Discovery and characterization of novel allosteric potentiators of M1 muscarinic receptors reveals multiple modes of activity

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Nonstandard abbreviations:

GPCR, G protein-coupled receptor; HTS, high-throughput screening; mGluR, metabotropic glutamate receptor; CHO, Chinese Hamster Ovary; DMSO, Dimethylsulfoxide;
ACh, acetylcholine; TBPB, 1-[(1’ -(2-Tolyl)-1,4’-bipiperidin-4-yl]-1,3-dihydro-2H-benzimidazol-2-one; PAM, positive allosteric modulator; 1,2-dipalmitoyl-sn-glycero-3-phosphomethanol (32:0 phosphatidylmethanol (PtdMeOH)); HTS, high throughput screening
Abstract

Activators of M₁ muscarinic acetylcholine receptors (mAChRs) may provide novel treatments for schizophrenia and Alzheimer’s disease. Unfortunately, the development of M₁-active compounds has resulted in nonselective activation of the highly related M₂-M₅ mAChR subtypes, which results in dose-limiting side effects. Using a functional screening approach, we identified several novel ligands that potentiated agonist activation of M₁ with low micromolar potencies and induced five-fold or greater leftward shifts of the ACh concentration-response curve. These ligands did not compete for binding at the ACh binding site, indicating that they modulate receptor activity by binding to allosteric sites. The two most selective compounds, VU0090157 and VU0029767, induced progressive shifts in ACh affinity at M₁ that were consistent with their effects in a functional assay, suggesting that the mechanism for enhancement of M₁ activity by these compounds is by increasing agonist affinity. These compounds were strikingly different, however, in their ability to potentiate responses at a mutant M₁ receptor with decreased affinity for ACh as well as in their ability to affect responses of the allosteric M₁ agonist, TBPB. Furthermore, these two compounds were distinct in their abilities to potentiate M₁-mediated activation of phosphoinositide hydrolysis and phospholipase D. The discovery of multiple structurally distinct positive allosteric modulators of M₁ is an exciting advance in establishing the potential of allosteric modulators for selective activation of this receptor. These data also suggest that structurally diverse M₁ potentiators may act by distinct mechanisms and differentially regulate receptor coupling to downstream signaling pathways.
Introduction

The psychotic and cognitive symptoms of neuropsychiatric disorders such as schizophrenia and Alzheimer’s disease (AD) remain serious unmet medical challenges. Patients suffering from schizophrenia exhibit a constellation of symptoms that include positive, negative, and cognitive symptom clusters. While current antipsychotic agents are effective in reducing positive symptoms such as hallucinations and delusions in most patients, negative symptoms such as anhedonia and blunted affect, as well as deficits in cognitive function, are not effectively treated with current medications (Vohora, 2007). In addition to the unmet medical needs of schizophrenia, the devastating cognitive and neuropsychiatric symptoms characteristic of AD present urgent needs for new therapeutic interventions (Saddichha and Pandey, 2008).

A large number of animal and clinical studies suggest that activation of muscarinic acetylcholine receptors (mAChRs) may provide a novel strategy for treatment of multiple symptoms of both schizophrenia and AD (Langmead et al., 2008). The mAChRs are G protein-coupled receptors (GPCRs) with five subtypes termed M₁ through M₅ that respond to the endogenous neurotransmitter acetylcholine (ACh); M₁, M₃, and M₅ couple to Gᵦ whereas M₂ and M₄ couple via Gᵣᵦo to downstream signaling pathways and associated effector systems (Langmead et al., 2008; Wess, 1996). Interestingly, mAChR agonists and acetylcholinesterase (AChE) inhibitors have established efficacy in improving cognitive performance in AD patients (Bodick et al., 1997; Davis et al., 1992; Raskind et al., 1999; Rogers et al., 1998). In addition, the mAChR agonist xanomeline has robust efficacy in reducing delusions, hallucinations, and other psychotic symptoms in patients suffering from AD (Bodick et al., 1997) and schizophrenia (Shekhar et al., 2008). mAChR agonists also reduce pathological processing of amyloid
precursor protein (APP), raising the exciting possibility that these agents may also have disease modifying effects in AD patients (Fisher, 2008; Jones et al., 2008).

Despite these advances, current mAChR agonists have dose-limiting adverse effects that preclude their use as therapeutic agents. The most prominent adverse effects of mAChR agonists (bradycardia, GI distress, salivation, sweating) are mediated by peripheral M2 and M3 mAChRs (Bymaster et al., 2003; Okamoto et al., 2002; Wess et al., 2007). In contrast, M1 is likely important for the effects of mAChR activation on cognition and sensory processing (Conn et al., 2008b; Fisher, 2008; Jones et al., 2008; Langmead et al., 2008). Unfortunately, efforts to develop selective orthosteric agonists for the M1 receptor have been unsuccessful. This is likely due to the fact that the ACh binding site is highly conserved, making it difficult to develop agonists for M1 that are devoid of activity at the other mAChR subtypes. In addition, there are a number of theoretical problems associated with the use of direct acting agonists of M1, including rapid desensitization that could lead to tolerance and induction of M1-mediated generalized seizures (Bymaster et al., 2003; Hamilton et al., 1997; Wess et al., 2007).

An alternative approach that has been highly successful for ligand-gated ion channels is development of positive allosteric modulators (PAMs) or allosteric potentiators of receptor function. Allosteric potentiators do not directly activate the receptor, but bind to an allosteric site removed from the orthosteric neurotransmitter binding site and potentiate the effects of the endogenous agonist. The classical example of this approach is the use of benzodiazepines to potentiate GABA-A receptor function and provide safe and effective drugs for treatment of insomnia and anxiety disorders without the potentially lethal effects of direct-acting GABA-A receptor agonists (Mohler et al., 2002). We and others have now expanded this approach to GPCRs (Conn et al., 2008a), including the discovery of highly selective allosteric potentiators of
M₄ (Brady et al., 2008; Chan, 2008; Shirey et al., 2008). These findings raise the possibility that allosteric potentiators of M₁ may provide a viable approach to selectively activate M₁. Brucine, an analog of strychnine, is a weak allosteric potentiator of M₁ (Birdsall et al., 1999; Lazarenko et al., 1998), providing proof-of-concept that M₁ PAMs can enhance receptor activity. However, brucine exhibits low potency as an M₁ PAM, induces only a slight increase in the potency of ACh, and is not selective for M₁. If identifying M₁ PAMs offers a viable approach to development of novel activators of this receptor, it will be critical to discover compounds that belong to structurally diverse chemical scaffolds that possess robust M₁ PAM activity. We now report discovery of multiple structurally distinct M₁ PAMs. These compounds induce significant increases in ACh potency and affinity and include compounds that are selective for M₁. Interestingly, detailed molecular pharmacological studies suggest that representative compounds are mechanistically distinct and can differentially regulate signal transduction downstream of M₁ activation.
Materials and Methods

Constructs, Cell Lines & Maintenance. Rat M1 cDNA, provided by T.I. Bonner (National Institutes of Health, Bethesda, MD), was used to generate the orthosteric-site mutant rat M1<sup>Y381A</sup> using the QuikChange site-directed mutagenesis kit. All tissue culture reagents were purchased from Invitrogen Corporation, Carlsbad, CA, unless otherwise noted. Cell lines used for these studies stably expressed rat M1, human M2, human M3, rat M4, or human M5 in the Chinese Hamster Ovary (CHO-K1) parental background. CHO-K1 cells expressing M1, M3, or M5 receptors were cultured in HAM’s F12 medium with 10% FBS, 20 mM HEPES, and 50 μg/ml G418. Cells expressing the M2 receptor along with the chimeric G protein, G<sub>qi5</sub>, were cultured in the same media with 500 μg/ml hygromycin. M2/G<sub>qi5</sub> cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) with 10% FBS, 20 mM HEPES, 1 mM Na pyruvate, 1X Nonessential amino acids, 2 mM L-glutamine, 400 μg/ml G418 (Mediatech, Inc., Herndon, VA) and 500 μg/ml hygromycin. Receptor densities for these cells lines were determined as described in Shirey et al. (Shirey et al., 2008) and were M1, 509 fmol/mg, M2-G<sub>qi5</sub>, 505 fmol/mg, M3, 2313 fmol/mg, M4-G<sub>qi5</sub>, 437 fmol/mg, and M5, 968 fmol/mg. Cells expressing the rat M1<sup>Y381A</sup> orthosteric-site mutant receptor were cultured in the same medium, except without hygromycin. Cells expressing the human mGluR4 receptor with G<sub>qi5</sub> were cultured in DMEM medium with 10% dialyzed FBS, 20 mM HEPES, 1 mM Na pyruvate, 20 μg/ml proline, 400 μg/ml G418, and 5 nM methotrexate (Calbiochem, EMD Chemicals, Gibbstown, NJ).

Primary high throughput screening. Rat M1/CHO cells (10,000 cells/20 μl/well) were plated in black-walled, clear-bottomed, TC treated, 384 well plates (Greiner Bio-One, Monroe, North Carolina) in Ham’s F12 medium with 10% FBS and 20 mM HEPES. The cells were
grown overnight at 37 °C in the presence of 5% CO2. The next day, the medium was removed using a VSpin (Agilent Technologies Velocity 11, Santa Clara, CA) fitted with a modified bucket allowing the 384 well plate to be mounted inverted over a catch basin and spun at 80xg for 10 sec with 40% acceleration and deceleration.

The medium was replaced, using a Thermo Fisher Combi (Thermo Fisher, Waltham, MA), with 20 µl of 2 µM Fluo-4AM (Invitrogen, Carlsbad, CA) for 45 minutes at 37 °C. Fluo-4AM was prepared as a 2.3 mM stock in DMSO, mixed in a 1:1 ratio with 10% (w/v) pluronic acid F-127 and diluted in Assay Buffer (Hank’s balanced salt solution, 20 mM HEPES and 2.5 mM Probenecid (Sigma-Aldrich, St. Louis, MO)). Dye solution was removed using the VSpin and replaced with 20 µl of Assay Buffer using a Combi. Test compounds were transferred to daughter plates using an Echo acoustic plate reformatter (Labcyte, Sunnyvale, CA) and then diluted into Assay Buffer, using a Combi, to generate a 20 µM stock. Ca2+ flux was measured using the Functional Drug Screening System 6000 (FDSS6000, Hamamatsu, Japan). Baseline readings were taken (10 images at 1 Hz, excitation, 470±20 nm emission, 540±30 nm) and then test compounds (20 µl/well) were added using the FDSS’s integrated pipettor. For the primary screen, cells were incubated with test compounds (final concentration 10 µM) for 3.2 minutes and then an EC20 concentration of carbachol was applied. The overall assay protocol was automated using the instruments noted above integrated with a Thermo Fisher F3 robotic arm (Thermo Fisher, Waltham, MA) under the control of a Polara scheduler (Thermo Fisher, Waltham, MA). All data were recorded to instrument local drives and later migrated to a network drive. FDSS data were analyzed using a custom analysis application and were associated with unique compound identifiers based on liquid handler transfer logs and plate barcode readings captured by the Echo and by Polara. “Hits” were selected by comparing the amplitude of the
responses at the time of EC\textsubscript{20} addition plus and minus test compounds. Wells with responses that differed from vehicle wells by 3 standard deviations or greater were selected as hits for further study.

**Concentration-response experiments and counterscreening.** Compounds were serially diluted 1:3 into 10 point concentration response curves in 100\% DMSO and were transferred to daughter plates using the Echo. Compounds were then diluted to a 2X final concentration using Assay Buffer; the final DMSO concentration in these experiments was 0.3\%. Test compounds were applied, followed 3 minutes later by an EC\textsubscript{20} concentration of agonist. For human mGlur4/G\textsubscript{qi5}/CHO counterscreening, cells were plated in DMEM, 10\% dialyzed fetal bovine serum, 20 mM HEPES, and 1 mM sodium pyruvate at 30,000 cells/well and the agonist glutamate was used rather than carbachol. Concentration-response experiments were performed in triplicate for each compound on M\textsubscript{1} and in singlicate for mGlur4. Compound activity at M\textsubscript{1} was then confirmed using ACh as the agonist. Similar calcium mobilization assays were used to confirm activity of the compounds highlighted here at M\textsubscript{1} in the presence of acetylcholine (ACh), and to test for concentration-dependent potentiation or antagonism of the other muscarinic subtypes as described in (Shirey et al., 2008). Curves were fit using a four parameter logistical equation in Microsoft with the XLfit add-in (IDBS, Bridgewater, NJ). Subsequent confirmations of concentration-response parameters were performed in triplicate or quadruplicate on multiple days using independent serial dilutions of source compounds. Data from each day were fit using a four parameter logistical equation in GraphPad Prism to generate Mean ± S.E.M. (GraphPad Software, Inc., La Jolla, CA).

**Competition binding.** Membranes were prepared from M\textsubscript{1}-expressing CHO cells as described (Shirey et al., 2008). Binding reactions contained 0.1 nM [\textsuperscript{3}H]-NMS, 20 μg of
membranes and compound or vehicle (0.3% DMSO, final, to define total binding) or 1 μM atropine (to define nonspecific binding) in a total volume of 500 μl. The $K_D$ of [3H]-NMS was determined empirically to be 0.21 nM. Compounds were serially diluted in DMSO, then diluted in assay buffer (100 mM NaCl, 10 mM MgCl2, 20 mM HEPES, pH 7.4) to give a final DMSO concentration of 0.3% in the binding reaction. Binding reactions were incubated for 2 hours at room temperature on a Lab-Line Titer plate shaker at setting 7 (~750 rpm). Reactions were stopped and membranes collected onto 96-well Barex microplates with GF/B filter (1μm pore size) using a Brandel harvester and washed 3X with ice-cold harvesting buffer (50mM Tris-HCl, 0.9% NaCl, pH 7.4). Filter plates were dried overnight and counted in a PerkinElmer TopCount scintillation counter (PerkinElmer Life and Analytical Sciences). For acetylcholine affinity experiments, the $K_I$ of an acetylcholine competition curve was determined in the absence and presence of fixed concentrations (3 to 30 μM, final) of test compound. Actual [3H]-NMS concentrations were back-calculated after counting aliquots of 5X [3H]-NMS added to the reaction. Radioligand depletion was routinely kept to approximately 5% or less.

**PI hydrolysis.** M1-expressing CHO cells were plated at 1.25x10^5 cells/well in the presence of 0.5 μCi [3H]-myo-inositol in 24-well tissue culture plates and allowed to incubate overnight at 37°C, 5% CO2. Krebs buffer (108 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO4, 1.2 mM KH2PO4, 2.5 mM CaCl2, 25 mM NaHCO3, 10 mM glucose, 20 mM LiCl) was prepared the morning of the assay, and allowed to equilibrate to 95:5 O2/CO2 at 37°C for at least 1 hour to obtain a pH of 7.4. After aspirating inositol-loading media, 0.5 mL of 2X test compound or vehicle (30 μM final concentration, 0.3% DMSO), and then 0.5 mL of 2X acetylcholine was added to M1 cells, followed by a 1 hour incubation at 37°C. Reactions were stopped and inositol phosphates were extracted for 40 minutes at room temperature by adding 10 mM formic acid to
each well. Inositol phosphates were separated on anion exchange columns containing AG 1-X8 resin (100-200 mesh, formate form, Bio-Rad, Hercules, CA). Columns were washed twice with H₂O prior to loading formic acid extracts. Columns were then washed once with H₂O, once with 5 mM myo-inositol (in H₂O), and once with H₂O before elution with 200 mM NH₄HCO₃/0.1 M H₂CO₂; counts were measured using a Beckman Coulter LS 6500 scintillation counter.

**Activation of Phospholipase D.** Phospholipase D activity in rat M₁ CHO cells was assessed using a mass spectrometry based assay as previously described in (Brown et al., 2007). Briefly, CHO cells expressing M₁ muscarinic receptors were plated at 5 x 10E5 cells/well in 6-well tissue culture plates in growth media (Ham’s F12 with 10% FBS, 20 mM HEPES) in a 37 °C humidified atmosphere with 5% CO₂. After 45 hours (~80-90% confluency) growth media was removed and cells were incubated in serum-free DMEM for 2 hours. The cells were challenged with either vehicle or the indicated concentrations of ACh and/or potentiators with and without 0.3% 1-BuOH-d₁₀. Cells were incubated in the indicated conditions for 30 minutes at 37 °C. After incubation, treatment media was removed, cells were washed with ice cold 1 x PBS, and 800 µl of ice cold 0.1N HCl:MeOH (1:1) and 400 µl CHCl₃ were added immediately. 10 µl of 10 µg/ml 1,2-dipalmitoyl-sn-glycero-3-phosphomethanol (32:0 phosphatidylmethanol (PtdMeOH)) was added as an internal standard. The samples were vortexed (1 min) and centrifuged (5 min, 18,000g). The lower phase was then isolated and evaporated, and extracted phospholipids were dissolved in 80 µl CH₃OH:CHCl₃ (9:1). Prior to analysis, 1 µl of NH₄OH was added to each sample to promote protonation of lipid species. Mass spectral analysis was performed on a Finnigan TSQ Quantum triple quadrupole mass spectrometer (Thermo Fisher, San Jose, CA) equipped with a Harvard Apparatus (Holliston, MA) syringe pump and an electrospray source. Samples were analyzed at an infusion rate of 10 µl/min. The lipid samples
were analyzed in negative ionization mode over the range of m/z 350 to 1200. The PLD activity was calculated as the ratio of the peak intensity of the produced PtdBuOH species to that of the internal standard (32:0 PtdMeOH, m/z 661 in negative mode MS). Specifically, 32:1 PtdBuOH-d9 at m/z 710, 34:1 PtdBuOH-d9 at m/z 738 and 36:2 PtdBuOH-d9 at m/z 764 were produced during stimulation and these species were used to calculate the enzyme activity. The PLD activity (PtdBuOH/PMeOH) of agonist treatment alone was normalized to a value of 1, and the ratio of PLD activity of the samples treated with agonist and potentiators to that of the agonist alone was expressed as the relative ratio.

**Compounds.** Carbamylcholine chloride (carbachol), acetylcholine chloride, and atropine were purchased from Sigma-Aldrich (St. Louis, MO). [3H]NMS was purchased from Amersham (Arlington Heights, IL). 1-BuOH-d10 was purchased from Acros Organics (Acros Organics Co., NJ, USA). 32:0 phosphatidyl methanol (Ptd MeOH) and 24:0 diacylglycerol (DAG), were purchased from Avanti Polar Lipids (Alabaster, AL).

The Vanderbilt High Throughput Screening Center compound collection was obtained from ChemBridge Corporation (San Diego, CA) and ChemDiv, Inc. (San Diego, CA) and stored in barcoded, Griener 384 well, Flat-bottom, polypropylene plates. The plates were thermally sealed with peelable seals using a PlateLoc (Velocity 11). Groups of ten plates were vacuum-packed in thermally sealed freezer bags (FoodSaver, Jarden Corp.) and stored frozen at -80°C. Primary hits identified in the screen were reordered from ChemBridge or ChemDiv as 10 mM DMSO stocks; these orders were accompanied by NMR spectra to confirm compound identity, and three of the compounds were resynthesized in house (below).

**Synthesis.** All NMR spectra were recorded on a Bruker 400 MHz instrument (Bruker BioSpin Ltd., Milton, ON). $^1$H chemical shifts are reported in δ values in ppm downfield from
DMSO as the internal standard in DMSO. Data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, br = broad, m = multiplet), integration, coupling constant (Hz). $^{13}$C chemical shifts are reported in δ values in ppm with the DMSO carbon peak set to 39.5 ppm. Low resolution mass spectra were obtained on an Agilent 1200 series 6130 mass spectrometer with electrospray ionization (Agilent Technologies, Santa Clara, CA). High resolution mass spectra were recorded on a Waters Q-TOF API-US (Waters Corporation, Milford, MA). Analytical thin layer chromatography was performed on Analtech silica gel GF 250 micron plates. Analytical HPLC was performed on an Agilent 1200 series with UV detection at 214 nm and 254 nm along with ELSD detection. Preparative purification was performed on an ISCO combi-flash companion (Teledyne ISCO, Lincoln, NE). Solvents for extraction, washing and chromatography were HPLC grade. All reagents were purchased from Sigma/Aldrich Chemical Co. (St. Louis, MO) and were used without purification. All polymer-supported reagents were purchased from Biotage, Inc. (Charlottesville, VA).

(E)-2-(4-ethoxyphenylamino)-N’-((2-hydroxynaphthalen-1-yl)methylene)acetohydrazide (VU0029767). To a solution of 4-ethoxyaniline (10.0 g, 72.9 mmol) in EtOH (100 mL) was added ethyl 2-bromoacetate (14.61 g, 87.0 mmol) and sodium acetate (8.97 g, 109.0 mmol) and stirred for 48 h at room temperature. Once complete, the reaction was then partitioned into H$_2$O and EtOAc and the organics washed with brine and dried over MgSO$_4$ before concentration in vacuo. The crude product was purified by passage through a silica plug eluting with 5% EtOAc in hexanes to afford the intermediate product ethyl 2-(4-ethoxyphenylamino) acetate (11.7 g, 72%) as a dark brown oil. To a solution of the acetate intermediate (4.0 g, 17.93 mmol) in MeOH (50 mL) was added hydrazine (177.9 mL, 177.9 mmol) and stirred for 16 h at 65 °C. Once complete, the reaction was then partitioned into H$_2$O
and EtOAc, and the organics washed with brine and dried over MgSO₄ before concentration in vacuo. The crude product was purified by column chromatography (silica gel) eluting with 5% MeOH in CH₂Cl₂ to afford the intermediate product 2-(4-ethoxyphenylamino)acetohydrazide (2.5 g, 67%) as a white solid. To a solution of the hydrazide intermediate (2.0 g, 9.56 mmol) in DMF (100 mL) was added 2-hydroxy-1-napthaldehyde (1.64 g, 9.56 mmol) and stirred for 16 h at room temperature. Once complete, the reaction was concentrated in vacuo and the crude product was purified by filtration with diethyl ether wash (3x250 mL) to afford title compound (3.41 g, 98%) as a yellow solid; ¹H NMR (400 MHz, DMSO-d₆) δ 9.38 (s, 1H), 8.09 (d, J = 4.2 Hz, 1H), 7.80 (dd, J = 8.8, 4.4 Hz, 2H), 7.50 (t, J = 7.2 Hz, 1H), 7.31 (t, J = 7.2 Hz, 1H), 7.12 (d, J = 8.8 Hz, 1H), 6.67 (d, J = 8.8 Hz, 2H), 6.49 (d, J = 8.8 Hz, 2H), 5.69 (d, J = 6.0 Hz, 1H), 3.80 (q, J = 6.8 Hz, 2H), 3.73 (d, J = 5.6 Hz, 1H), 3.32 (s, 2H), 1.18 (t, J = 6.8 Hz, 3H). ¹³C NMR (100 MHz, DMSO-d₆) δ 167.2, 157.8, 150.6, 146.3, 142.7, 132.6, 131.6, 129.8, 127.8, 123.5, 120.7, 118.8, 115.4, 113.5, 113.3, 108.4, 63.3, 47.4, 14.8.; LC-MS (214 nm) 3.09 min (>98%); m/z 364.2 [M+H].

1-(4-bromobenzyl)indoline-2,3-dione (VU0119498). To a solution of isatin (250 mg, 1.70 mmol) in ACN (8 mL) was added potassium carbonate (470 mg, 3.40 mmol), potassium iodide (28.2 mg, 0.17 mmol), and 4-bromobenzyl bromide (632 mg, 2.55 mmol) and irradiated at 160 °C for 10 min. The reaction was judged complete and partitioned into H₂O and CH₂Cl₂ and passed through a phase separation column. The organics were then concentrated in vacuo. The crude product was purified by filtration with diethyl ether wash (3x30 mL) to afford title compound (500 mg, 93%) as a bright orange solid; ¹H NMR (400 MHz, DMSO-d₆) δ 7.58 (m, 2H), 7.53 (d, J = 8.4 Hz, 2H), 7.40 (d, J = 8.4 Hz, 2H), 7.12 (t, J = 7.6 Hz, 1H), 6.95 (d, J = 3.2 Hz, 1H), 4.89 (s, 2H). ¹³C NMR (100 MHz, DMSO-d₆) δ 182.9, 158.3, 150.1, 137.9, 135.0,
Cyclopentyl 1,6-dimethyl-4-(6-nitrobenzo[d][1,3]dioxol-5-yl)-2-oxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate (VU0090157). To a solution of 6-nitrobenzo[d][1,3]dioxole-5-carbaldehyde (195 mg, 1.0 mmol) in MeOH (100 mL) was added 2-cyanoethyl 3-oxobutanoate (186 mg, 1.2 mmol), 1-methylurea (111 mg, 1.5 mmol), and p-TsOH (17 mg, 0.1 mmol) and refluxed for 4 hr. Once complete, the reaction was filtered and the crude product was purified by recrystallization with MeOH and H2O to afford the intermediate product 2-cyanoethyl 1,6-dimethyl-4-(6-nitrobenzo[d][1,3]dioxol-5-yl)-2-oxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate (283 mg, 78%) as a white crystalline solid. To a solution of the carboxylate intermediate (1.0 g, 2.57 mmol) in acetone/H2O (6/2 mL) was added 2 M NaOH (4 mL) at 0°C, and the solution was warmed to room temperature and stirred for 2 h. Once complete, the reaction was acidified with 2 N HCl and partitioned into H2O and CH2Cl2 and the organics were concentrated in vacuo. The crude product was purified by recrystallization with MeOH and H2O to afford the intermediate product 1,6-dimethyl-4-(6-nitrobenzo[d][1,3]dioxol-5-yl)-2-oxo-1,2,3,4-tetrahydropyrimidine-5-carboxylic acid (790 mg, 92%) as a white crystalline solid. To a solution of the acid intermediate (500 mg, 1.49 mmol) in DMF (12 mL) was added cyclopentanol (385 mg, 4.48 mmol), EDC (428 mg, 2.24 mmol), and polystyrene-bound DMAP (1.5 mmol/g, 5.96 mmol) and irradiated at 100°C for 20 min. Once complete, the reaction was partitioned into 1N HCl in H2O and CH2Cl2 and the organics were concentrated in vacuo. The crude product was purified by recrystallization in EtOAc and hexanes to afford the title compound (200 mg, 33%) as a brown crystalline solid. 1H NMR (400 MHz, DMSO-d6) δ 7.60 (s, 1H), 7.58 (d, J = 1.6 Hz, 1H), 6.95 (s, 1H), 6.23 (s, 1H), 6.20 (s, 1H), 5.81 (d, J = 1.4 Hz, 1H),
4.93 (m, 1H), 3.16 (s, 3H), 2.57 (s, 3H), 1.66 (m, 1H), 1.55 (m, 1H), 1.40 (m, 1H), 1.19 (m, 1H), 1.09 (m, 1H).  

$^{13}$C NMR (100 MHz, DMSO-d$_6$) $\delta$ 164.5, 152.6, 152.3, 151.8, 147.0, 141.3, 135.3, 106.9, 104.8, 103.4, 100.4, 75.9, 48.5, 32.0, 29.6, 23.0, 15.9; LC-MS (214 nm) 3.14 min (>98%); m/z 404.2
Results

Novel M₁ PAMs were identified via a high throughput screening approach. To search for novel positive allosteric modulators (PAMs) of the M₁ muscarinic receptor, we performed a high-throughput screen. For these studies, we used a Chinese Hamster Ovary (CHO) cell line expressing the rat M₁ muscarinic receptor and measured intracellular calcium mobilization. The screening protocol monitored fluorescence in each well at baseline and changes in fluorescence after addition at 4 seconds of either vehicle or test compound (10 µM final nominal concentration). After an approximately 3.2 minute incubation period, a submaximally effective concentration (EC₂₀) of the muscarinic agonist, carbachol, was added.

Raw data were normalized by dividing the fluorescence traces by the minimum fluorescence reading obtained two to five seconds prior to the EC₂₀ agonist addition. This allowed us to retain information for compounds that were slightly fluorescent or that induced small baseline changes. Potential PAMs were defined as compounds that increased the EC₂₀ carbachol response by at least 3 standard deviations above the control EC₂₀ response. Vehicle, EC₂₀, and EC₅₀ controls were included on each plate and data were analyzed on a plate-by-plate basis. Plates were randomly spot checked to assure the quality of the data, validate this criterion for choosing PAMs, and prevent the loss of compounds with weak activity. The primary screen of M₁ yielded 1,634 hits that increased the carbachol EC₂₀ response by at least 3 standard deviations over control.

Initial retests focused on single-point (30 µM) screening of 961 compounds (with responses 3 standard deviations above control) and concentration-response screening of 327 compounds that were deemed to have stronger potentiator activity (at least 5 standard deviations above control) or mixed agonist/PAM activity. The remaining compounds (346) were
unavailable for commercial reorder and have not yet been confirmed for activity. Of the 961 compounds in the weaker category, 34 compounds potentiated the acetylcholine response when tested at a 30 µM concentration. These 34 compounds, as well as the 327 compounds mentioned above, were formatted into ten point concentration-response curves and tested for concentration-dependent ability to potentiate M₁-mediated calcium responses. Compounds were also screened against a metabotropic glutamate receptor 4 (mGluR4)/CHO line to determine if compounds raised calcium levels by a non-specific mechanism. 105 compounds were confirmed as potentiating the acetylcholine response in a concentration-dependent manner. 30 of these compounds potentiated (28 compounds) or antagonized (2 compounds) the mGluR4 response, indicating that approximately 71% of the compounds were selective for M₁ over mGluR4. Four compounds that exhibited M₁ PAM activity, but were devoid of activity at mGluR4, were chosen for further characterization. The structures of these compounds are shown in Figure 1. Figure 2A shows the effects of multiple concentrations of a representative M₁ PAM hit, VU0090157 (corresponding to ChemDiv ID 3058-5056), on the calcium fluorescence response to an EC₂₀ concentration of the endogenous M₁ agonist, ACh. Figure 2B shows the resulting concentration-response relationship for VU0090157 (white boxes) and the three additional PAMs shown in Figure 1 (VU0119498, ChemDiv ID 0924-0042; VU0027414, ChemBridge ID 5661730; and VU0029767, ChemBridge ID 5723320) for potentiation of an EC₂₀ concentration of ACh. Each of these compounds exhibited potencies in the low micromolar range for potentiating M₁-mediated calcium responses.

**M₁ PAMs induce robust shifts in the ACh concentration-response curves.** The effects of the four representative M₁ PAMs on potency and efficacy of ACh at activating M₁ were determined by evaluating the concentration-response relationship for ACh in the absence
and presence of each compound. Pre-incubation of M₁/CHO cells with a 30 µM concentration of each M₁ PAM prior to the addition of increasing concentrations of ACh revealed that each compound induced a robust leftward shift in the ACh concentration-response curve (Figure 3). Shifts in ACh potency were most pronounced for VU0029767 (14.9 ± 5.9 fold, Mean±S.E.M.) and VU0119498 (14.0 ± 2.8 fold). VU0090157 also induced a robust shift in the ACh concentration-response relationship (10.1 ± 1.6 fold), whereas the increase in ACh potency induced by VU0027414 (5.3 ± 0.3 fold) was somewhat less pronounced than that observed for the other M₁ PAMs. The relative efficacies of these compounds in shifting the ACh CRCs corresponded with the maximal effects of these compounds on responses to an EC₂₀ concentration of ACh, where VU0027414 induced a more modest effect than did the other M₁ PAMs (Figure 2B). The level of potentiation of M₁ responses induced by these compounds is comparable to the efficacies that have been observed for some of the most robust allosteric potentiators of other GPCRs (Conn et al., 2008a) and represents a major advance relative to the 2-fold shift induced by brucine (Birdsall et al., 1999; Lazareno et al., 1998).

M₁ PAMs do not compete with an orthosteric antagonist for binding at M₁ receptors. To determine if these PAMs interact with M₁ at the ACh (orthosteric) binding site, we performed competition binding studies with the orthosteric antagonist, l-[N-methyl-³H]scopolamine ([³H]NMS) (Figure 4). Control experiments performed with the orthosteric antagonist atropine demonstrated a classical competitive interaction with [³H]NMS with a Kᵢ of 1.1±0.09 nM (Mean±S.E.M.). In contrast, none of the four new M₁ PAMs significantly competed with equilibrium binding of [³H]NMS at concentrations that maximally potentiated M₁ responses to ACh. These data suggest that these compounds do not directly interact with the orthosteric ACh site on M₁.
Novel M₁ PAMs exhibit differential selectivity profiles for the different mAChR subtypes. It has been difficult to develop orthosteric mAChR ligands that are truly selective for individual mAChR subtypes. Allosteric sites may be less highly conserved, providing potential strategies for the development of more selective GPCR modulators. Examination of the effects of the four compounds presented here on each of the mAChR subtypes revealed clear distinctions in their selectivity profiles (Figure 5). For example, VU0119498 potentiated ACh responses at each of the three G₉ coupled muscarinic receptors (M₁, M₃, and M₅), but had no activity at the Gᵯₒ-coupled mAChR subtypes, M₂ and M₄ (Figure 4A). VU0027414 potentiated M₁ and M₅ responses, antagonized M₂ and M₃, and had no effect at M₄. Interestingly, VU0090157 and VU0029767 were more selective in potentiating responses to M₁ relative to the other mAChR subtypes. VU0090157 weakly potentiated responses at M₄ at the highest concentration, but displayed significantly lower potency for M₄ than for M₁ and did not potentiate any of the other receptor subtypes. VU0029767 was the most selective M₁ PAM and exhibited only a slight potentiation of the four other muscarinic subtypes at the highest concentrations tested. Based on these selectivity profiles and the finding that VU0090157 and VU0029767 were among the most robust and potent of the M₁ PAMs, these two compounds were chosen for further studies.

Novel M₁ PAMs increase affinity of the orthostERIC site for ACh. Theoretically, allosteric potentiators of GPCRs can act by increasing affinity of the receptor for orthosteric agonists or by increasing efficiency of coupling of the receptor to G proteins (Conn et al., 2008a), and GPCR PAMs have been identified that act by a combination of these mechanisms (Shirey et al., 2008). To gain insight into the mechanisms of action of the novel M₁ PAMs, we assessed effects of these compounds on affinity of ACh for the orthosteric site by performing
competition binding studies (Figure 6). ACh exhibited a competitive interaction with \(^3\text{H}\)NMS with an estimated \(K_I\) value of approximately 9 \(\mu\)M. Increasing concentrations of each of the novel M\(_1\) PAMs progressively shifted the ACh competition curves to the left suggesting that the compounds induced concentration-dependent increases in ACh affinity for the orthosteric site. The maximal affinity shifts of ACh measured in the presence of a 30 \(\mu\)M concentration of each compound were similar to the maximal shifts in ACh potency as measured in the functional calcium mobilization assays. For example, a maximally effective concentration of VU0090157 induced a 17.9 ± 3.5 (Mean ± S.E.M.) fold shift in ACh affinity and VU0029767 induced an increase in ACh affinity of 8.8 ± 1.9 fold (Mean ± S.E.M.). These results suggest that the increase in ACh affinity induced by these two M\(_1\) PAMs can account for the increase in ACh potency measured in the studies of ACh-induced calcium mobilization.

**VU0029767 and VU0090157 differentially modulate a form of M\(_1\) bearing a mutation in the orthosteric site.** Previous studies aimed at mapping the orthosteric binding site on M\(_1\) suggest that a tyrosine residue at amino acid position 381 plays a critical role in ACh binding within the orthosteric binding pocket of this receptor. Mutation of this single tyrosine residue to alanine (M\(_1^{Y381A}\)) dramatically reduces the affinity and potency of ACh and other orthosteric ligands at M\(_1\) (Ward et al., 1999). Although the potency of ACh is greatly reduced by mutation of this residue, high concentrations of ACh or other orthosteric ligands can activate M\(_1^{Y381A}\) and induce concentration-dependent activation of the mutant receptor (Figure 7A and C, ACh concentration range shifted roughly 1000-fold to the right for M\(_1^{Y381A}\) relative to WT M\(_1\)). This mutant, therefore, serves as a valuable tool to begin probing the interaction of novel compounds with the M\(_1\) receptor. Since the new M\(_1\) PAMs are structurally unrelated, such a strategically located mutation of M\(_1\) may differentially affect their ability to increase the affinity
of ACh for the orthosteric site, and thus may provide insight into whether compounds act in a mechanistically distinct manner. VU0029767 induced a leftward shift in the ACh concentration response curve at M$_1^{Y381A}$ in a manner similar to that observed for the WT receptor (Figure 7A). Furthermore, the potency of VU029767 at potentiating the response to an EC$_{20}$ concentration of ACh was similar at M$_1^{Y381A}$ and WT M$_1$ receptors (Figure 7B). Interestingly, VU0090157 did not increase the potency of ACh for M$_1^{Y381A}$ activation (Figure 7C) and did not significantly potentiate responses to an EC$_{20}$ concentration of ACh at any concentration tested (Figure 7D). This is in striking contrast to the ~15-fold potentiation of ACh potency induced by VU0090157 at the WT receptor and suggests that VU029767 and VU0090157 may potentiate ACh responses at different sites and/or by stabilizing different conformations of the receptor.

**VU029767 and VU0090157 differentially modulate responses of M$_1$ to a recently discovered allosteric agonist.** We recently reported that a compound termed TBPB is a selective allosteric agonist of M$_1$ that directly activates the M$_1$ receptor (Jones et al., 2008). This is in contrast to the novel M$_1$ PAMs reported here, which increase M$_1$ activity by potentiating the effects of ACh. Combined Pharmacological and mutational analyses suggest that TBPB activates M$_1$ by a mechanism that is distinct from ACh and acts via an allosteric site or a site partially overlapping the ACh binding site. Since VU029767 and VU0090157 act by increasing the affinity of ACh for the orthosteric site, it is not clear whether these compounds would potentiate the agonist response to TBPB in a manner similar to that seen with ACh.

Furthermore, if these compounds stabilize slightly different conformations of the receptor, they may have different effects on responses of M$_1$ to TBPB. Consistent with previous results, TBPB elicited a concentration-dependent activation of M$_1$, as measured in the calcium fluorescence assay (Figure 8A). VU0029767 potentiated the response of TBPB as manifested by a leftward
shift in the TBPB concentration response curve and an increase in the maximal response to
TBPB (Figure 8A). VU0029767 also potentiated the response to an EC$_{20}$ concentration of TBPB
in a concentration-dependent manner, with a potency consistent with the potency of this
compound for potentiating the response to ACh (Figure 8B). In contrast to VU0029767,
VU0090157 did not shift the TBPB concentration response relationship (Figure 8A) and had
little effect on the response to an EC$_{20}$ concentration of TBPB (Figure 8B).

VU0090157 and VU0029767 differentially modulate coupling of M$_1$ to activation of
phospholipase C and phospholipase D. In recent years it has become clear that traditional
orthosteric agonists of GPCRs can differentially activate distinct signaling pathways of a single
GPCR, a phenomenon referred to as agonist directed trafficking or functional selectivity
(reviewed in (Urban et al., 2007)). This implies that different orthosteric agonists can stabilize
unique conformations of the GPCR that favor coupling to distinct downstream signaling
pathways. In the simplest case, the novel M$_1$ PAMs VU029767 and VU0090157 will increase
activity of M$_1$ regardless of the specific effector system being monitored. Since these
compounds appear to act by increasing ACh affinity, one might expect this to be the case, and
the M$_1$ PAMs might simply increase the potency of ACh for all responses normally elicited by
ACh action via M$_1$. However, the finding that VU029767 and VU0090157 have differential
effects on ACh activation of M$_1^{Y381A}$ and on responses to TBPB suggests that these compounds
do not lead to stabilization of identical activity states of M$_1$ when activated by ACh.
Consequently, it is possible that these M$_1$ PAMs will not have uniform effects on coupling of M$_1$
to all signaling pathways, but might differentially alter coupling of M$_1$ to various downstream
effector systems. To begin to address this, we determined the effects of VU029767 and
VU0090157 on M$_1$-mediated activation of phosphoinositide hydrolysis (a measure of coupling to
phospholipase C) and activation of phospholipase D (PLD). Consistent with previous reports, ACh stimulated phosphoinositide hydrolysis in M1-CHO cells in a concentration-dependent manner (Figure 9). As in the calcium mobilization studies, VU0090157 and VU0029767 induced leftward shifts in the ACh concentration response curves (Figure 9). These studies confirmed that the two most selective PAMs were able to increase ACh potency in a second assay that is thought to require activation of the same signaling pathway as that involved in the calcium mobilization response. However, it is interesting to note that VU0090157 induced a shift in ACh potency that was almost identical to that observed in the calcium assay (15.1 ± 1.2-fold), whereas VU0029767 had considerably lower efficacy in potentiating ACh activation of phosphoinositide hydrolysis (2.1 ± 0.1-fold shift of ACh potency) than in potentiating ACh-induced calcium mobilization or enhancing ACh affinity.

We next determined the effects of VU0090157 and VU0029767 on ACh-induced increases in PLD activity as a measure of a fundamentally distinct signaling pathway activated by this receptor in M1-CHO cells (Lopez De Jesus et al., 2006; Rumenapp et al., 1996). ACh induced a robust, concentration-dependent increase in PLD activity, as assessed by measurement of agonist-induced formation of PtdBuOH, with a potency of approximately 150 nM (Figure 10A). After establishing the concentration-response relationship for ACh, we selected an EC20 concentration of ACh and determined the effects of increasing concentrations of VU0090157 and VU0029767 (Figure 10B). Consistent with its effects in the calcium mobilization and phosphoinositide hydrolysis assays, VU0090157 induced a robust potentiation of the response to ACh with a potency in the low μM range. In contrast, VU0029767 had only a weak positive effect on the ACh response (Figure 10B). This experiment further differentiates the actions of
these two novel M1 PAMs and suggests that these compounds may differentially regulate coupling of M1 to various signaling pathways.
Discovery of multiple chemical classes of novel allosteric potentiators of M₁ builds on major advances that have been achieved in recent years in developing highly selective allosteric modulators of GPCRs (Bridges and Lindsley, 2008; Conn et al., 2008a; May et al., 2007). Unlike recently discovered allosteric agonists of M₁, such as AC42 and TBPB (Jones et al., 2008; Spalding et al., 2002; Sur et al., 2003), the allosteric potentiators described here do not activate M₁ directly, but rather potentiate responses to the orthosteric agonist ACh. Altogether, these foundational studies suggest that the identification of both PAMs and allosteric agonists is a viable approach in the discovery of novel activators of M₁ that may have higher selectivity than has been possible with traditional orthosteric M₁ agonists.

The M₁ PAMs described here, VU0029767 and VU0090157, were similar in multiple respects. Both induced similar increases in M₁-mediated mobilization of intracellular calcium and both compounds appeared to act by increasing ACh affinity for the orthosteric site. Furthermore, neither compound reduced equilibrium binding of an orthosteric antagonist radioligand, [³H]NMS, suggesting that they do not bind to the orthosteric site or regulate this orthosteric antagonist’s affinity. Considering these similarities, it is especially interesting that these two M₁ PAMs appear to act by subtly different mechanisms. For instance, while VU0029767 induced similar potentiation of ACh responses at WT M₁ and at a form of M₁ in which a single point mutation known to reduce ACh affinity was introduced into the orthosteric site, VU0090157 was inactive in potentiating ACh-mediated responses of the mutant receptor. Furthermore, VU029767 induced similar potentiation of responses to the orthosteric agonist ACh and the allosteric agonist TBPB, whereas VU0090157 had no effect on activation of M₁ by TBPB. Although the reasons for these differences are not yet clear, these data suggest that the
two compounds may stabilize different conformational states of the receptor, thereby increasing ACh responses through different mechanisms. We previously identified multiple allosteric potentiators for the metabotropic glutamate receptor (mGluR) mGluR5 and have shown that these mGluR5 PAMs interact with at least two distinct allosteric sites on this receptor and can differentially modulate mGluR5 responses (Chen et al., 2008; Chen et al., 2007; O'Brien et al., 2003; O'Brien et al., 2004; Zhang et al., 2005). Likewise, we have provided evidence for the existence of multiple allosteric sites on mGluR1 (Hemstapat, 2006), mGluR2 (Hemstapat, 2007), and mGluR4 (Niswender et al., 2008). It is possible that M1 is similar to the mGluRs in having multiple allosteric sites and that VU0090157 and VU029767 act at different sites to regulate receptor function. However, it is also possible that these compounds interact with a single allosteric region, but have distinct activities. Full characterization of the sites at which these compounds act will require detailed mutagenesis studies, as well as discovery of high affinity allosteric modulators that can be developed as radioligands for the allosteric sites. In recent years, concepts about GPCR function have dramatically changed from a view of these receptors as simple on/off switches to a view in which receptors are seen as complex signaling molecules in equilibrium between inactive and active states that couple to different pathways – an equilibrium that is differentially regulated by ligands (Liggett, 2002). Studies of multiple GPCRs show that members of each of the major subfamilies of GPCRs can exhibit multiple subsets of active conformations which link to different and independent signaling pathways. Furthermore, traditional orthosteric agonists can differentially activate distinct signaling pathways of a single GPCR, a phenomenon referred to as agonist receptor trafficking or functional selectivity (reviewed in (Urban et al., 2007)). Since VU0090157 and VU029767 appear to act by increasing affinity of ACh for M1, one might expect that these M1 PAMs would
not alter the signaling profile of ACh action on M1 but would simply increase ACh potency regardless of the response measured. However, the finding that VU0090157 and VU029767 have differential effects on the orthosteric site mutant form of M1 and on responses to TBPB suggests that these compounds may stabilize different active states of the receptor and could differentially modulate ACh actions on different signaling pathways. Consistent with this, VU0090157 and VU029767 similarly potentiated ACh-induced calcium mobilization, but differed in their impact on coupling of M1 to activation of phospholipase D and phosphoinositide hydrolysis. VU0090157 potentiated ACh-induced activation of PLD and PI hydrolysis in a manner similar to that observed with calcium mobilization assays. In contrast, VU0029767 had almost no effect on M1-mediated increases in PLD activity and a very small effect on ACh-induced PI hydrolysis. While the precise mechanism by which M1 activates PLD in these cells has not been studied in detail, previous studies suggest that mAChR-induced activation of PLD (but not PLC) involves coupling to Ga12 or small G proteins such as R-Ras (Lopez De Jesus et al., 2006; Rumenapp et al., 2001; Rumenapp et al., 1996). It is possible, therefore, that VU0029767 does not stabilize productive coupling complexes with non-Gaq signaling partners. The differences between the calcium and PI hydrolysis results for VU0029767, however, are interesting as the classical pathway activating both of these pathways stems from Gaq activation of PLCβ. One potential explanation is the temporal differences in the assays; calcium assay responses are monitored kinetically, while PI hydrolysis is measured in an endpoint assay. It is possible that VU0029767 induces very rapid desensitization of the receptor thereby preventing dramatic accumulation of phosphoinositides but allowing for a strong calcium response. Future studies will address desensitization kinetics of M1 responses induced in the presence of the various M1 PAMs.
Differential effects of VU0029767 are reminiscent of the distinct effects of allosteric modulators of other GPCRs on various signaling pathways that have recently been described. For instance, CPPHA, a selective mGluR5 PAM, induces robust potentiation of mGluR5-mediated mobilization of intracellular calcium in rat astrocytes, but can inhibit coupling of mGluR5 to activation of ERK1/2 phosphorylation at the highest concentrations of orthosteric agonist (Zhang et al., 2005). Likewise, an allosteric modulator of the chemo-attractant-receptor-homologous molecule on T helper (Th)2 cells (CRTH2), was recently described that is inactive with respect to coupling to G protein-linked pathways, but is a potent antagonist of G protein-independent, β-arrestin coupling to the same receptor (Mathiesen et al., 2005). The finding that VU0090157 and VU0029767 have differential effects on M1 coupling to various signaling pathways raises the possibility that these compounds could have fundamentally different abilities to modulate M1 activity in the CNS. The ability to selectively modulate certain signaling pathways associated with activation of M1 introduces an additional mode for fine-tuning intracellular signaling with allosteric modulators. It is conceivable that allosteric regulators could be developed to preferentially regulate a specific pathway to achieve a desired response. However, in the absence of a clear understanding of the signaling pathways involved in potential therapeutic effects of M1 activation, this also raises a potential problem of inadvertently optimizing M1 PAMs that do not impact coupling of the receptor to the most important downstream signaling events. These possibilities suggest that it is critical to focus continued studies on developing a better understanding of the signaling pathways involved in different responses to M1 activation and to carefully analyze the effects of different classes of allosteric ligands on these pathways in native systems.
In addition to the implications of the current studies for the molecular pharmacology of M1 and allosteric modulators of GPCRs, these findings represent a further advance in developing a framework for discovery of novel drug leads and, ultimately, therapeutic agents for treatment of AD and schizophrenia. The therapeutic activity of mAChR agonists for the treatment of Alzheimer’s disease and schizophrenia is thought to be mediated by activation of the M1 or M4 subtypes. In contrast, activation of peripheral M2 and M3 leads to adverse effects and has resulted in discontinuation of the development of compounds that are not selective among the mAChRs (Conn et al., 2008b; Langmead et al., 2008; Wess et al., 2007). A major advance in discovery of M1-selective activators came with discovery of AC42 as an allosteric M1 agonist that fully activates the receptor in the absence of orthosteric agonist and is highly selective for M1 relative to M2-M5 mAChRs. While providing valuable proof-of-concept at the molecular level, AC42 lacked the potency, solubility, and ancillary pharmacology needed for use as a tool to probe M1 function. More recently, we reported characterization of TBPB as another highly selective allosteric agonist for the M1 mAChR with no agonist activity at any of the other mAChR subtypes (Jones et al., 2008). TBPB has properties that make it highly useful for in vitro and in vivo studies in native systems and was found to potentiate NMDA receptor currents by activation of M1 in hippocampal pyramidal cells, but did not alter responses thought to be mediated by M2 and M4 mAChRs in these neurons. Furthermore, TBPB was efficacious in multiple rodent models predictive of antipsychotic-like activity in rats. Finally, TBPB had effects on processing of the amyloid precursor protein towards the non-amyloidogenic pathway and decreased Aβ production in vitro in a manner similar to that previously shown for a less selective mAChR agonist (Caccamo et al., 2006). These studies, combined with the clinical studies outlined above,
provide strong proof-of-concept suggesting that selective activation of M1 may represent a novel strategy for the treatment of symptoms associated with schizophrenia and AD.

It is conceivable that allosteric potentiators of M1 could provide the needed selectivity for this receptor and also afford further advantages relative to allosteric agonists. For instance, there are a number of problems that are often associated with the use of direct acting agonists as drugs. These include adverse effects associated with excessive activation of the receptor, greater receptor desensitization than may occur with more indirect approaches, and loss of activity dependence of receptor activation. Potential adverse effects of M1 agonists are a particular concern as excessive activation of this receptor has been shown to lead to generalized seizures in animal models (Hamilton et al., 1997). While the relative utilities of allosteric agonists versus allosteric potentiators of M1 are not yet known, the present findings suggest that it will be possible to develop selective allosteric potentiators of M1. In future studies, it will be critical to optimize these compounds for use in behavioral studies and to make a direct comparison between M1-selective agonists and allosteric potentiators with respect to efficacy and adverse effects.
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**Footnotes:**

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**Figure Legends**

**Figure 1.** Structures of novel M1 PAMs VU0119498, VU0027414, VU0090157, VU0029767.

**Figure 2.** Novel M1 PAMs potentiated agonist responses in M1 CHO cells. A. Fluorescence ratio trace generated by a potentiator identified via HTS, VU0090157, in the Ca²⁺ assay. Each point of the raw trace was divided by the fluorescence at time 0 to generate a ratio. Compound was added at 4 seconds, followed by an EC20 concentration of acetylcholine at 196 seconds. Traces reflect the increase in fluorescence obtained in the presence of increasing concentrations of compound. B. Maximum fluorescence ratio values in the EC20 window (196-225 seconds)
were plotted as a function of compound concentration to generate a concentration-response curve. Potencies of compounds were as follows (Mean±S.E.M.): VU0119498, 3.1 ± 0.2 µM, VU0027414, 2.1 ± 0.5 µM, VU0090157, 5.3 ± 0.7 µM, VU0029767, 9.4 ± 4.4 µM. Data represent the Mean ±S.E.M. of 3 independent experiments performed in quadruplicate.

**Figure 3.** Novel M<sub>1</sub> PAMs shifted the concentration-response curve for ACh at least five fold to the left. DMSO-matched vehicle or 30 µM VU0119498 (A), VU0027414 (B), VU0090157 (C), VU0029767 (D) were applied to M<sub>1</sub>-CHO cells prior to the addition of increasing concentrations of ACh and calcium mobilization was measured. Shifts of the ACh curve were (Mean±S.E.M.): VU0119498, 14.0 ± 2.8 fold (A), VU0027414, 5.3 ± 0.3 fold (B), VU0090157, 10.1 ± 1.6 fold (C), and VU0029767, 14.9 ± 5.9 fold (D). Data represent the Mean ±S.E.M. of 3 independent experiments performed in triplicate.

**Figure 4.** Novel M<sub>1</sub> PAMs exhibited minimal effects on [³H]NMS binding to the M<sub>1</sub> receptor. Competition curves for PAMs, VU0119498 (filled squares), VU0027414 (filled circles), VU0090157 (open squares), VU0029767 (open circles), were obtained in the presence of 0.1nM [³H]NMS using membranes from M<sub>1</sub>-expressing CHO-K1 cells. The K<sub>I</sub> for atropine competition (filled triangles) was determined to be 1.08 ± 0.09 nM. Data represent the Mean ±S.E.M. of 3 independent experiments performed in triplicate.

**Figure 5.** Different muscarinic subtype selectivity profiles were observed for various PAMs. Increasing concentrations of each PAM, VU0119498 (A), VU0027414 (B), VU0090157 (C), VU0029767 (D), were added prior to the application of an appropriate EC<sub>20</sub> concentration of
ACh determined individually for each muscarinic receptor subtype, and calcium-generated fluorescence changes were measured. Average ACh EC$_{20}$: M$_1$ (filled squares, 1.4 nM); M$_2$ (open squares, 13.2 nM); M$_3$ (filled circles, 0.12 nM); M$_4$ (open circles, 23.7 nM); M$_5$ (filled triangles, 0.20 nM). The M$_2$ and M$_4$ receptors were co-expressed with the chimeric G protein, G$_{qi5}$, to permit coupling of these G$_{i/o}$-coupled receptors to calcium mobilization. Data represent the Mean ±S.E.M. of at least three independent experiments performed in quadruplicate.

**Figure 6. Novel M$_1$ PAMs altered the affinity of ACh for M$_1$.** Membranes from M$_1$-expressing CHO-K1 cells were incubated with 0.1nM $[^3]$H]NMS. ACh competition curves were generated in the absence and presence of increasing concentrations of VU0090157 and VU0029767. The K$_I$ of ACh in the presence of DMSO control was 9.7 ± 2.3 µM (VU0090157-matched experiments) and 8.7 ± 1.3 µM (VU0029767-matched experiments). 3, 10 and 30 µM of VU0090157 (A) shifted the ACh competition curve by 2.1 ± 0.2, 7.9 ± 1.3 and 17.9 ± 3.5 fold, respectively. 3, 10 and 30 µM of VU0029767 (B) shifted the ACh competition curve by 1.7 ± 0.8, 4.9 ± 2.0 and 8.8 ± 1.9 fold, respectively. Data represent the Mean ±S.E.M. of 3 independent experiments performed in triplicate.

**Figure 7. VU0029767 potentiated responses of a mutant M$_1$ receptor with greatly reduced affinity for ACh whereas VU0090157 did not.** Compound concentration-response curves of VU0090157 (A) and VU0029767 (B) were generated in the presence of an EC$_{20}$ concentration of acetylcholine (1.4 nM for WT and 7.5 µM for the M$_1^{Y381A}$ receptor) in a calcium assay using cells expressing either the WT M$_1$ receptor (filled squares) or the M$_1^{Y381A}$ receptor (open squares). In panels C and D, DMSO vehicle or 30 µM VU0090157 (C) or VU0029767 (D) were
pre-applied to M₁<sup>Y381A</sup> cells prior to the addition of increasing concentrations of ACh. Data represent the Mean ±S.E.M. of 3 independent experiments performed in quadruplicate.

**Figure 8. VU0029767 potentiated responses to the selective M₁ agonist, TBPB, whereas VU0090157 did not.** A, A 30 µM concentration of VU0029767 shifted the concentration-response curve for TBPB 5.4 ± 1.3 fold to the left while 30 µM VU0090157 did not induce responses significantly different from vehicle control (potency of TBPB plus DMSO, 210 ± 30 nM, TBPB plus 30 µM VU0090157, 145 ± 23 nM; 1.4 ± 0.1 fold shift). B. Compound concentration-response curves of VU0090157 (black squares) and VU0029767 (white squares) were generated in the presence of an EC<sub>20</sub> concentration of TBPB (53 nM) in a calcium assay using cells expressing the wildtype M₁ receptor. VU0029767 potentiated TBPB-mediated responses in a concentration-dependent manner with a potency and efficacy similar to its effects in potentiating ACh responses in the same cell line. In contrast, VU0090157 only weakly potentiated TBPB-mediated responses. Data represent the Mean ±S.E.M. of 3 independent experiments performed in quadruplicate.

**Figure 9. M₁ PAM compounds potentiated PI hydrolysis in M₁-expressing cells.** ACh concentration-response curves were generated in the absence and presence of 30µM VU0090157 (A) and VU0029767 (B). VU0090157 and VU0029767 shifted the ACh concentration-response 15.1 ± 1.2-fold and 2.1 ± 0.1-fold to the left, respectively. Data represent the Mean ±S.E.M. of 3 independent experiments performed in quadruplicate.
Figure 10. VU0090157 and VU0029767 differentially affected the ACh-mediated PLD activation in M1-CHO cells. A. Acetylcholine induced a dose-dependent increase in PLD activity with a potency of 148 ± 39 nM. B. Increasing concentrations of VU0090157 and VU0029767 were incubated with an EC_{20} concentration of ACh and PLD activity was measured. Data represent Mean ± S.E.M. and are representative of 3 independent experiments performed in triplicate.
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