Pharmacological Characterization of LY593093, an M1 Muscarinic Acetylcholine Receptor-selective Partial Orthosteric Agonist

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mAChR, muscarinic acetylcholine receptor
M1-5AChR, muscarinic
acetylcholine receptor subtype 1-5
ACh, acetylcholine
NMS, 3H-N-methylscopalamine
oxo-m, oxotremorine-m
PAM, positive allosteric modulator

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Abstract

Alzheimer’s disease and schizophrenia are characterized by expression of psychotic, affective and cognitive symptoms. Currently, there is a lack of adequate treatment for the cognitive symptoms associated with these diseases. Cholinergic signaling, and M1 muscarinic acetylcholine receptor (m1AChR) signaling in particular, have been implicated in the regulation of multiple cognitive domains. Thus, the M1AChR has been identified as a therapeutic drug target for diseases such as schizophrenia and Alzheimer’s that exhibit marked cognitive dysfunction as part of their clinical manifestation.

Unfortunately, the development of selective M1 agonist medications has not been successful, mostly due to the highly conserved orthosteric acetylcholine binding site among the five muscarinic receptor subtypes. More recent efforts have focused on the development of allosteric M1AChR modulators that target regions of the receptor distinct from the orthosteric site that are less conserved between family members. However, orthosteric and allosteric ligands may differentially modulate receptor function and ultimately downstream signaling pathways. Thus, the need for highly-selective M1AChR orthosteric agonists still exists, not only as a potential therapeutic, but also as a pharmacological tool to better understand the physiologic consequences of M1AChR orthosteric activation. Here, we describe the novel, potent and selective M1AChR orthosteric partial agonist LY593093 (N-[(1R,2R)-6-{{(1E)-1-[(4-fluorobenzyl)(methyl)amino]ethylidene}}amino)-2-hydroxy-2,3-dihydro-1H-inden-1-yl)biphenyl-4-carboxamide). This compound demonstrates modest to no activity at the other muscarinic receptor subtypes, stimulates Gαq-coupled signaling events as well as β-arrestin recruitment and displays significant efficacy in in vivo models of cognition.
Introduction

Schizophrenia is characterized by positive (e.g., hallucinations and delusions) and negative (e.g., apathy and social withdrawal) psychotic symptoms, as well as cognitive deficits (e.g., inattention and memory loss). Current antipsychotic treatments are most effective against the positive symptoms associated with schizophrenia, but are considerably less effective in treating the negative symptoms and cognitive deficits (Vohora, 2007). One rationale for the lack of observed cognitive improvement may be the anti-cholinergic activity exerted by many of the current antipsychotics (Stahl, 2000). Acetylcholine signaling has been implicated in cognition, and muscarinic acetylcholine receptor (mAChR) agonists have demonstrated anti-psychotic efficacy in pre-clinical studies (McArthur et al., 2010). The neurotransmitter acetylcholine, along with the two receptor families to which it binds, ionotropic nicotinic receptors and metabotrophic muscarinic receptors, comprise the cholinergic system. The mAChRs are class A members of the G protein-coupled receptor superfamily and consist of five subtypes designated M1-M5AChR (Kristiansen, 2004; Wess, 1996). These subtypes are separated into two distinct classes based on the G-proteins to which they preferentially couple. M1AChR, M3AChR and M5AChR signal through Gαq/11 and mobilize intracellular calcium, whereas M2AChR and M4AChR couple to Gαi/o and inhibit adenylate cyclase and cAMP production (Caulfield, 1998).

An imbalance in cholinergic synaptic transmission has been associated with the cognitive decline apparent in both Alzheimer’s disease and schizophrenia (Langmead et al., 2008; Raedler et al., 2007). Pharmacological activation of the M1AChR subtype may ameliorate the cognitive decline associated with these and other neurological illnesses (Langmead et al., 2008). The M1AChR subtype is predominantly expressed in the forebrain including the hippocampus and cortex, and these brain regions have been implicated in learning and memory deficits associated with the pathophysiology of Alzheimer’s disease and schizophrenia (Dean et al., 2002; Levey, 1996; Porter et al., 2002).

The discovery and development of M1AChR-selective agonists has been challenging due to the high sequence similarity and identity shared between all five receptor subtypes at their respective orthosteric acetylcholine binding sites (Heinrich et al., 2009; Hulme et al., 1990). Although a number of arecholine-based muscarinic agonists with M1 partial agonist activity entered the clinic, only xanomeline showed efficacy in the cognitive domain in both Alzheimer’s disease (Bodick et al., 1997) and
schizophrenia (Bodick et al., 1997; Shekhar et al., 2008). However, the muscarinic subtype selectivity and drug-like qualities of xanomeline and related compounds were less than ideal leading to discontinuation of further development.

More recently, allosteric mAChR agonists and modulators have been investigated as a means to gain greater selectivity for individual subtypes (Chan et al., 2008; Conn et al., 2009b). Allosteric site sequences are less conserved among the MAChR subtypes, allowing for greater potential compound specificity. Moreover, allosteric modulation allows retention of spatial and temporal signaling patterns critical for normal neurophysiology (Conn et al., 2009a; Valant et al., 2009). Allosteric binding sites are distinct from orthosteric sites and either regulate receptor-mediated signaling directly or through cooperativity with simultaneous orthosteric ligand binding and signaling.

We sought new chemotypes with improved prospects for selectivity, and conducted a high throughput functional screen for M1AChR agonists against a sample collection comprised of Lilly archival compounds and libraries prepared via parallel synthesis. We describe here the pharmacological characterization of the novel, potent and highly selective M1AChR partial agonist LY593093 (N-[(1R,2R)-6-(((1E)-1-[(4-fluorobenzyl)(methyl)amino]ethylidene)amino)-2-hydroxy-2,3-dihydro-1H-inden-1-yl]biphenyl-4-carboxamide) from a 1,6-diamino-indane-2-ol chemical platform. LY593093 competes with ACh for $^3$H-N-methylscopalamine ($^3$H-NMS) binding and selectively stimulates M1AChR-mediated G-protein activation and calcium mobilization. Additionally, LY593093 stimulates $\beta$-arrestin recruitment, a signaling pathway postulated to be pertinent for behavioral and cognitive efficacy, as well as inositol phosphate turnover in vivo (Ma et al., 2009). Finally, we demonstrate that the compound facilitates spatial learning in rats following oral administration, suggesting therapeutic potential in disorders involving cognitive dysfunction.
Methods

Materials

All reagents were purchased from Sigma Aldrich (St. Louis, MO) unless otherwise indicated. All compounds were synthesized at Eli Lilly & Co. unless otherwise noted. Acetylcholine chloride was purchased from MP Biomedicals (Solon, OH). Oxotremorine-m was purchased from Tocris Bioscience (Ellisville, MO). PathHunter Detection Kit and β-arrestinized M1AChR CHOK1 cells were licensed from DiscoveRx (Fremont, CA). M1-M5AChR-expressing CHO cell membrane preparations, GTPγ-[35S], [3H]-myoinositol and MeltiLex A scintillation sheets were purchased from Perkin Elmer (Waltham, MA). Anti-Gαi,3 and anti-Gαq antibodies were generated by Eli Lilly & Co. Scintillation proximity assay (SPA) beads were purchased from GE Healthcare (Piscataway, NJ). Fluo-4 dye, probenecid, OptiMEM I and tissue culture reagents were purchased from Invitrogen (Carlsbad, CA) unless otherwise indicated. Ready Solv HP scintillation fluid was purchased from Beckman-Coulter (Brea, CA). Wild-type, M1AChR knock-out and M3AChR knock-out mice were purchased from Taconic Farms Inc. (Hudson, NY).

Inhibition of [3H]-NMS Binding at Equilibrium

[3H]-NMS binding assays were performed as described previously (Chan et al., 2008) with the following modifications. Briefly, frozen membrane preparations were thawed and resuspended in assay buffer (20 mM HEPES, 100 mM NaCl, 10 mM MgCl2; pH 7.4). Twenty-five µg protein/well was incubated with [3H]-NMS and unlabeled test compounds for 2 h at 22°C in a total volume of 200 µL in polypropylene 96-deep well plates. Membranes were collected by rapid filtration, using a Tomtec cell harvester (Tomtec, Inc., Hamden, CT, USA) through GF/A filters that had been presoaked in 0.3% polyethyleneimine. The filters were washed with 5 mL ice-cold 50 mM Tris buffer (pH 7.4) and air-dried overnight. The dried filters were treated with MeltiLex A melt-on scintillator sheets, and the radioactivity retained on the filters was counted using a Wallac 1205 Betaplate scintillation counter (Perkin-Elmer, Wallac, Gaithersburg, MD, USA). Kᵢ values were determined from the Cheng-Prusoff relationship $K_i = IC_{50}/ 1 + [\text{ligand}] / K_d$, where IC₅₀ is determined from a four parameter fit of displacement curves, [ligand] = 1 nM [3H] NMS and K_d is the equilibrium dissociation constant of [3H] NMS for each receptor determined by saturation binding experiments carried out by the membrane supplier. Non-specific binding was determined using 1 or 10 µM atropine, as indicated in the figure legend.

Cell-based and Native Tissue GTPγ-[35S] Binding Assays

The GTP-γ-[35S] binding assay measures the level of G-protein activation following agonist treatment by determining the amount of non-
hydrolysable GTP-γ-[35S] bound to Gα subunit subtypes of interest (see Harrison & Traynor, 2003, for a thorough review). GTPγ-[35S] binding in cells was determined using the antibody capture technique previously described by DeLapp et al. (DeLapp et al., 1999) in a 96 well plate format. Briefly, 100 µl (20-40 fmol/well) of membrane preparation from CHO cells that over-express human M1, M2, M3, M4 or M5 muscarinic receptors were incubated for 30 min with 50 µl of test compound. The receptor densities (Bmax) for the ectopically-expressed M1-M5AChRs were 9, 3, 0.7, 0.6, 0.9 and 4.8 pmol/mg membrane protein, respectively. Representative receptor densities for the M1- and M3AChR knockout animals were reported previously (Oki et al., 2005). In the case of the M2 and M4 receptor membranes, 1 µM GDP (final concentration) was added to the membranes. (No GDP was added to M1, M3, or M5 membranes.) Following the incubation period, 50 µl/ well (500 pM) diluted GTPγ-[35S] was added to each well and the mixture allowed to incubate for 30 min. The labeled membranes were then solubilized with 0.27% Nonident-P-40 for 30 min followed by the addition of 20 µl (final dilution of 1:400) of the appropriate anti-Gαq antibody. For the M1-, M3-, or M5AChR membranes, anti Gαq/11 antibody was used, whereas anti Gαi-3 antibody was used for the M2- and M4AChR membranes. Following the 60 min incubation, 50 µl/well (1.25 mg/well) scintillation proximity assay (SPA) beads were added and the plates incubated for an additional 3 h. The plates were centrifuged for 10 min at 1000 rpm using an Accuspin 1R centrifuge (Fisher Scientific, Pittsburgh, PA) and counted for 1 min/well using a Wallac MicroBeta TriLux scintillation counter (Perkin-Elmer, Wallac, Gaithersburg, MD, USA). All incubations took place at room temperature in GTP-binding assay buffer (20mM HEPES, 100mM NaCl, 5 mM MgCl2, pH 7.4). GTPγ-[35S] binding in native tissue was performed essentially as described previously (Overk et al., 2010).

**Calcium Mobilization Assay** Calcium mobilization was measured using assay guidance according to the NIH’s Chemical Genomics Assay Guidance Manual for FLIPR™ Assays to Measure GPCR and Ion Channel Targets (http://www.ncgc.nih.gov/guidance/section9.html). CHO cells stably over-expressing either M1-, M3- or M5AChR were plated at 50,000 cell/well in poly-D-lysine coated 96-well black, clear-bottom plates and grown overnight at 37°C with 5% CO2 and 95% humidity in growth media (DMEM/F-12 (3:1), 10% FBS, 20 mM HEPES, 2 mM Glutamax, 40 µg/mL L-proline, 250 µg/mL genetecin, 0.5% Pen-Strep). Twenty-four hours later, media was removed and cells were pre-loaded with 50 µL of Fluo-4 Dye (5 µg/µL) for 45 minutes prior to replacing with 100 µL of fresh assay buffer (1x Hank’s Balanced Salt
Solution, 20 mM HEPES, 250 µM propenecid. Cells were allowed to equilibrate at room temperature in buffer for 6 minutes prior to addition of compounds using FLIPR™ instrument.

**β-arrestin Recruitment Assay** β-arrestin 3 recruitment was measured using the PathHunter™ β-Arrestin complementation assay system, including a β-arrestinized CHOK1 cell line stably-expressing M1AChR and two fragments of β-galactosidase, one fused to M1AChR and the other fused to β-arrestin 3 (DiscoveRx, Fremont, CA). Briefly, 40,000 cells/well were treated in OptiMEM I with ligand for 90 minutes @ 37°C with 5% CO2. β-arrestin 3 recruitment, as determined by β-galactosidase complementation, was measured after the addition of PathHunter Detection Reagent for 60 minutes at room-temperature followed by luminescence signal detection using a GENiosPro (Tecan, Switzerland).

**Animal Experiments**

Rats used were maintained in facilities fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC). All research protocols were approved by the institutional animal care and use committee and are in accordance with the guidelines of the Institutional Care and Use Committee of the National Institute on Drug Abuse, National Institutes of Health, and the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council 1996).

**In Vivo Phosphoinositide Hydrolysis** LY593093 was assessed for its ability to induce phosphoinositide (PI) hydrolysis in rats using methods described previously (Bymaster et al., 1998). Briefly, six colony-acclimated male Sprague-Dawley rats (95-110 g) were anesthetized and prepared for stereotaxic administration of 2 μCi of [³H]-myoinositol (18.6 Ci/mmol) in 8 μL saline injected into the ventricle (1.5 mm lateral, 1.4 mm posterior from bregma, and 4 mm deep from the surface of the skull). Twenty-four hours later, rats received saline or lithium chloride (10 mmol/kg; subcutaneous route). Vehicle (water + Labrisol 10%) or LY593093 (3 or 10 mg/kg; oral route) was administered 1 hour after lithium administration. Three hours later, rats were sacrificed and the hippocampus was dissected and placed on dry ice. After weighing, hippocampal tissues were rapidly homogenized by sonication in 1.25 mL of 10 mM lithium chloride. After centrifugation at 10,000 x g for 10 minutes, the [³H]-inositol monophosphates in 1 mL of supernatant were determined by chromatography. Total radioactivity in the tissue was determined in an 80 µL aliquot of the homogenate. Aqueous aliquots or column eluates were added to scintillation vials.
containing 15 mL of Ready Solv HP scintillation fluid, and radioactivity was determined by liquid scintillation spectrometry with about 40% counting efficiency. Effects of in vivo phosphoinositide hydrolysis were calculated using a within-animal design by determining percent conversion of [³H]-radioactivity to inositol monophosphates and treatment differences analyzed via ANOVA.

**Radial Arm Maze Acquisition** Six male Sprague-Dawley rats, approximately 200-225 g, were tested for 8-arm radial maze learning using food reward. Rats were single-housed and acclimated for at least 4 days to a 12:12 light:dark colony with light onset at 0600 hr. During this time, rats were reduced to and maintained at 85% of their arrival body weight. Rats received a single maze session each day for a total of 4 consecutive days. A session began with an individual rat being placed into the hub of the maze (Coulbourn Instruments) and then all guillotine doors were raised, allowing free access to all areas of the maze. A food hopper was located at the end of each of the 8 runway arms and a single food pellet (45 mg Bio-serv) was placed in each food hopper. Each daily session terminated when either all 8 food-hoppers had been visited or when the rat timed out (15 min on Day 1, 5 min on Days 2-4). The number of arm entries was recorded. Errors were counted as repeat arm entries or failures to visit an arm in the session period. An animal was excluded from the study if it failed to visit at least one arm on Day 1, 2 arms on Day 2, and at least 4 arms on Days 3 & 4. Each rat was pseudo-randomly assigned to either a vehicle or drug group and received the same treatment throughout the experimental period. Vehicle consisted of 10% labrasol within sterile water. Injections were administered orally 3 hours prior to each daily session.
Results

**LY593093 is selective for the M1AChR** The selectivity of LY593093 (Figure 1A) for the various mAChR subtypes was assessed *in vitro* by three methods: functional stimulation of both G-protein activation and calcium mobilization as well as Kᵢ determination following displacement of NMS binding.

Ca²⁺ Mobilization

Among the three Gα₉-coupled subtypes (M1AChR, M3AChR and M5AChR), LY593093 preferentially stimulated M1AChR-mediated calcium mobilization (85.3 ± 2.86% control) (Figure 1C). LY593093 displayed 100- and 2,000-fold better potency at M1AChR (EC₅₀ = 49.9 ± 6.32 nM) versus either M3AChR (EC₅₀ = 6,053 ± 2,490 nM) or M5AChR (EC₅₀ = > 100,000 nM), respectively, distinguishing itself from the pre-existing molecules in terms of M1AChR functional selectivity (Figures 1C and 2; Table 1). While both alvameline and talsaclidine displayed relatively no activity in terms of M5AChR-mediated calcium mobilization, none of the control compounds showed selectivity for M1AChR over both M3- and M5AChR with the combined affinity and percent Eₘₐₓ demonstrated by LY593093 (Figure 2 and Table 1). EC₅₀ (log₁₀, nM) values for the various compounds at the M1-/M3-/M5AChR were observed as follows:

LY593093 = -7.3/-5.2/-4.0; acetylcholine = -7.6/-8.8/-7.4; alvameline = -6.4/-5.8/-4.0; sabcomeline = -8.3/-8.4/-7.4; talsaclidine = -6.6/-6.0/-4.0; xanomeline = -7.2/-6.6/-5.9. The percent Eₘₐₓ values at the M1-/M3-/M5AChR for those compounds listed above with the highest affinity at M1AChR versus the M3- or M5AChR (LY593093, alvameline and xanomeline) were observed as follows (% Eₘₐₓ): LY593093 = 85.3 ± 2.86/43.9 ± 0.98/NA; alvameline = 46.9 ± 7.72/44.0 ± 8.99/NA; xanomeline = 115 ± 4.32/92.4 ± 9.20/64.2 ± 8.77. Interestingly, xanomeline behaved as a partial agonist at M5AChR (Figure 2), indicating that it may also act as a partial antagonist at this receptor subtype. As was expected, the M1AChR allosteric agonist TBPB, was a high-affinity (EC₅₀ = 2.8 ± 1.1 nM) full agonist (118 ± 6.6 % Eₘₐₓ) at M1AChR in terms of calcium mobilization (Jones et al., 2006; Jones et al., 2008) (Figure 2). Surprisingly, the M1AChR PAM BQCA also displayed weak partial agonist activity (EC₅₀ = 1.8 ± 1.7 µM; 73 ± 7.2 % Eₘₐₓ) with respect to M1AChR-mediated calcium mobilization (Figure 2).

GTP-γ[S] Binding
The concentration response curves for LY593093-stimulated GTP-γ[^35S] binding in cells heterologously expressing the various receptor subtypes demonstrate that LY593093 is a functionally-selective partial agonist for the M1AChR subtype, with modest (M2AChR, M4AChR) or no (M3AChR, M5AChR) agonist activity at the other muscarinic subtypes (Figure 1B). In terms of G-protein activation, at M1AChR, LY593093 (EC₅₀ = 219 ± 31.9 nM) demonstrated ~95% of the control maximal efficacy (Eₘₐₓ) observed with the full orthosteric agonist oxotremorine-m (oxo-m; 30 µM), whereas LY593093 displayed less than 25% Eₘₐₓ at both M2AChR and M4AChR, (EC₅₀ = 1.07 ± 0.17 µM and > 10 µM, respectively) (Figure 3 and Table 2). These functional results contrast those of the M1AChR-preferring control agonists, which failed to exhibit strong functional selectivity for M1AChR over both M2AChR and M4AChR (Figure 3 and Table 2). Xanomeline displayed relatively no functional selectivity for the M1AChR subtype (EC₅₀ = 43.6 ± 7.33 nM) over either M2- or M4AChR (EC₅₀ = 78.4 ± 10.4 and 63.1 ± 16.9 nM, respectively), but displayed the greatest degree of G-protein activation at M2AChR (82.6 ± 3.12% Eₘₐₓ) as compared to M1AChR or M4AChR in these studies (63.7 ± 3.48% and 46.1 ± 4.02% control, respectively) (Table 2). Alvameline, sabcomeline and talsaclidine also demonstrated activity at M2AChR (EC₅₀ = 296 ± 5.69, 56.3 ± 6.58 and 848 ± 69.0 nM, respectively) with sabcomeline and talsaclidine exhibiting high partial agonist activity at M2AChR (89.9 ± 2.85 and 94.4 ± 2.47% Eₘₐₓ, respectively) (Table 2). The augmented M3AChR activity observed with the calcium mobilization assay (Figures 1C and 2; Table 2) relative to the GTP-γ[^35S] binding assay (Figure 1B and 4) likely demonstrates the amplified downstream calcium mobilization response measured with the FLIPR assay, whereas the GTP-γ[^35S] binding assay measures initial receptor activation.

Native GTP-γ[^35S] binding studies performed using membrane preparations of hippocampi (Hippo) and frontal cortex (FCTX) isolated from the brains of wild-type, M1AChR knockout and M3AChR knockout mice further define the functionally selective nature of LY593093 for M1AChR (Figure 4).

Frontal cortex membrane preparations from M3AChR knockout and wild-type animals treated with oxo-m displayed similar EC₅₀ and Eₘₐₓ values with respect to Ga₉ activation (EC₅₀FCTX-oxo(WT) = 99.7 ± 23.8 nM, % EₘₐₓFCTX-oxo(WT) = 102.4 ± 3.2; EC₅₀FCTX-oxo(M3KO) = 69.9 ± 22.1 nM, % EₘₐₓFCTX-oxo(M3KO) = 99.2 ± 5.2).

Ga₉ activation in M3AChR knockout and wild-type hippocampal membrane preparations treated with oxo-
m was relatively similar, both in terms of percent E_{max} and potency (EC_{50Hippo-oxo(WT)} = 70.5±17.4 nM, % E_{maxHippo-oxo(WT)} = 102.8±3.3; EC_{50Hippo-oxo(M3KO)} = 66.8±21.3 nM, % E_{maxHippo-oxo(M3KO)} = 101.7±6.5) (Figure 4, A and C). Oxo-m signaling in M1AChR knockout membranes (both hippocampal and frontal cortex) was dramatically decreased (>80% reduction of wild-type efficacy in both tissues) (Figure 4, A and C). LY593093-mediated G\alpha_q signaling was nearly identical in M3AChR and wild-type membrane preparations of both hippocampal and frontal cortex (EC_{50FCTx-LY593093(WT)} = 3.6±3.3, % E_{maxFCTx-LY593093(WT)} = 92.9±4.5; EC_{50FCTx-LY593093(M3KO)} = 2.7±1.6 nM, % E_{maxFCTx-LY593093(M3KO)} = 90.9±6.6; EC_{50Hippo-LY593093(WT)} = 3.2±1.6 nM, % E_{maxHippo-LY593093(WT)} = 93.5±4.5; EC_{50Hippo-LY593093(M3KO)} = 1.6±0.2 nM, % E_{maxHippo-LY593093(M3KO)} = 95.0±5.9) (Figure 4, B and D). LY593093-mediated G\alpha_q signaling in M1AChR knockout tissues was dramatically diminished in both frontal cortex and hippocampus, relative to wild-type (Figure 4, B and D).

[^3H]-NMS Displacement

LY593093 dose-dependently competed with the non-selective mAChR antagonist[^3H]-NMS for binding at the M1AChR and M2AChR orthosteric sites (K_i = 622±23.2 nM and 890±28.6 nM, respectively; Figure 5A). However, LY593093 displayed no appreciable affinity for receptor subtypes M3AChR-M5AChR, distinguishing itself from the other M1AChR-preferring agonists tested (Table 3). Whereas sabcomeline, talsaclidine and xanomeline all displayed relatively similar binding affinities for the different mAChR subtypes, only LY593093 exhibited no appreciable affinity for receptor subtypes 3-5.

Curveshift analysis was performed to determine if LY593093 had any modulatory role in ACh functional activity. Curveshift analyses indicated that LY593093 does not potentiate ACh-mediated displacement of[^3H]-NMS as does the prototypical M1AChR-selective PAM BQCA (Figures 5B and 5C), but rather competed with acetylcholine for binding to the M1AChR (Figure 5B). Interestingly, BQCA did not modulate the competitive nature of LY593093 binding (Figure 5D), indicating that the interaction between LY593093 and M1AChR is different than the interaction of the receptor with the endogenous ligand ACh.

In addition to determining the affinity of LY593093 for the various MAChR subtypes, the affinity of LY593093 against a panel of 64 various human proteins, including neurotransmitter receptors and transporters, ion channels, second messenger receptors, enzymes and peptides was determined
At 10 µM, LY593093 was relatively inefficient at displacing the binding of radioligand selective for any of the non-mAChR human proteins in the panel. LY593093 (10 µM) inhibited the binding of radioligand by greater than 50% at only three of the > 60 proteins tested: dopamine transporter (58.8% inhibition); 5HT2A receptor (53.99% inhibition); glutamate receptor, chloride-dependent site (54.06% inhibition) (Supplemental Table 1).

**LY593093 stimulates β-arrestin 3 recruitment at M1AChR** Paralleling the effect on GTPγS binding and calcium mobilization, LY593093 displayed both partial agonist and antagonist activity with respect to M1AChR-mediated β-arrestin 3 recruitment, as did the M1/M4AChR-preferring orthosteric agonist xanomeline (Figures 6A and 6B and data not shown). LY593093 and xanomeline partially stimulated β-arrestin 3 recruitment at M1AChR (EC50 = 1.95 µM and 19.91 nM, respectively). The M1AChR-selective PAM BQCA potentiated the effect of ACh on M1AChR-mediated β-arrestin 3 recruitment in a dose-dependent fashion similar to its effect on ACh-stimulated calcium mobilization (Figure 6A) and confirming the observation made previously by Ma et al. (2009). The EC20 concentration of ACh used when testing BQCA in potentiator mode was determined based on previous experiments and not immediately prior to the potentiator assay being run. Assay-to-assay variability likely explains why, at low concentrations of BQCA, < 20% Emax was observed in the presence of what we previously observed in this assay to be an EC20 concentration of ACh.

Unlike xanomeline and BQCA, not every control compound tested uniformly activated all M1AChR-coupled signaling pathways. For example, TBPB did not stimulate β-arrestin 3 recruitment at M1AChR, as had been demonstrated previously (Figure 6C) (Ma et al., 2009). Surprisingly, the full agonists oxo-m and talsaclidine also exhibited partial agonist activity at M1ACh with respect to β-arrestin 3 recruitment (Figure 6C). This observation was independent of the supplier or lot number of oxo-m tested (data not shown; multiple lot numbers not tested for talsaclidine).

**LY593093 is Active at M1AChR in vivo**

**LY593093 Effects on In Vivo Hippocampal Phosphoinositide Hydrolysis**

Conversion of [3H]-radioactivity to [3H]-inositol monophosphates was increased in a dose-related manner with the 10 mg/kg dose of LY593093 producing a 165% increase compared to the lithium alone group (Figure 7; p < 0.034 versus lithium alone control). These data suggest that LY593093 activates...
muscarinic M1 receptors and stimulates formation of inositol triphosphate in a brain region rich in m1AChRs.

**LYS93093 Effects on Spatial Learning**

In this 4-day training paradigm, untreated rats typically do not show significant improvement in maze performance (i.e., fewer errors), whereas nootropic agents accelerate learning acquisition and statistical separation in number of errors committed can be observed by Day 4 of training. Planned independent-group t-test, using total errors on Day 4, was conducted on test groups for 2 separate studies. In Experiment 1 (Figure 8, left panel), 3 and 10 mg/kg LY593093 significantly reduced errors relative to vehicle controls \[t(13) = 2.11, p < 0.03\] and \[t(14) = 2.23, p < 0.02,\] respectively. In Experiment 2 (Figure 8, right panel), the top dose of 3 mg/kg LY593093 significantly reduced error \[t(13) = 2.73, p < 0.01\]. These data show that consistent efficacy of LY593093 within a 3-10 mg/kg dose range under the present experimental conditions.

**Discussion**

The deregulation of the cholinergic system has been implicated in the development of cognitive symptoms associated with mental illnesses such as Alzheimer’s disease and schizophrenia (Langmead et al., 2008; Raedler et al., 2007; Rainer and Mucke, 1998). The cholinergic M1AChR receptor subtype is widely expressed in the cortical and limbic brain regions, and extensive evidence indicates a mediating role in cognitive processes (Anagnostaras et al., 2003; Buckley et al., 1988; Conn et al., 2009b). Thus, the M1AChR receptor has emerged as a potential pharmacological target for diseases characterized by cognitive impairment. Unfortunately, the high degree of conservation between muscarinic receptor subtypes at their orthosteric ligand binding sites has hindered the development of M1AChR-selective drugs. The M1/M2-/M4AChR-prefering muscarinic agonist xanomeline showed symptom reduction efficacy in both Alzheimer’s disease and schizophrenic patients; however, parasympathetic side-effects, particularly in elderly patients, has limited its therapeutic potential (Bodick et al., 1997; Heinrich et al., 2009; Shekhar et al., 2008). Results of previous studies performed with M1-M5AChR knockout mice indicate that the M2AChR and M3AChR receptor subtypes are responsible for mediating the physiological processes involved in many of the side-effects observed during testing of the pre-existing of M1AChR-preferring agonists, including xanomeline (Wess et al., 2007). Thus, significant improvement in M1
selectivity over M2- and M3AChR receptor subtypes is likely essential in the successful development of a tolerable M1AChR orthosteric agonist (Heinrich, Butera et al 2009). While a role for the M3AChR in cognition has recently emerged (Poulin et al., 2010), its role in peripheral parasympathetic regulation of smooth muscle and exocrine glands precludes it as an attractive drug target.

We have demonstrated that LY593093 is a potent, functionally-selective partial orthosteric agonist at the M1AChR receptor subtype. When comparing the ability of LY593093 to stimulate calcium mobilization at the endogenously Gαq-coupled subtypes, LY593093 displayed selectivity for M1AChR, exhibiting extremely modest or no activity at M3AChR and M5AChR, respectively (Figure 1C). Using the GTP-γ[35S] binding assay to assess mAChR-coupled G-protein activation, LY593093 displayed potent partial activation of M1AChR (while demonstrating only modest (M2AChR, M4AChR) or no (M3AChR, M5AChR) activity at the other subtypes) (Figure 1B). These results were further supported by a decrease in LY593093-stimulated GTP-γ[35S] Gαq activation in M1AChR knockout, but not M3AChR or wild-type, hippocampal and frontal cortex membrane preparations (Figure 4). Similar results were shown previously for the non-selective full agonist oxo-m (Felder et al., 2001; Porter et al., 2002). The rationale for the initial modest, yet, seemingly dose-dependent increase in Gαq activation in LY593093-treated M1AChR knockout tissues followed by a decrease to near zero stimulation at higher concentrations is unknown. While the results clearly show that genetic ablation of M1AChR drastically affects LY593093-mediated Gαq activation, other non-obvious signaling pathways may also be modulated by LY593093 treatment in the absence of the M1AChR that impact G-protein activation and the results of this native tissue-based assay, but with modest effect.

Varying assay methods to evaluate LY593093 activity at the various mAChR subtypes may also explain the discrepancy that exists between our results here and those published previously by Heinrich et al. In an initial pharmacological characterization of LY593093 functional selectivity, Heinrich et al. observed LY593093 affinity at M2AChR (Kᵢ = 0.79 µM) to be similar to that which we report here (0.62 µM) (Heinrich JN et al., 2009). However, in terms of functional selectivity, they reported modest LY593093 activity (> 35-fold less potent compared to M1AChR) at the M2- and M4AChRs, which conflicts our observations reported here (Heinrich JN et al., 2009). Once again, these differences may be explained by the assay method chosen to assess LY593093 activity. For their experiments, Heinrich et
al. utilized assays measuring amplified downstream responses (cAMP production) to assess mAChR activity instead of an initial event such as G-protein activation, measured here using the GTP-γ-S binding assay. Assaying calcium mobilization, Heinrich et al. also reported modest activation of M3AChR by LY593093 (EC$_{50}$ = 1.42 µM; 102% E$_{max}$), which is similar to our results (6.05 ± 2.5 µM; Table 1), despite the fact that in our hands, maximal agonist effect of LY593093 (43.9% E$_{max}$; Table 1) was two-fold less (102% E$_{max}$) than that observed by Heinrich et al. While these data highlight the importance of investigating receptor activation by more than one detection method, these discrepancies do not negate the highly selective and potent activity of LY593093 for the M1AChR subtype observed here and previously (Heinrich JN et al., 2009).

A CEREP (Paris, France) screen of LY593093 against various human proteins, including neurotransmitter receptors and transporters, ion channels and enzymes indicated that LY593093 has little-to-no affinity for off-target proteins (Supplemental Table 1). The selective nature of LY593093 for M1AChR over the other mAChR subtypes suggests improved tolerability without the undesirable off-target effects that have been observed with many of the pre-existing muscarinic compounds (Bymaster et al., 2003). LY593093 was competitive with acetylcholine and displaced binding of the antagonist [³H]-NMS at M1AChR with no effect on [³H]-NMS binding at the M3-M5AChRs (Figures 5A, 5B and Table 3). Furthermore, the observation that LY593093 binding was unaffected by the M1AChR-selective PAM BQCA further supports the likelihood that LY593093 binds at additional sites distinct from ACh binding determinants (Figure 5D). LY593093 also displaced [³H]-NMS binding at M2AChR (Table 3). However, in terms of functional activity, LY593093 displayed between 5- and 120-fold better potency and demonstrated at least 2-fold higher E$_{max}$ values at M1AChR versus any of the other subtypes, including M2AChR (Tables 1-2).

Ma et al. recently suggested that ligand-stimulated β-arrestin recruitment at M1AChR may be an important indicator of pharmacological efficacy in improved cognition (Ma et al., 2009). The requirement for this pathway in memory and learning was further supported in studies with wild type and mutant M3AChRs in a fear conditioning behavioral paradigm (Poulin et al., 2010). Here, we show that LY593093 acts as a partial agonist with respect to M1AChR-mediated β-arrestin 3 recruitment, indicating that LY593093 may induce the signaling events proposed to be pertinent for cognitive improvement. To that
end, our in vivo studies demonstrate pharmacological activity of LY593093 in stimulating PI turnover following oral dosing in brain regions important for learning and memory – an effect shown to be M1AChR-dependent (Bymaster et al., 2003). PI turnover is used as a measure of Gαq-mediated stimulation of phospholipase C signaling. The doses of LY593093 that produced PI hydrolysis effects were consistent with the dose range that improved acquisition of a spatial learning task. These data support the premise that β-arrestin recruitment at M1AChR may indeed be essential for cognitive restoration and continue to highlight a role for M1AChR-mediated signaling in cognitive processing.

Allosteric agonists and positive allosteric modulators (PAMs) have shown promise as a means of selectively modulating M1AChR signaling (Ma et al., 2009; Shirey et al., 2009; Spalding et al., 2002). However, controversy exists concerning the possibility that differences in G-protein coupling occur when the M1AChR receptor is stimulated by allosteric versus orthosteric agonists (Thomas et al., 2009). For example, the M1AChR-selective allosteric agonist 4-n-butyl-1-[4-(2-methylphenyl)-4-oxo-1-butyl] (AC-42) was shown to cause slightly altered G-protein coupling to M1AChRs compared to orthosteric agonists and exhibits a decreased likelihood to induce receptor desensitization and internalization (Thomas et al., 2009). Additionally, the M1AChR-selective PAM benzyl quinolone carboxylic acid (BQCA) was shown to potentiate ACh-mediated β-arrestin recruitment, whereas the selective M1AChR allosteric agonist TBPB did not, despite similar stimulation of other M1AChR-mediated signaling pathways (Figures 6A and 6C) (Ma et al., 2009). We also show that, in our hands, the prototypical full orthosteric agonist oxo-m behaves as only a partial agonist in terms of stimulating β-arrestin recruitment at M1AChR (Figure 6C). These phenomena may be explained by a “bitopic” mode of ligand binding, whereby allosteric compounds differentially interact with both the allosteric and orthosteric sites, possibly inferring unique receptor conformations and subsequently, differential signaling events (Avlani et al., 2010). Such “bitopic” interactions have been previously proposed regarding 77-LH-28-1 (1-[3-(4-butyl-1-piperidinyl)propyl]-3,4-dihydro-2(1H)-quinolinone) at M1AChR and McN-A-343 (4-(N-(3-chlorophenyl)carbamoyloxy)-2-butynyltrimethylammonium chloride) at M2AChR (Avlani et al., 2010; Valant et al., 2008). Such ligand-specific signaling bias at M1AChR may help to illuminate particular amino acid residues responsible for the successful coupling of M1AChR activation and its various associated downstream signaling events, as has been explored previously for M2AChR (Gregory et al.,
2010). Future studies should focus on determining the specific amino acids responsible for M1AChR ligand-specific signaling bias.

In summary, we have presented here the pharmacological characterization of the partial M1AChR orthosteric agonist LY539039. Compared to the other orthosteric agonists tested here, LY539039 displays selectivity for M1AChR versus the remaining four mAChR subtypes (Figures 1-3 and Tables 1-2). Furthermore, LY593093 stimulates β-arrestin recruitment at M1AChR and displays in vivo activity in pharmacological and cognition animal models (Figures 6-8). We propose that LY539039 may be worthy of subsequent clinical investigation as a therapeutic agent in illnesses characterized, at least in part, by cognitive decline, including Alzheimer’s disease and schizophrenia. Additionally, the highly selective nature of this ligand lends itself as a pharmacological tool to better evaluate the role of M1AChR signaling in cognition and other physiological processes.
Acknowledgements

The authors would like to acknowledge Frank Bymaster, Mary Wolff, Robert Crile and Petra Carter for their technical contributions.
Authorship Contributions

*Participated in research design:* MLW, DAS, SH, AKC, DM, CCF

*Contributed new reagents or analytical tools:* SH, BL, CCF

*Conducted experiments:* MLW, DAS, AKC, DM

*Performed data analysis:* MLW, DAS, AKC, DM, CCF

*Wrote or contributed to writing of the manuscript:* MLW, DM, CCF

*Other:* BL (designed LY593093)
References


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Legends for Figures

**Figure 1 Legend:** LY593093 is functionally selective for M1AChRs.  
A) Structure of LY593093;  
B) LY593093-induced GTP-γ-[35S]-binding activity at the M1-M5AChRs (receptor densities (B_max) for the M1-M5AChRs were 9.3, 0.7, 0.6, 0.9 and 4.8 pmol/mg membrane protein, respectively). Data are expressed as a percentage of response in presence of 30 µM oxotremorine-m;  
C) Effect of LY593093 on stimulation of calcium mobilization in whole cells (CHO) stably-expressing human M1, M3 or M5 muscarinic receptors. Data are expressed as a percentage of response in presence of 10 µM oxotremorine-m. All data were analyzed using nonlinear regression for a sigmoidal concentration response curve (GraphPad Prism v. 4.0) and are displayed as the mean ± S.E.M. from N ≥ 3 separate experiments.

**Figure 2 Legend:** Effect of LY593093 and reference muscarinic agonists on stimulation of calcium mobilization in whole cells (CHO) stably expressing human M1-, M3-, or M5AChRs. Data were analyzed using nonlinear regression for a sigmoidal concentration response curve (GraphPad Prism v. 4.0) and are expressed as a percentage of response in presence of either maximal acetylcholine (50 µM ACh, for BQCA and TBPB) or maximal oxotremorine-m (10 µM oxotremorine-m, for remaining compounds). Data are displayed as the mean ± S.E.M. from n ≥ 3 separate experiments.

**Figure 3 Legend:**  
Effects of LY593093 and reference muscarinic agonists on stimulation of GTP-γ-[35S] binding by Gαi3 in membranes prepared from CHO cells stably expressing human M1- (B_max = 9 pmol/mg protein), M2- (B_max = 3 pmol/mg protein), or M4AChRs (B_max = 0.6 pmol/mg protein). Data were analyzed using nonlinear regression for a sigmoidal concentration response curve (GraphPad Prism v. 4.0) and are expressed as the mean ± S.E.M. from n ≥ 3 separate experiments. Data are displayed as a percentage of response in presence of 30 µM oxotremorine-m.

**Figure 4 Legend:**  
Effect of LY593093 and oxotremorine-m on stimulation of GTP-γ-[35S] binding by Gαq in membranes prepared from the hippocampi and frontal cortex of wild-type, M1AChR knockout and M3AChR knockout mice. Data were analyzed using non-linear regression for a sigmoidal concentration response curve with the minimum value set to zero (GraphPad Prism v. 4.0) and are graphed as the mean ± S.E.M. from n ≥ 2 separate experiments.
separate experiments. Data are displayed as a percentage of the signal observed in wild-type membrane preparations from each tissue type using oxotremorine-m and LY593093, respectively.

**Figure 5 Legend:** Displacement of n-methylscopalamine (NMS) binding to membranes prepared from CHO cells stably-expressing human M1AChR (B_max = 1.8 pmol/mg protein). A) LY593093, unlike the PAM BQCA, dose-dependently competes with NMS for M1AChR binding; B) LY593093 dose-dependently competes with acetylcholine for an orthosteric binding site on M1AChR; C) BQCA dose-dependently potentiates acetylcholine binding at M1AChR; D) BQCA has no effect on LY593093 binding at M1AChR, indicating that LY593093 binding cannot be allosterically modulated by compounds acting at the BQCA-interacting allosteric site. Non-specific binding was determined in the presence of 10 µM atropine. Data were analyzed using nonlinear regression for a sigmoidal concentration response curve (GraphPad Prism v. 4.0) and are expressed as the mean ± S.E.M. from n=2 experiments (A) and n ≥ 3 separate experiments (B-D), each performed in duplicate.

**Figure 6 Legend:** Ligand-induced signaling bias for M1AChR-coupled β-arrestin recruitment. A) LY593093 and xanomeline behave as partial agonists for β-arrestin recruitment at M1AChR, and BQCA dose-dependently augments ACh stimulated β-arrestin recruitment when incubated with an empirically-determined EC_{20} concentration of ACh; B) LY593093 displays both partial agonist and partial antagonist (when incubated with an empirically-determined EC_{90} concentration of ACh); C) TBPB, oxotremorine-m and talsaclidine behave as partial agonists in terms of M1AChR-mediated β-arrestin recruitment. Data were analyzed using nonlinear regression for a sigmoidal concentration response curve (GraphPad Prism v. 4.0) and are expressed as a percentage of response at 1.0 mM ACh. Data are displayed as the mean ± S.E.M. of n ≥ 3 separate experiments (except for BQCA (agonist mode), for which n = 2), each performed in either duplicate or quadruplicate.

**Figure 7 Legend:** Stimulation of in vivo phosphoinositide hydrolysis by oral LY593093 administration. LY593093 (3 or 10 mg/kg) was given to rats 1 hour after lithium (10 mmol/kg) and hippocampus collected 2 hours later. Data represent percent conversion of [^3H]-radioactivity to [^3H]-inositol monophosphates. * p < 0.034 versus Lithium alone group.

**Figure 8 Legend:** Total errors on Day 4 radial arm maze performance in 2 independent studies. Errors are plotted on the ordinate and dose of LY593093 (LY) on the abscissa. LY was administered orally, 3
hours prior to each training session. LY was tested across a range of doses to establish the effective
dose range and establish repeatability. * signifies p < 0.05 versus vehicle control group for each
experiment.
Tables

**Table 1.** Summary of effects of LY593093 and reference muscarinic agonist compounds on stimulation of calcium mobilization in whole cells (CHO) stably expressing human M1, M3, or M5 muscarinic receptors.

<table>
<thead>
<tr>
<th>Compound</th>
<th>M1AChR</th>
<th>M3AChR</th>
<th>M5AChR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC\textsubscript{50} (log\textsubscript{10} M)</td>
<td>E\textsubscript{max} (%)</td>
<td>EC\textsubscript{50} (log\textsubscript{10} M)</td>
</tr>
<tr>
<td>LY593093</td>
<td>-7.30 ± 8.20</td>
<td>85.3 ± 2.86</td>
<td>-5.21 ± 5.60</td>
</tr>
<tr>
<td>Acetylcholine</td>
<td>-7.62 ± 8.64</td>
<td>130 ± 3.90</td>
<td>-8.77 ± 9.82</td>
</tr>
<tr>
<td>Alvameline</td>
<td>-6.36 ± 7.28</td>
<td>46.9 ± 7.72</td>
<td>-5.82 ± 6.43</td>
</tr>
<tr>
<td>Sabcomeline</td>
<td>-8.27 ± 9.30</td>
<td>87.1 ± 4.41</td>
<td>-8.39 ± 9.30</td>
</tr>
<tr>
<td>Talsaclidine</td>
<td>-6.57 ± 7.42</td>
<td>104 ± 4.37</td>
<td>-5.96 ± 6.64</td>
</tr>
<tr>
<td>Xanomeline</td>
<td>-7.22 ± 7.76</td>
<td>115 ± 4.32</td>
<td>-6.58 ± 7.42</td>
</tr>
</tbody>
</table>

*Indicates < 20% efficacy at highest dose tested (100 nM).

Data were analyzed using nonlinear regression for a sigmoidal concentration response curve (GraphPad Prism v. 4.0) and are expressed as mean ± S.E.M. from n ≥ 3 separate experiments. Potency data are expressed in log\textsubscript{10} molar (M) units and efficacy data are expressed as a percentage of response at 10 \textmu M oxotremorine-m.
**Table 2:** Summary of effects of LY593093 and reference muscarinic agonist compounds on stimulation of GTPγS binding activity in membranes prepared from CHO cells stably expressing human M1, M2, or M4 muscarinic receptors.

<table>
<thead>
<tr>
<th>Compound</th>
<th>M1AChR EC$<em>{50}$ (log$</em>{10}$ M)</th>
<th>M1AChR $E_{\text{max}}$ (%)</th>
<th>M2AChR EC$<em>{50}$ (log$</em>{10}$ M)</th>
<th>M2AChR $E_{\text{max}}$ (%)</th>
<th>M4AChR EC$<em>{50}$ (log$</em>{10}$ M)</th>
<th>M4AChR $E_{\text{max}}$ (%)</th>
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<tr>
<td>LY593093</td>
<td>-6.66 ± 7.50</td>
<td>94.3 ± 2.29</td>
<td>-5.97 ± 6.76</td>
<td>23.2 ± 1.39</td>
<td>&gt; -5.00</td>
<td>*</td>
</tr>
<tr>
<td>Acetylcholine</td>
<td>-7.59 ± 8.61</td>
<td>103 ± 1.86</td>
<td>-8.16 ± 9.31</td>
<td>101.7 ± 2.20</td>
<td>-7.28 ± 7.87</td>
<td>87.4 ± 3.88</td>
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<tr>
<td>Alvameline</td>
<td>&gt; -4.52</td>
<td>*</td>
<td>-6.53 ± 8.24</td>
<td>49.4 ± 2.33</td>
<td>&gt; -4.52</td>
<td>*</td>
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<tr>
<td>Sabcomeline</td>
<td>-7.25 ± 8.03</td>
<td>38.9 ± 2.18</td>
<td>-7.25 ± 8.18</td>
<td>89.9 ± 2.28</td>
<td>-7.17 ± 7.95</td>
<td>20.0 ± 1.41</td>
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<tr>
<td>Talsaclidine</td>
<td>-6.09 ± 7.04</td>
<td>69.3 ± 2.42</td>
<td>-6.07 ± 7.16</td>
<td>94.4 ± 2.47</td>
<td>-6.10 ± 7.03</td>
<td>21.6 ± 0.78</td>
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<tr>
<td>Xanomeline</td>
<td>-7.36 ± 8.13</td>
<td>63.7 ± 3.48</td>
<td>-7.11 ± 7.98</td>
<td>82.6 ± 3.12</td>
<td>-7.20 ± 7.77</td>
<td>46.1 ± 4.02</td>
</tr>
</tbody>
</table>

*Indicates < 20% efficacy at highest does tested (10 µM FOR LY593093 and 30 µM for Alvameline).

Data were analyzed using nonlinear regression for a sigmoidal concentration response curve (GraphPad Prism v. 4.0) and are expressed as mean ± S.E.M. from n ≥ 3 separate experiments. Potency data are expressed in log$_{10}$ molar (M) units and efficacy data are expressed as a percentage of response at 10 µM oxotremorine-m.
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<table>
<thead>
<tr>
<th>Compound</th>
<th>M1AChR (log₁₀ M)</th>
<th>M2AChR (log₁₀ M)</th>
<th>M3AChR</th>
<th>M4AChR</th>
<th>M5AChR</th>
</tr>
</thead>
<tbody>
<tr>
<td>LY593093</td>
<td>-6.21 ± -7.63</td>
<td>-6.05 ± -7.54</td>
<td>&gt; -5.00</td>
<td>&gt; -5.00</td>
<td>&gt; -5.00</td>
</tr>
<tr>
<td>alvameline</td>
<td>-5.72 ± -6.67</td>
<td>-6.49 ± -7.48</td>
<td>-5.96 ± -6.88</td>
<td>-5.33 ± -6.25</td>
<td>-5.53 ± -6.48</td>
</tr>
<tr>
<td>xanomeline</td>
<td>-6.81 ± -7.92</td>
<td>-7.16 ± -8.11</td>
<td>-7.36 ± -8.23</td>
<td>-7.24 ± -8.33</td>
<td>-6.88 ± -8.10</td>
</tr>
</tbody>
</table>

Non-specific binding was measured in the presence of 1 μM atropine. Kᵢ values were determined from the Cheng-Prusoff relationship \( K_i = \frac{IC_{50}}{1 + [\text{ligand}]/K_d} \) where IC₅₀ is determined from a four parameter fit of displacement curves, [ligand] = 1 nM [³H] NMS, and K_d is the equilibrium dissociation constant of [³H] NMS for each receptor determined by saturation binding experiments carried out by the membrane supplier. Data are expressed as the mean ± S.E.M. from \( n \geq 3 \) separate experiments in log₁₀ molar (M) units.
Figure 1

A. LY593093

B. 

GTP-γ-[35S] Binding (% Maximum Signal)

log [LY593093], (M)

M1  M2  M3  M4  M5

C. 

Ca²⁺ Mobilization (% Maximum Signal)

Log [LY593093], M
Figure 2

M1AChR

![Graph showing Ca\(^{2+}\) Mobilization vs. Log [compound], M for M1AChR with various compounds represented by different symbols.]

M3AChR

![Graph showing Ca\(^{2+}\) Mobilization vs. Log [compound], M for M3AChR with various compounds represented by different symbols.]

M5AChR

![Graph showing Ca\(^{2+}\) Mobilization vs. Log [compound], M for M5AChR with various compounds represented by different symbols.]
Figure 3

M1AChR

GTP-$\gamma$-$[\text{35S}]$ Binding (% Maximum Signal)

Log [compound], M

-10.5 -9.5 -8.5 -7.5 -6.5 -5.5 -4.5 -3.5

Acetylcholine

Alvameline

Sabcomeline

Talsaclidine

Xanomeline

LY593093

M2AChR

GTP-$\gamma$-$[\text{35S}]$ Binding (% Maximum Signal)

Log [compound], M

-10 -9 -8 -7 -6 -5 -4

M4AChR

GTP-$\gamma$-$[\text{35S}]$ Binding (% Maximum Signal)

Log [compound], M

-10 -9 -8 -7 -6 -5 -4
Figure 8

Experiment 1

Day 4 Errors

Vehicle  3 mg/kg LY  10 mg/kg LY  30 mg/kg LY

Experiment 2

Vehicle  0.3 mg/kg LY  1 mg/kg LY  3 mg/kg LY

* indicates statistical significance.