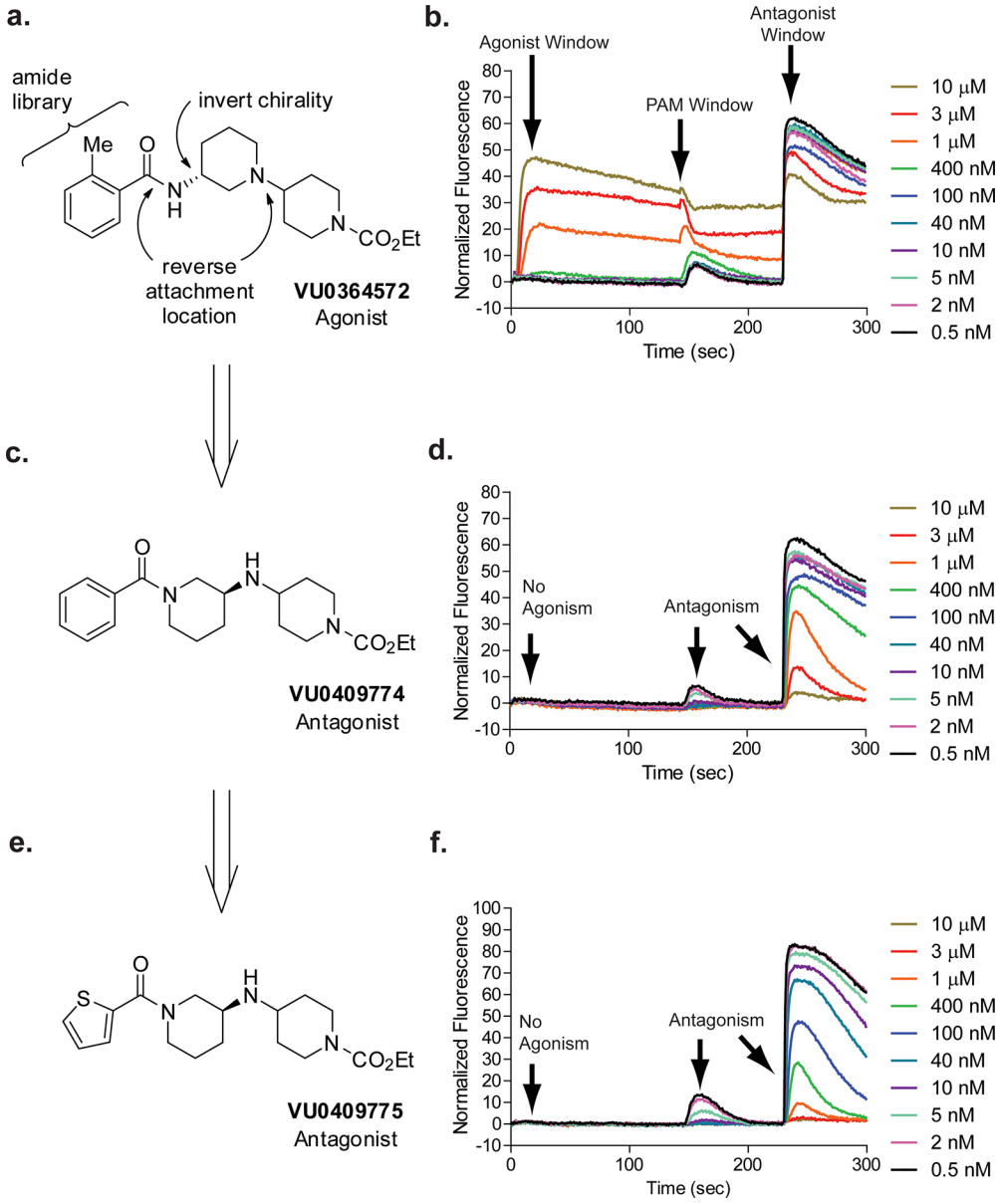

Figure 1. Structural exploration of the M_1 agonist VU0364572 leads to the development of the mAChR antagonist series, represented by VU0409774. (a) Structure of VU0364572 that includes topographical sketch of the chirality inversion and reverse attachment location. (b) Raw traces of VU0364572-induced Ca^{2+} responses using a triple add assay protocol. VU0364572 shows activity in the agonist window. (c) Structure of the antagonist VU0409774. (d) Raw traces of VU0409774-induced effects in triple add Ca^{2+} assays. VU0409774 has no activity in the agonist window but rather inhibits both EC_{20} and EC_{80} concentrations of ACh. (e) Structure of VU0409775 showing the introduction of the thiophene group on the western portion of the molecule. (f) Raw traces of VU0409775-induced effects in triple add Ca^{2+} assays. In a manner similar to VU0409774, VU0409775 inhibits EC_{20} and EC_{80} ACh concentrations, suggesting this compound functions as an antagonist.

and it is thought that this is due to the high conservation of the orthosteric site across all five mAChR subtypes.

A major breakthrough in the development of M_1-selective compounds came with the discovery of allosteric agonists and positive allosteric modulators (PAMs) of M_1 receptors. M_1 PAMs and M_1 allosteric agonists have fundamentally different modes of efficacy in increasing M_1 activity. M_1 PAMs act at an allosteric site that is clearly distinct from the orthosteric ACh site to potentiate responses to ACh. In contrast, M_1 allosteric agonists do not potentiate ACh responses but have intrinsic agonist activity. Multiple M_1-selective agonists have recently been discovered, including AC-42, TBPB, and 77-LH-28-1, which can induce M_1-mediated responses in cellular systems and native tissues.[13−15] More recently, we reported discovery of VU0364572 and VU0357017 as highly selective M_1 agonists that appear to act at an allosteric site to activate the receptor.[7,16,17] Extensive profiling of VU0364572 and VU0357017 across a large panel of GPCRs revealed that these compounds have little or no activity at other GPCRs and are the most selective M_1 agonists reported to date.[7,16,18] It is thought that the allosteric mechanism of action of these novel M_1 agonists is critical for achieving high selectivity for M_1 relative to other mAChR subtypes.

Previous studies with allosteric modulators of other GPCRs suggest that close structural analogues based on a single chemical scaffold can have varying activities, including allosteric agonists, PAMs, and allosteric antagonists, also referred to as negative allosteric modulators (NAMs).[18] We and others have

taken advantage of these "molecular switches" in pharmacological activity within a given scaffold to develop highly selective allosteric activators and allosteric antagonists for a single GPCR subtype.[18] Based on this and the high selectivity of VU0364572 and VU0357017 relative to other GPCRs, we initiated a chemistry effort aimed at the discovery of M_1-selctive allosteric antagonists based on the VU0364572 chemical scaffold. We now report discovery of close structural analogues of VU0364572 that act as mAChR antagonists. Surprisingly, the lead antagonists developed from this scaffold proved to be nonselective orthosteric mAChR antagonists when profiled against M_1–M_5 receptor subtypes. This prompted further studies with VU0364572 and VU0357017 in which we found that these compounds also have properties consistent with actions as orthosteric site weak partial agonists. However, VU0364572 and VU0357017 significantly slowed the rate of [^{3}H]-NMS from CHO-rM_1 cell membranes, a finding consistent with previous results suggesting activity of these compounds at an allosteric site on M_1. These data suggest that VU0364572 and VU0357017 do not act as traditional orthosteric agonists of muscarinic receptors but bind at both allosteric and orthosteric sites and act as bitopic ligands. These findings are similar to previous reports with at least two other M_1 allosteric agonists, 77-LH-28-1 and AC-42 which bind M_1 at orthosteric and allosteric sites.[19]

RESULTS

Identification of Two Novel Antagonists of mAChRs through the Structural Exploration of VU0364572. In an effort to develop novel mAChR ligands, we initiated a diversity-oriented structural exploration around the M_1 agonist VU0364572 (Figure 1a) and used a triple add protocol Ca^{2+} assay which allows for detection of agonists, positive allosteric modulators (PAMs), and antagonists. When added alone, VU0364572 induced a concentration-dependent increase in Ca^{2+} mobilization in Chinese hamster ovary (CHO) cells expressing rat M_1 receptors (rM_1Rs) (Figure 1b), a finding similar to our previously published reports.[7,16] The amino piperidine with the opposite chirality of VU0364572 was then reversed and attached via its secondary amine. This rearrangement yielded compound VU0409774 (Figure 1c). Unlike VU0364572, VU0409774 did not induce Ca^{2+} responses when added alone but rather induced a concentration-dependent inhibition of responses to EC_{20} and EC_{80} concentrations of ACh. Thus, these structural modifications to VU0364572 had generated a compound with antagonist activity (Figure 1d). A further exchange of the western benzene portion of VU0409774 with a thiophene modification yielded the compound VU0409775 (Figure 1e). Similar to VU0409774, VU0409775 functioned as an antagonist of ACh responses and induced a concentration-dependent inhibition of responses to ACh with no agonist activity (Figure 1f). Based on these findings, it appears that modifications of this class of M_1-selective agonists can cause major shifts in their functional properties to yield novel M_1 antagonists.

To determine if VU0409774 and VU0409775 are selective antagonists of M_1 relative to other mAChR subtypes, we tested the effects of both compounds by measuring their ability to inhibit ACh-induced Ca^{2+} responses across the rat M_{1-5} receptor subtypes. Ca^{2+} assays were performed in CHO cells stably expressing rM_1, rM_3, or rM_5 receptors or stably expressing the chimeric G protein G_{qi5} together with rM_2 and rM_4 receptors.[20] VU0409775 and VU0409774 inhibited ACh-induced responses at all five muscarinic receptor subtypes (Figure 2a, b). Both compounds were found to be potent

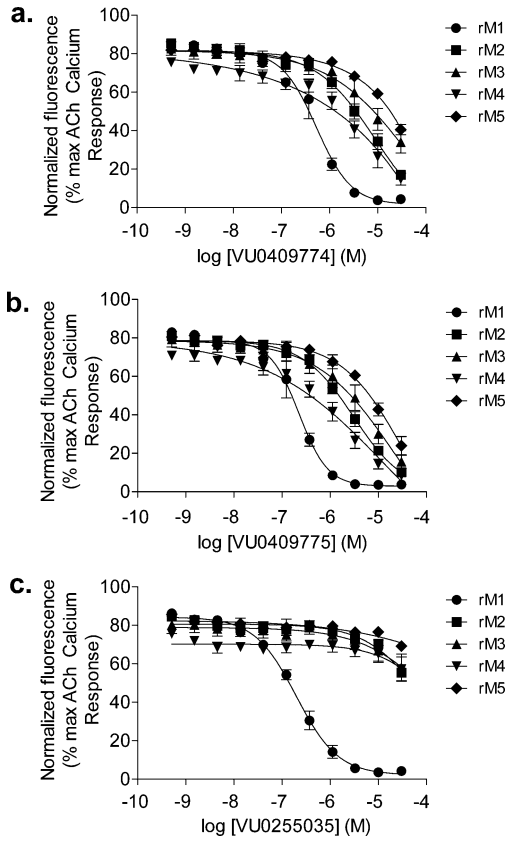

Figure 2. VU0409774 and VU0409775 inhibit ACh-induced calcium responses when profiled against all five muscarinic receptor subtypes. (a and b) Concentration–response curves (CRCs) of VU0409774 and VU0409775 antagonism of ACh-induced responses in CHO cells expressing rat M_{1-5} mAChRs. (c) CRCs of VU0255035-induced antagonism across M_{1-5} mAChRs. VU0255035 selectively antagonizes responses in cells expressing rM_1 mAChRs relative to M_{2-5} mAChRs. CRCs were performed in the presence of an EC_{80} concentration of ACh in a calcium mobilization assay. Data are normalized to the maximal response elicited by ACh and are presented as a percentage of the EC_{80} response. Data represent the mean ± SEM of two to three experiments conducted in triplicate.

antagonists at M_1 (pK_b values at rM_1 receptors = 7.02 ± 0.13 and 6.63 ± 008, respectively) than at other mAChR subtypes, and VU0409774 exhibited only minimal functional effects at M_3 and M_5 (K_b values > 10 μM; see Table 1 for calculated pK_b values of both antagonists across M_{1-5} expressing cells) (see Lazareno and Birdsall for determination of pK_b values[21]). Based on this, both compounds are thought to function as relatively nonselective mAChR antagonists. Neither compound functioned as an agonist when tested against rat M_{1-5} mAChR subtypes. In contrast, the M_1-selective antagonist VU0255035[11] inhibited ACh-induced responses in cell lines expressing rM_1 receptors, but not in cells expressing the remaining mAChR subtypes (Figure 2c, pK_b at rM_1 receptor = 7.05 ± 0.07). Together, these results suggest that VU0409774 and VU0409775 function as antagonists of ACh-induced Ca^{2+} responses, but have limited selectivity for M_1 relative to other mAChRs.

VU0409774 and VU0409775 are Competitive Antagonists of mAChRs. Based on previous studies suggesting that

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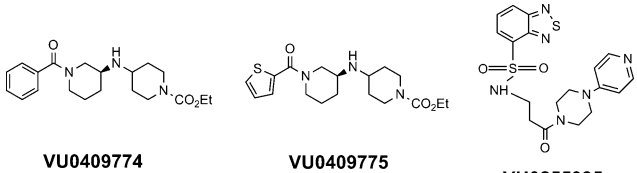
Table 1. pK_b and IC_{50} values of VU0409775, VU0409774, and VU0255035 When Profiled Across All Five Muscarinic Receptor Subtypes[a]



VU0409774 VU0409775 VU0255035

	rM_1		rM_2		rM_3		rM_4		rM_5	
compd	pK_b (±SEM)	pIC_{50} (±STD)	pK_b (±SEM)	pIC_{50} (±STD)	pK_b (±SEM)	pIC_{50} (±STD)	pK_b (±SEM)	pIC_{50} (±STD)	pK_b (±SEM)	pIC_{50} (±STD)
VU0409775	7.02 (0.13)	6.44 (0.26)	5.99 (0.07)	5.74 (0.16)	5.78 (0.19)	5.49 (0.13)	6.28 (0.15)	5.93 (0.14)	5.38 (0.08)	5.24 (−)
VU0409774	6.63 (0.08)	6.18 (0.12)	5.64 (0.09)	5.74 (−)	5.29 (0.19)	–	5.80 (0.17)	–	4.99 (0.06)	–
VU0255035	7.05 (0.07)	6.58 (0.11)	inactive	inactive	inactive	inactive	4.64 (0.02)	inactive	inactive	inactive

[a]Data represents values determined from the inhibition of ACh-induced EC_{80} Ca^{+2} responses in CHO cells stably expressing $rM_{1\text{-}5}$ receptor subtypes. pK_b values represent the mean ± SEM of two to three independent experiments performed in triplicate. pK_b values were derived from the method previously described in Lazareno and Birdsall.[21] pIC_{50} values represent the mean ± STD of same experiments.

VU0364572 acts at a site that is distinct from the orthosteric ACh binding site, we anticipated that antagonists based on this scaffold may act as NAMs rather than competitive antagonists at the orthosteric site. To determine if VU0409775 and VU0409774 displace the orthosteric radioligand 1-[*N*-methyl-^{3}H] scopolamine ([^{3}H]-NMS), we performed radioligand binding studies on membranes isolated from CHO cells expressing rM_1 or rM_3 mAChRs. In these experiments, membranes were incubated with a nonsaturating concentration of [^{3}H]-NMS (0.3 nM) together with increasing concentrations of atropine, VU0409774, or VU0409775. Atropine displaced [^{3}H]-NMS binding in membranes from both receptor subtypes, a finding that is consistent with previous reports that atropine is a nonselective antagonist of muscarinic receptors[11,22] (Figure 3a and b). The calculated K_i value of atropine at rM_1 CHO membranes (2.51 ± 0.48 nM) is consistent with previous reports and is similar to the pK_b value of atropine for inhibition of functional responses to ACh (data not shown). VU0409774 and VU0409775 also completely displaced [^{3}H]-NMS in rM_1 CHO membranes (Figure 3a and b; for complete list of K_i values for all antagonists, see Table 2) and the calculated K_i values of VU0409774 and VU0409775 at M_1 are consistent with the pK_b and pIC_{50} values of these compounds (Table 1) at inhibiting functional responses to ACh. At M_3, VU0409774 showed micromolar K_i values, a finding similar to its weak activity (>10 μM) for inhibition of ACh-induced functional responses in Ca^{2+} assays. While these data alone are not sufficient to suggest a competitive mechanism of action of these compounds, they are consistent with an action of VU0409774 and VU0409775 as competitive antagonists that are not selective for M_1 relative to other mAChR subtypes. We also performed dissociation kinetic experiments and found that VU0409774 and VU0409775 have slight/no effects on [^{3}H]-NMS dissociation suggesting these compounds function as pure orthosteric antagonists (Figure 3c).

To more directly assess the mechanism by which VU0409774 and VU0409775 inhibit functional responses to ACh in M_1-expressing cells, we performed Schild analyses[23] to determine whether these compounds act in a competitive manner with ACh at the orthosteric site. Ca^{2+} responses of multiple ACh concentrations were assessed in rM_1-expressing CHO cells in the absence and presence of increasing concentrations of VU0409774 or VU0409775 (Figure 4a, b). Both compounds induced parallel rightward shifts in the ACh CRC with no effect on the maximal response to ACh. Schild regression analysis revealed linear regression lines with slope values that were not significantly different from unity (Figure 4c) for both VU0409774 (slope = 0.924 ± 0.012) and VU0409775 (slope = 0.948 ± 0.007). This suggests that inhibition of functional responses to ACh by both compounds is mediated by competitive interaction with the orthosteric ACh binding site. In addition, the affinities estimated from Schild analysis for both VU0409774 (K_d = 21.0 nM) and VU0409775 (K_d = 51.0 nM) are consistent with the affinities of these compounds as assessed in radioligand binding studies at rM_1 (82.5 and 160 nM, respectively). The prototypical competitive antagonist atropine was used as a control and showed a similar profile in Schild analysis experiments (data not shown) but had a higher affinity (5 nM) than either VU compound. As was observed with the novel antagonists VU0409774 and VU0409775, the affinity of atropine estimated from Schild analysis (K_d = 5 nM) was consistent with its K_i value (2.51 ± 0.48 nM) as determined from radioligand binding experiments in rM_1 CHO membranes.

Detailed Characterization of VU0364572 and VU0357017 Reveals Binding at an Orthosteric Site of Muscarinic Receptors. The findings that VU0409774 and VU0409775 are nonselective orthosteric antagonists prompted further characterization of VU0364572 and the recently developed M_1 agonist VU0357017. Previous molecular pharmacology and mutagenesis studies suggest that VU0357017 is not a typical orthosteric agonist but interacts with M_1 in a manner that is distinct from that of ACh.[17] VU0357017 also fails to significantly inhibit [^{3}H]-NMS binding to the orthosteric site at concentrations up to 3 μM. In more recent studies, we assessed activity of these compounds in a system where we could vary the levels of M_1 expression and found that VU0357017 and VU0364572 can act as weak partial agonists in systems expressing low levels of M_1 so that there is little or no receptor reserve.[7] In the low receptor reserve systems, both compounds behaved as weak partial agonists and displayed micromolar potency values. On the other hand, both compounds were found to be very potent (nanomolar potency values) in systems with high receptor reserve (see Digby et al.[7] for details). These findings, coupled with the present results showing that close analogues of VU0364572 behave as competitive orthosteric antagonists of M_1, raise the possibility that VU0364572 and VU0357017 could act as very weak partial

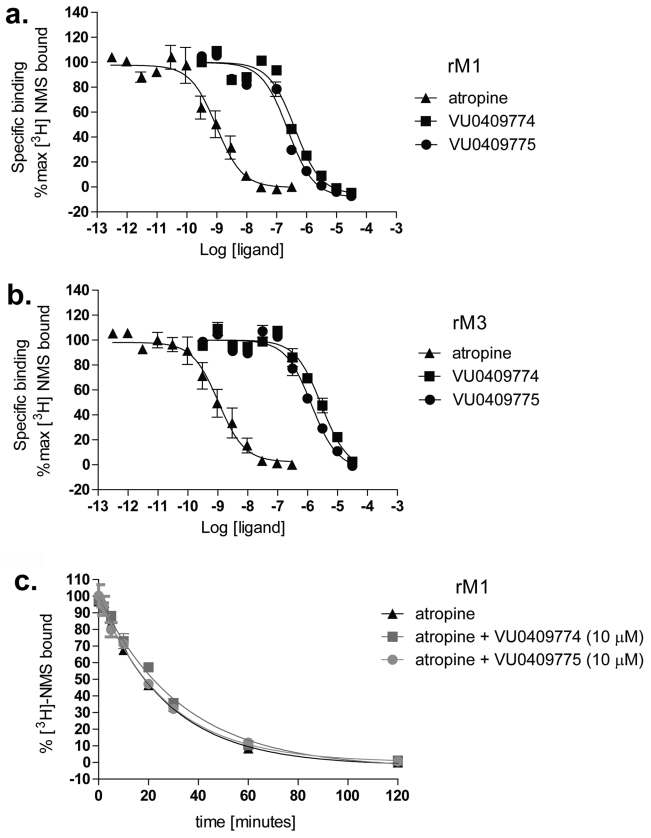


Figure 3. VU0409774 and VU0409775 displace [^{3}H]-NMS at rM_1 and rM_3 receptors and do not slow the dissociation of radioligand at rM_1 receptors. Antagonists VU0409774 and VU0409775 displace radioligand binding at rM_1 (a) and rM_3 (b) mAChRs. Membranes were prepared from CHO cells stably expressing rM_1 receptors or rM_3 receptors and were incubated with the radiolabeled NMS analogue [^{3}H]-NMS (0.3 nM final concentration in 100 mM NaCl and 20 mM HEPES, pH = 7.4) for 3 h at room temperature in the presence of varying concentrations of VU0409774, VU0409775, or atropine. Data are plotted as a percentage of specific [^{3}H]-NMS binding and represent three or four separate experiments performed in duplicate or triplicate. (c) Effects of VU0409774 (10 μM) and VU0409775 (10 μM) on atropine-induced dissociation of [^{3}H]-NMS binding to membrane preparations isolated from CHO cells stably expressing rat M_1 receptors. The k_{off} values measured in the presence of VU0409774 (0.030 ± 0.00/min) and VU0409775 (0.037 ± 0.00/min) are comparable to the k_{off} values derived from membranes treated with atropine alone (0.036 ± 0.00/min). Equilibrium binding was terminated by rapid filtration. Graphs are expressed as the mean ± SEM.

Table 2. K_i Values of VU0409775, VU0409774, and Atropine Derived from Competition Binding Experiments[a]

compd	rM_1 K_i, nM (±SEM)	rM_3 K_i, nM (±SEM)
atropine	2.51 (0.48)	1.97 (1.07)
VU0409775	82.5 (23.4)	1360 (700.0)
VU0409774	160 (66.0)	3240 (1800)

[a]Data represents values determined from displacement of [^{3}H]-NMS (0.3 nM) in cell membranes isolated from CHO cells stably expressing rM_1 or rM_3 receptors. Values represent the mean ± SEM of three independent experiments performed in duplicate.

agonists with low affinity at the orthosteric site. Thus, we performed a series of studies to further test the hypothesis that VU0364572 and VU0357017 also interact with the orthosteric

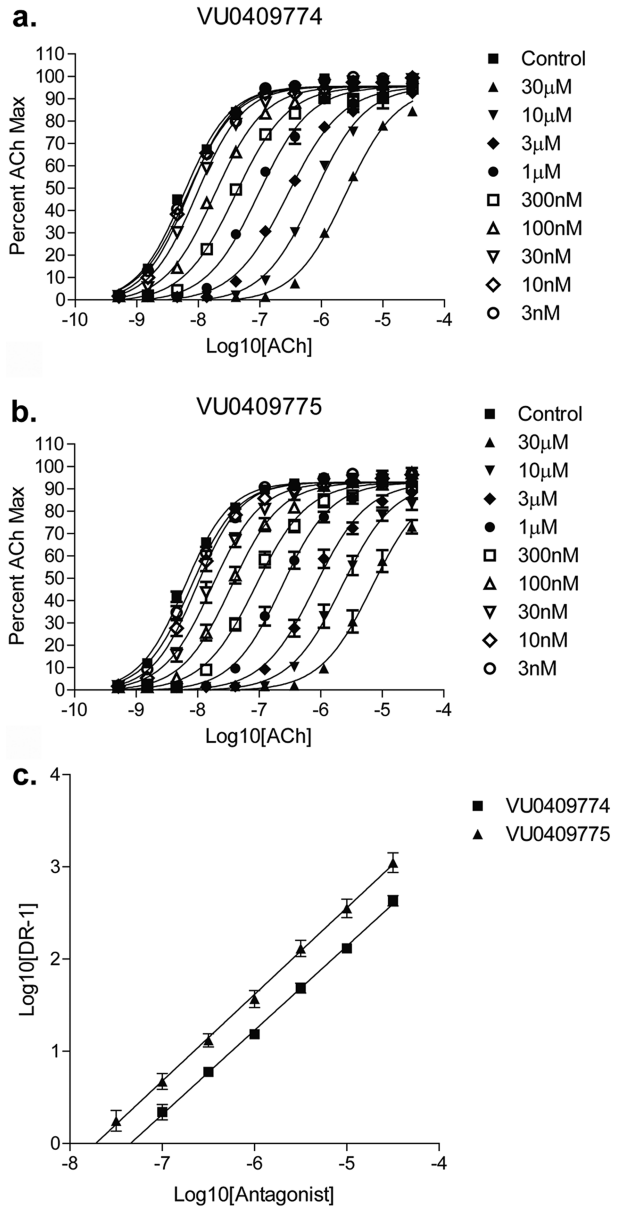


Figure 4. VU0409774 and VU0409775 competitively antagonize ACh functional responses at the rM_1 receptor. ACh CRCs were performed in the absence or presence of 30 μM, 10 μM, 3 μM, 1 μM, 300 nM, 100 nM, 30 nM, 10 nM, or 3 nM VU0409774 (a) or VU0409775 (b). (c) Schild regression of the dose ratios plotted against EC_{50} values derived from ACh-induced Ca responses across a range of VU0409774 or VU0409775 concentrations. Graphs represent the mean ± SEM of three experiments conducted in triplicate.

site and that this contributes to the agonist activity of these compounds.

We first evaluated the ability of VU0364572 and VU0357017 to bind to the orthosteric binding site at each of the mAChR subtypes by assessing their effects on [^{3}H]-NMS binding. Consistent with our previous studies, neither compound significantly inhibited [^{3}H]-NMS binding in rat M_1 CHO membranes at concentrations up to 3 μM (Figure 5). However, when the concentration of either compound was "pushed" to millimolar values, both VU0364572 and VU0357017 completely displaced [^{3}H]-NMS at each of the mAChR subtypes (Figure 5). Thus, both compounds have weak orthosteric binding activity at all mAChR subtypes. High micromolar range

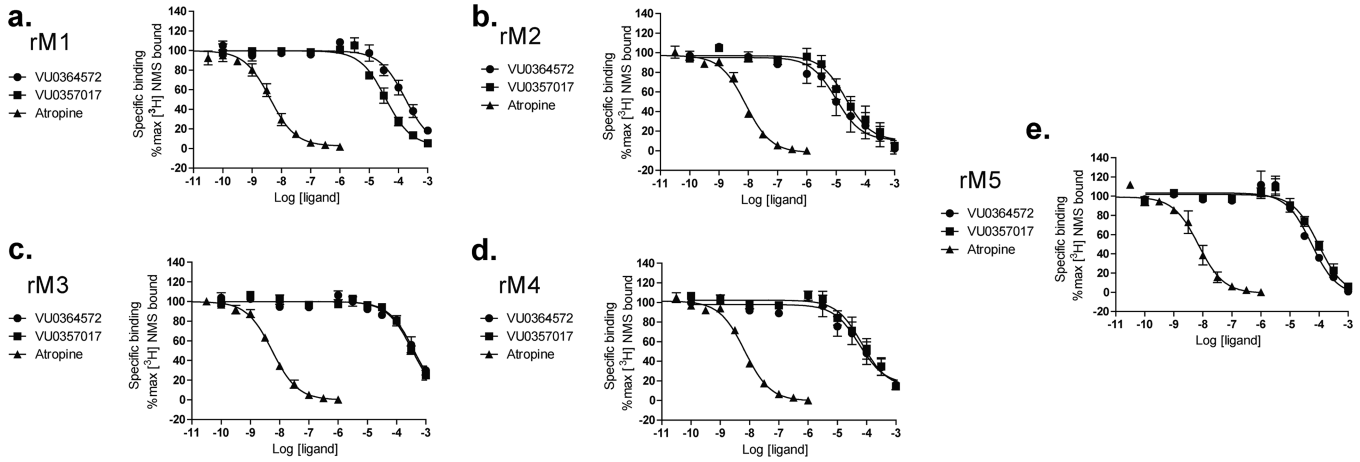
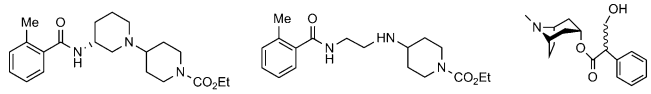


Figure 5. VU0364572 and VU0357017 displace [^{3}H]-NMS at rM_{1-5} mAChRs, suggesting they can occupy the orthosteric binding site. Agonists VU0357017 and VU0364572 displace radioligand binding in membranes isolated from CHO cells expressing rM_1 (a), rM_2 (b), rM_3 (c), rM_4 (d), or rM_5 receptors (e). Membranes were incubated with [^{3}H]-NMS (0.3 nM final concentration in 100 mM NaCl and 20 mM HEPES, pH = 7.4) for 3 h at room temperature in the presence of varying concentrations of atropine, VU0357017, or VU0364572. Equilibrium binding was terminated by rapid filtration. Data are plotted as a percentage of specific [^{3}H]-NMS binding and represent three or four separate experiments performed in duplicate or triplicate. Graphs are expressed as the mean ± SEM.

Table 3. K_i values of Atropine, VU0364572, and VU0357017 Derived from Competition Binding Experiments[a]



VU0364572 VU0357017 atropine

compd	rM_1 K_i μM (±SEM)	rM_2 K_i μM (±SEM)	rM_3 K_i μM (±SEM)	rM_4 K_i μM (±SEM)	rM_5 K_i μM (±SEM)
VU0364572	45.9 (10.1)	19.7 (16.9)	77.5 (8.41)	30.3 (13.2)	26.6 (0.56)
VU0357017	9.91 (1.6)	21.4 (15.7)	55.3 (21.5)	35.0 (12.9)	50.0 (13.6)
atropine	0.001 (0.000)	0.003 (0.000)	0.001 (0.000)	0.002 (0.000)	0.003 (0.001)

[a]Data represents values determined from displacement of [^{3}H]-NMS (0.3 nM) in cell membranes isolated from CHO cells stably expressing rM_1–rM_5 receptors. Values represent the mean ± SEM of three to five independent experiments performed in duplicate.

K_i values were found for both compounds at each receptor subtype (see Table 3).

Next we confirmed that VU0357017 (EC_{50} = 477 ± 172 nM; pEC_{50} = 6.37 ± 0.15) and VU0364572 (EC_{50} = 287 ± 147 nM; pEC_{50} = 6.60 ± 0.240) are potent agonists of Ca^{2+} responses in CHO cells expressing rat M_1 receptors (Figure 6a), a finding consistent with previous reports.[7,16,17] This cellular system expresses moderate to high levels of rM_1Rs and abundant receptor reserve, as suggested by a potent CCh response (EC_{50} = 73.0 ± 16.0 nM; pEC_{50} = 7.33 ± 0.08). Both compounds were also found to be inactive at inducing Ca^{2+} responses at M_{2-5} receptors at concentrations up to 30 μM (data not shown). In light of our findings that VU0357017 and VU0364572 can bind to the orthosteric site at micromolar concentrations, we further evaluated these compounds by measuring effects on inositol monophosphate (IP) accumulation in the same CHO cells that were used to measure calcium mobilization responses. In contrast to the calcium mobilization assay, the IP accumulation assay is not characterized by high receptor reserve and orthosteric agonist potencies more closely reflect their affinities at M_1.[14] Consistent with this, the orthosteric mAChR agonist carbachol (CCh) induced an increase in IP accumulation with an EC_{50} value (EC_{50}= 4.05 ± 0.492 μM) that is similar to its previously determined affinity at M_1 (12.5 ± 5.00 μM; see ref 24). VU0364572 induced a modest increase in IP accumulation with a maximal response that was approximately 30% of the response to CCh (Figure 6b). In this low receptor reserve system, the EC_{50} value for VU0364572 (23.2 ± 11.5 μM) is similar to its K_i value at binding to the orthosteric site as assessed using [^{3}H]-NMS binding (45.9 ± 10.1 μM; Table 3). In contrast, VU0357017 was without effect in this assay (Figure 6b), suggesting that agonist activity of VU0357017 can only be observed in settings with higher receptor reserve.

The finding that the potency of VU0364572 in this low receptor reserve assay is similar to its K_i value at the orthosteric site is consistent with activity as a weak orthosteric site partial agonist. The lack of measurable activity of VU0357017 in this assay does not rule out weak partial agonist activity at the orthosteric site but suggests that some level of receptor reserve is necessary for weak partial agonist activity to be observed. If VU0364572 and VU0357017 act as weak partial orthosteric site agonists, these compounds should also inhibit the response to the orthosteric site full agonist CCh in a system with low receptor reserve with potencies similar to the calculated K_i values of these compounds at the orthosteric site. Thus, we evaluated the effects of these compounds on responses to a submaximal concentration of CCh. As shown in Figure 6c, both VU0364572 and VU0357017 inhibited an EC_{60} concentration of CCh in PI hydrolysis assays with IC_{50} values (see Table 3) that are similar to their estimated orthosteric site affinities as measured by inhibition of [^{3}H]-NMS binding (VU0364572 IC_{50} = 7.58 μM ± 1.54 μM; VU0357017 IC_{50} = 3.67 μM ± 1.78 μM). Also, consistent with expected actions as a partial

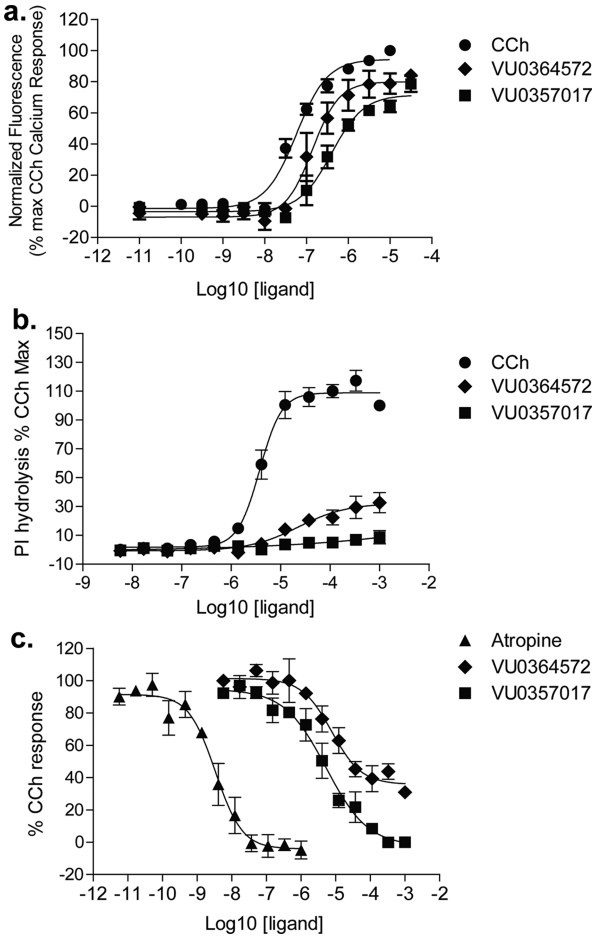


Figure 6. Previously developed M_1 partial agonists induce responses in Ca^{++} and PI hydrolysis assays and can function as antagonists of orthosteric ligands. (a) CCh, VU0364572, and VU035717 induce calcium responses in CHO cells expressing M_1 mAChRs. Data were normalized to the maximal response elicited by CCh. Data represent 3−10 independent experiments performed in triplicate. (b) CCh and VU0364572 induced responses in PI hydrolysis assays whereas VU0357017 was without effect. In this assay, phosphoinositides were measured following addition of each compound. Data for each assay was obtained from three separate experiments, each performed in duplicate, and expressed as the mean ± SEM. Data were normalized to the maximum CCh response. (c) Partial agonists VU0364572 and VU0357017 antagonize CCh-induced responses in PI hydrolysis assays. CRCs were performed by adding increasing concentrations of atropine or each M_1 partial agonist in the presence of an EC_{60} concentration of CCh. Data were normalized to the maximal response elicited by CCh and presented as a percentage of the EC_{80} response.

agonist, VU0364572 failed to completely inhibit this response but inhibited the response to CCh to approximately 30% of the response to CCh alone (see Figure 6c).

To fully evaluate the role for binding to the orthosteric ACh site for functional agonist activity of VU0364572 and VU0357017 at M_1, we assessed the activities of these compounds using the Furchgott analysis.[25,26] This quantitative analysis of relative potencies of agonists using functional assays of receptor activity under conditions of varying levels of receptor expression provides an estimate of affinities of partial agonists at the site responsible for functional agonist activity. To accomplish this, we took advantage of a tetracycline (TET)-inducible M_1 mAChR stable cell line that allows precise control of levels of M_1 expression.[7] Figure 7a shows an example of the concentration response relationship of CCh for the induction of calcium mobilization in cells that were not treated with 25 ng tet which induces modest receptor expression tetracycline and in cells incubated with 1 μg of tetracycline to induce robust M_1 expression. As can be seen, induction of M_1 expression induces an increase in the maximal response to CCh and shifts the concentration response relationship to the left. Figure 7d shows a double reciprocal plot of equiactive concentrations in cells exposed to 1 μg of TET for 24 h and cells that were exposed to 25 ng test. A pK_d value of CCh (5.08 ± 0.20; K_d = 10.1 μM) was calculated from the fitted straight line (see Methods) and was found to be consistent with previously published results.[24] Using this same approach, we performed full concentration response curve analysis of both VU0364572 and VU0357017 (Figure 7b and c). pK_d values for VU0364572 (6.67 ± 0.29.; K_d = 0.95 μM) and VU0357017 (5.66 ± 0.34; K_d = 2.88 μM) were estimated from these analysis and were in agreement with the calculated K_i values for each compound as assessed in measures of inhibition of [^{3}H]-NMS binding (Table 3). Furthermore, upon alternative analysis of these data using an operational model of receptor depletion,[27] we found that the operationally derived pK_a values derived were in agreement with pK_a values estimated from double reciprocal plots of the Furchgott analysis (see Figure 7e and f).

VU0364572 and VU0357017 also Exhibit Activity via an Allosteric Site. The data outlined above suggest that VU0364572 and VU0357017 act as weak partial agonists at M_1 and that their actions could be accounted for by low affinity binding to the orthosteric ACh binding site. However, previous studies, including site-directed mutagenesis, suggest that functional activity of these compounds do not behave as traditional orthosteric M_1 agonists.[17] While the previous studies were suggestive of an allosteric effect, they do not provide definitive evidence that these compounds act at an allosteric site. For instance, the calcium mobilization assays used in previous Schild analyses relied on a high affinity antagonist (atropine) which may not have allowed sufficient time for low affinity agonists to establish equilibrium. Thus, these studies are entirely consistent with VU0364572 and VU0357017 functioning as low affinity orthosteric partial agonists (see Christopoulos et al. for additional examples of nonclassical antagonism by competitive antagonists under conditions of transient response kinetics[28]). If VU0364572 and VU0357017 act only as fully competitive ligands at the orthosteric site on M_1, they should displace [^{3}H]-NMS binding under equilibrium conditions (as shown in Figure 5) and should not alter dissociation kinetics of [^{3}H]-NMS. However, if these compounds also interact with a distinct site on M_1, they may alter dissociation kinetics of [^{3}H]-NMS binding.[29] Thus, to further determine whether these compounds interact with an allosteric site on M_1 receptors, we performed dissociation kinetic experiments. [^{3}H]-NMS dissociation was performed on membranes isolated from CHO cells expressing rM_1 receptors. Figure 8 shows the rate of dissociation of [^{3}H]-NMS binding after addition of the orthosteric antagonist atropine (1 μM; $t_{1/2}$ = 18.0 ± 2.09 min; k_{off}= 0.041 ± 0.001/min). As expected for a traditional orthosteric mAChR agonist, CCh did not alter the dissociation kinetics of [^{3}H]-NMS binding ($t_{1/2}$ = 19.8 ± 3.22 min; k_{off} = 0.042 ± 0.009/min). In contrast, gallamine (1 mM), a previously characterized negative allosteric modulator of M_1, drastically slowed the dissociation of [^{3}H]-NMS in membranes ($t_{1/2}$ = 51.5 ± 14.9 min; k_{off} = 0.015 ± 0.004/min). This is consistent with previous reports of gallamine effects on [^{3}H]-

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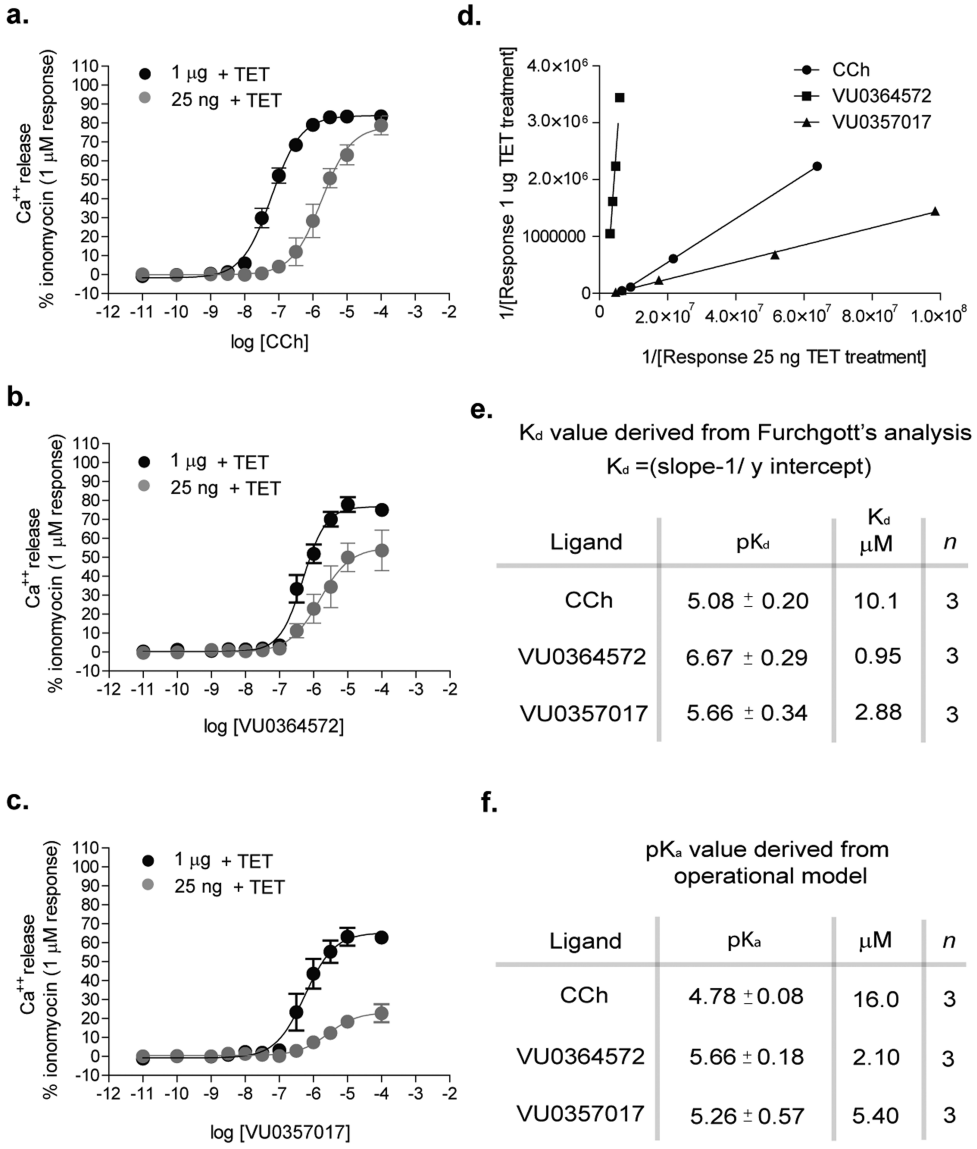

e.

K_d value derived from Furchgott's analysis

K_d =(slope-1/ y intercept)

Ligand	pK_d	K_d μM	*n*
CCh	5.08 ± 0.20	10.1	3
VU0364572	6.67 ± 0.29	0.95	3
VU0357017	5.66 ± 0.34	2.88	3

f.

pK_a value derived from operational model

Ligand	pK_a	μM	*n*
CCh	4.78 ± 0.08	16.0	3
VU0364572	5.66 ± 0.18	2.10	3
VU0357017	5.26 ± 0.57	5.40	3

Figure 7. Affinity estimates of CCh, VU0364572, and VU0357017 determined from Furchgott analysis. (a) Example traces showing log concentration−response curves for CCh in cell lines treated with 1 μg of tetracycline (TET) or treated with 25 ng/mL TET. (b) Example traces showing log concentration−response curves for VU0364572 in cell lines treated with 1 μg of TET or treated with 25 ng/mL TET. (c) Example traces showing log concentration−response curves for VU0357017 in cell lines treated with 1 μg of TET or treated with 25 ng/mL TET. Values for agonist-induced responses in both conditions were obtained from three independent experiments and represent the mean ± SEM. (d) Double reciprocal plots of equiactive concentrations in cells exposed to 1 μg of TET for 24 h and cells that were treated with 25 ng/mL TET. (e) Equilibrium dissociation constants (pK_d and K_d) of CCh, VU0364572, and VU0357017 that were calculated from the fitted straight line and were determined from the method previously described in Besse and Furchgott (1976).[26] (f) Affinity (pK_a) values derived from the operational model of receptor depletion previously describe in Leff and Black.[27]

NMS dissociation time constants.[29] Interestingly, VU0364572 and VU0357017 dose-dependently slowed dissociation of [^{3}H]-NMS from M_1Rs. At a 10 μM concentration, VU0364572 and VU0357017 had slight effects on this response (VU0364572, $t_{1/2}$ = 22.6 ± 0.185 min; k_{off} = 0.031 ± 0.000/min; VU0357017, $t_{1/2}$ = 23.9 ± 4.67 min; k_{off} = 0.030 ± 0.006/min), whereas a more prominent effect was observed at 1 mM of either agonist (VU0364572, $t_{1/2}$ = 32.7 ± 7.30 min; k_{off} = 0.025 ± 0.005/min; VU0357017, $t_{1/2}$ = 34.1 ± 8.97 min; k_{off} = 0.025 ± 0.006/min). These data provide strong support for the hypothesis that these compounds also have activity at an allosteric site that is distinct from the orthosteric site that is occupied by [^{3}H]-NMS and CCh.

DISCUSSION

Over the past decade, there have been major advances in discovery and optimization of novel highly selective ligands for individual mAChR subtypes. The major conceptual and practical shift that has allowed these unprecedented advances in developing subtype-selective ligands has been the move away from ligands that interact with the highly conserved orthosteric ACh site and discovery of compounds that act at allosteric sites to modulate mAChR function.[12,30] A priority for these studies has been the development of selective allosteric activators for the M_1 mAChR subtype because of their potential for treatment of schizophrenia and other CNS disorders that involved impaired cognitive function.[12,30] Allosteric activators

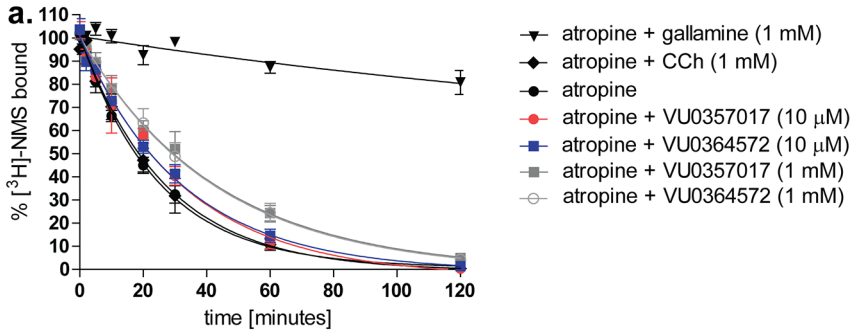

Figure 8. VU0364572 and VU0357017 slow the dissociation of [^{3}H]-NMS, suggesting that they can induce negative cooperativity with orthosteric ligands via an allosteric site. (a) Effects of VU0364572 (10 μM), VU0357017 (10 μM), VU0364572 (1 mM), VU0357017 (1 mM), CCh (1 mM), and gallamine (1 mM) on atropine-induced dissociation of [^{3}H]-NMS binding to membrane preparations isolated from CHO cells stably expressing rat M_1 receptors. The k_{off} values measured in the presence of 10 μM VU0364572 (0.031 ± 0.000/min), 10 μM VU0357017 (0.030 ± 0.006/min), 1 mM VU0364572 (0.025 ± 0.005/min), 1 mM VU0357017 (0.025 ± 0.006/min), CCh (0.042 ± 0.009/min) and gallamine (0.015 ± 0.004/min) are compared to k_{off} values derived from membranes treated with atropine alone (0.041 ± 0.001/min). Data represent five separate experiments which were performed in duplicate. Data are expressed as the mean ± SEM.

of M_1 and other GPCRs include allosteric agonists and positive allosteric modulators (PAMs). The primary distinguishing property of GPCR PAMs is that simultaneous binding of the PAM and orthosteric agonists to distinct sites has a positive cooperative effect such that the PAMs increase responses to the orthosteric agonist.[12] Thus, while some PAMs can activate GPCRs in the absence of agonist (ago-PAMs), a defining property of classical GPCR PAMs is that they potentiate responses only in the presence of the endogenous agonists and induce leftward shifts in the orthosteric agonist concentration response relationship. A large number of highly selective M_1 PAMs have now been identified, including BQCA, VU0405652 (ML169), VU0405645, VU0029767, and others.[31−35] These M_1 PAMs have proven to be highly selective and have provided important new insights into the functional roles of M_1 in defined CNS circuits.[8,36]

Unlike the PAMs, allosteric agonists do not potentiate responses to orthosteric agonists but activate the receptor directly by actions at a site that is distinct from the orthosteric site. Based on previous studies, VU0357017 and VU0364572 have been viewed as allosteric agonists of M_1.[7,16,17] For instance, single point mutations that reduce binding and activity of ACh have no effect on responses to VU0357017 and VU0364572, and other mutations were identified that eliminate effects of these novel M_1 agonists but do not alter responses to ACh. The current findings that VU0357017 and VU0364572 both reduce the rate of dissociation of [^{3}H]-NMS from M_1 are consistent with a view that these agonists do not bind to M_1 in a manner that is identical to that of traditional orthosteric agonists. However, the finding that these compounds bind to the orthosteric site of mAChRs with micromolar affinities along with results from the current functional studies of M_1 activation suggest that these compounds behave in a manner that is consistent with activity as weak orthosteric agonists. Most notably, both compounds have partial agonist activity in systems with reduced receptor reserve and in the absence of receptor reserve (see Digby et al.[7]); their agonist and antagonist potencies are also consistent with their affinities at the orthosteric site on M_1. Furthermore, both compounds also show predictable shifts in agonist efficacy and potency across multiple levels of M_1 expression, and Furchgott analysis provides estimates of affinities based on functional assays that are closely aligned with measured affinities at the orthosteric site.

Based on these findings, we propose a bitopic mechanism of action of VU0364572 and VU0357017. Both compounds dose-dependently slowed NMS dissociation when added at 10 μM and 1 mM concentrations, suggesting that high concentrations of compound are not required to detect an allosteric effect. VU0357017 or VU0364572 may also bind to the multiple residues of the orthosteric pocket including Y381, and it is possible that allosteric binding site exists above the Y381. In agreement with this, mutagenesis and molecular modeling of the M_1 agonist VU0184670 (see Lebois et al.[17]) suggests that the western aryl moiety of this compound (which is shared between VU0184670, VU0364572, and VU0357017) orients itself toward the extracellular space. At this time, the role of the orthosteric D3.32 residue in TM3 is unclear. Mutagenesis experiments of D3.32 will be required for a definitive determination of this residue in the binding of M_1 agonists.

Interestingly, multiple recent findings suggest that other M_1 allosteric agonists bind M_1 by novel mechanisms that are not consistent with mutually exclusive allosteric or orthosteric binding. For example, the recently developed M_1 agonists 77-LH-28-1 and AC-42 have been shown to bind M_1 at both orthosteric and allosteric sites.[19,37] Similarly, the M_1 agonist TBPB has multiple properties consistent with an allosteric mechanism of action[13,29] and slows dissociation kinetics of [^{3}H]-NMS binding to M_1[29] but also displaces [^{3}H]-NMS at all five muscarinic receptor subtypes, suggesting it may have additional activity at orthosteric sites of muscarinic receptors.[38] Our studies are in agreement with these, and it is likely that VU0357017 and VU0364572, as well as other compounds classified as allosteric agonists of M_1, are actually bitopic othosteric/allosteric agonists which interact with multiple sites on M_1 receptors.[39] One of the most important implications of this finding is that these compounds may suffer from many of the same challenges that hindered previous efforts to develop highly selective orthosteric agonists as viable drug candidates. For instance, unlike M_1 PAMs, bitopic M_1 agonists can suffer from the same issues of variable activity depending on varying levels of receptor reserve that has been a major challenge for orthosteric M_1 agonists. Furthermore, these data suggest that the selectivity of VU0357017 and VU0364572 and other bitopic mAChR ligands is based on functional selectivity rather

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than selective binding to M_1 relative to other mAChR subtypes. For mAChRs, functional selectivity with similar affinities at multiple mAChR subtypes has proven to be problematic in clinical studies because of the high receptor reserve of M_2 and other mAChR subtypes in peripheral systems.[12] Because of this high receptor reserve, low affinity binding and weak efficacy at M_2 and M_3 were sufficient to induce dose limiting adverse effects. These properties complicate efforts to chemically optimize M_1 allosteric agonists in a manner similar to the challenges observed with previous orthosteric agonist efforts. Thus, structural changes that increase affinities for M_1 may also increase affinities for other mAChR subtypes and erode selectivity.[38] In contrast, traditional PAMs do not act by binding to the orthosteric site of GPCRs, and we have found that selective PAMs for individual mAChR and other GPCR subtypes have similar potencies and efficacies at potentiating agonist responses across a range of systems and levels of receptor expression.[7,40]

In addition to our findings of a bitopic mode of binding for VU0364572 and VU0357017, we also developed a novel series of muscarinic antagonists. The lead compound, VU0409774, was shown to be nonselective for muscarinic receptors and functioned as an orthosteric antagonist in multiple assays, including Schild analysis and receptor binding experiments. Interestingly, these antagonists were formed from rather modest changes in the structure of VU0364572, such as the reversal of pipredine group and the inversion of its chirality. Based on this, care must be taken when making structural modifications to this series of bitopic agonists. Furthermore, the finding that subtle changes can fundamentally alter the activity of these compounds raises the importance of monitoring potential metabolites when performing in vivo studies to unsure that metabolites are not formed that have different actions than the parent compound.[41]

In summary, the current findings provide critical new insights into issues surrounding chemical lead optimization efforts focused on developing and optimizing novel M_1 bitopic agonists. The present findings suggest that while VU0357017 and VU0364572 have multiple properties associated with allosteric agonists, the binding of these compounds to the orthosteric site is critical for their activity. While the bitopic nature of these compounds may confer advantages relative to orthosteric agonists, many of the challenges associated with efforts to optimize orthosteric M_1 agonists, including variable activity depending on receptor reserve and functional selectivity, are not necessarily mitigated in efforts to optimize bitopic agonists as drug candidates.

METHODS

Compounds. Muscarinic agonist carbachol and the antagonist atropine were purchased from Sigma-Aldrich (St. Louis, MO). Chemical synthesis of VU0364572 and VU0357017 was performed at the Vanderbilt Center for Neuroscience Drug Discovery (VCNDD) (Vanderbilt University, Nashville, TN). Negative allosteric modulator Gallamine Triethiodide was purchased from MP Biomedicals (Solon, OH).

Cell Lines. Stable CHO cell lines constitutively expressing rat M_1, M_2, M_3, M_4, or M_5 mAChRs were used in binding and Ca^{2+}assays. For experiments where activity was measured at M_2 and M_4 receptors, CHO cells stably expressing each were used in combination with the chimeric G protein G_{qi5}. Generation of a tetracycline (TET)-inducible (TRex) human M_1 mAChR stable cell line was previously described (see Digby et al.[7]).

Furchgott Analysis. To determine K_d values of CCh, VU0364572, and VU0357017 via Furchgott's analysis, we measured responses at equieffective concentrations of ligand in a TET-inducible cell line. Agonist-induced Ca^{2+} responses from TET-treated cells (1 μg treatment for 24 h) and untreated cells were obtained. The reciprocal of the concentrations of agonist in TET-treated cells (1/[response 1 μg TET treatment]) were plotted against the reciprocal of the corresponding equieffective concentrations with 25 ng TET treatment (1/[response 25 ng treatment]). Slope and y-intercept values were obtained from line fitting points which were used to determine K_d estimates according to the following equation K_d = (slope − 1/intercept).[25,26] For determination of log K_a values, an operational model of receptor depletion expressed as operate = $((10^{\log K_a} + 10^{X})/10^{\text{logtau}+X})^{n}$ and Y = basal + (effectmax − basal)/(1 +operate).[27] All analysis was performed using Prism software. See Digby et al.[7] for characterization of receptor densities following TET treatments in hM_1 TRex cell line.

Calcium Mobilization Assays. CHO cells were seeded at a density of 15 000 cells/well in 384-well plates (Greiner). Cells were treated for 45 min with calcium indicator dye, fluo-4 (2 μM) dissolved in Hank's balanced salt solution (HBSS, containing 20 mM HEPES and 2.5 mM probenecid, pH = 7.4) followed by a wash step. Agonist stocks were added to each well, and fluorescent signals were measured at λ_{525} nm fluorescence emission after λ_{480} nm excitation using an FDSS (Hammamatsu). For triple add experiments, a single add of agonist or antagonists at different doses was added to cells within 10 s of beginning of the read followed by a 1.5 min delay prior to the addition of an EC_{20} concentration of ACh to determine potential positive modulation of muscarinic mediated responses. After an additional minute, an EC_{80} concentration of ACh was added to cells to determine potential antagonism of a compound. Baseline values were subtracted from the maximally induced responses and responses were plotted CRC format. pK_b values were derived from a method previously described in Lazareno and Birdsall.[21]

[^{3}H]-NMS Inhibition and Saturation Binding Assays. Competition binding studies were performed by incubating membranes isolated from ratM_1-CHO cells or membranes isolated from CHO cells expressing one of the other five muscarinic receptor subtypes with 300 pM [^{3}H]-NMS (GE Heathcare) dissolved in radioligand binding buffer containing 100 mM NaCl, 20 mM HEPES, and 10 mM $MgCl_2$ (pH = 7.4). An amount of 10 μg of protein was used per well, and serial dilutions of test compounds were added to 96-well deep-well plates. A final volume of 0.5 mL was used for all assays. Nonspecific binding was determined in the presence of 10 μM atropine. Binding reactions were performed at room temperature for 3 h, and reactions were terminated by rapid filtration through GF/B filter plates. Following termination, plates were washed three times with ice-cold harvesting buffer using a 96-well Brandel harvester. Plates were allowed to dry overnight and radioactivity was determined using a TopCount NXT microplate scintillation and luminescence counter (PerkinElmer Life and Analytical Sciences).

Phosphoinositol Hydrolysis Assays. Measurements of phosphoinositol (PI) accumulation were performed on rat M_1 CHO cells that were plated in 24 well plates at a density of 3.0×10^5 cells/well in antibiotic-free media containing 0.5 μC/mL [^{3}H]-inositol (PerkinElmer). Prior to addition of antagonists or test compounds, cells were washed with HBSS containing LiCl (30 mM). For agonist-induced experiments, agents were serially diluted into HBSS for a 2× stock concentration in 2% DMSO; 0.5 mL of HBSS and 0.5 mL of stock compound was added to each well yielding a final DMSO concentration of 1%. For antagonist experiments, 0.5 mL of (2×) antagonist was added to cells for the equilibration phase; a re-equilibration phase followed with the addition of 0.5 mL of (2×) agonist. Agonist/antagonist solutions were removed from cells after a 1 h incubation period (37 °C), and a formic acid (10 mM) stop buffer solution was added to each well. Stop reactions were allowed to sit at room temperature for 40 min and were either stored overnight at 4 °C or were added to prewashed anion exchange columns (AG 1-X8 Resin, 100−200 mesh, formate form, BIO-RAD). myo-inositol (5 mM) was added to each column; each column's contents were eluted into

scintillation vials using elution buffer (200 mM ammonium formate, 100 mM formic acid) and were measured by liquid scintillation counting.

Kinetic Off-Rate Studies. Kinetic studies were performed using 100 pM [^{3}H]-NMS and membranes isolated from cells expressing rat M_1 mAChRs (10 μg/well concentration). Nonspecific binding was determined by incubating membranes with 10 μM atropine. Membranes and radioligand were allowed to equilibrate for 3 h prior to addition of atropine or atropine + test lignds. At the end of the equilibrium incubation, 1 μM atropine in the absence or presence of 1 mM VU0364572, 1 mM VU0357017, 10 μM VU0364572, 10 μM VU0357017, 10 μM VU0409774, 10 μM VU0406775, 1 mM CCh, or 1 mM gallamine was added, and bound [^{3}H]-NMS was captured after incubation for 2 h, 1 h, 30 min, 20 min, 10 min, 5 min, 2 min, and 0 min. At the 0 min time point, membranes were immediately harvested using ice-cold harvesting buffer and a 96-well Brandel harvester. Specific [^{3}H] NMS bound was calculated using Prism software, and K and $t_{1/2}$ values were calculated using one-phase exponential decay.

AUTHOR INFORMATION

Author Contributions

G.J.D. and T.J.U. planned and performed experiments. C.S. and A.L. performed experiments. D.J.S., M.R.W., C.M.N., E.P.L., T.M.B., and C.W.L. helped with experiment planning and manuscript editing. P.J.C. directed multiple parts of the project and cowrote the manuscript with G.J.D.

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The authors declare no competing financial interest.

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NOTE ADDED AFTER ASAP PUBLICATION

This paper published on the Web on October 2, 2012, with incorrect weight units in Figure 7 and in the text. Weight units were amended from microgram (μg) to nanogram (ng). The corrected version was reposted on November 30, 2012.