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

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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, in particular, M1 muscarinic acetylcholine receptor (m1AChR) signaling 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 disease, that exhibit marked cognitive dysfunction as part of their clinical manifestation. Unfortunately, the development of selective M1 agonist medications has not been successful, mostly because of 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 Goαq coupled signaling events as well as β-arexin 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 anticholinergic 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 antipsychotic efficacy in preclinical studies (McArthur et al., 2010). The neurotransmitter acetylcholine, along with the two receptor families to which it binds, ionotropic nicotinic receptors and metabotropic 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 (Wess, 1996; Kristiansen, 2004). These subtypes are separated into two classes based on the G proteins to which they preferentially couple. M1AChR, M3AChR, and M5AChR signal through Goαq and mobilize intracellular calcium, whereas M2AChR and M4AChR couple to Goα1 and inhibit adenylyl cyclase and cAMP production (Caulfield, 1998).

ABBREVIATIONS: mAChR, muscarinic acetylcholine receptor; M1–5AChR, muscarinic; acetylcholine receptor subtypes 1–5; CHO, Chinese hamster ovary; GTPγS, guanosine 5'-O-(3-[35S]thiotriphosphate); ACh, acetylcholine; NMS, N-methylscopolamine; oxo-m, oxotremorine M; FLIPR, fluorometric imaging plate reader; 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]; P1, phosphoinositide; PAM, positive allosteric modulator; FCTX, frontal cortex; AC-42, 4-n-butyl-1-[(4-methylphenyl)-4-oxo-1-butyI]; 77-LH-28-1, 1-[3-(4-butyl-1-pipendinyl)propyl]-3,4-dihydro-2(1H)-quinolimine; McN-A-343, 4-(N-[3-chlorophenyl]carbamoyloxy)-2-butynyltrimethylammonium chloride; TBPB, [1-(1'-2-methylbenzyl)-1,4-bipiperidin-4-yl]-1H-benzof[1]imidazol-2(3H)-one.
An imbalance in cholinergic synaptic transmission has been associated with the cognitive decline apparent in both Alzheimer’s disease and schizophrenia (Raedler et al., 2007; Langmead et al., 2008). 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 (Levey, 1996; Dean et al., 2002; Porter et al., 2002).

The discovery and development of M1AChR-selective agonists has been challenging because of the high sequence similarity and identity shared among all five receptor subtypes at their respective orthosteric acetylcholine binding sites (Hulme et al., 1990; Heinrich et al., 2009). Although a number of arecoline-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 composed of Lilly archival compounds and libraries prepared via parallel synthesis. Here we describe the pharmacological characterization of the novel, potent and highly selective M1AChR partial agonist LY593093 [(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 material. The receptor densities (max) for the ectopically expressed M1AChR-mediated G protein activation and calcium mobilization. Furthermore, LY593093 stimulates β-arrestin recruitment, a signaling pathway postulated to be pertinent for behavioral and cognitive efficacy, as well as inositol phospholipid turnover in vivo (Ma et al., 2009). Finally, we demonstrate that the compound facilitates spatial learning in rats after oral administration, suggesting therapeutic potential in disorders involving cognitive dysfunction.

Materials and 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 CHO-K1 cells stably expressing both the β-arrestin fusion protein and M1AChR-ProLink fusion protein were licensed from DiscoveRx (Fremont, CA). M1-M5AChR-expressing CHO cell membrane preparations, GDP-β[S], [3H]mioinositol, and MultiLex A scintillation sheets were purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA). Anti-Gαq and anti-Gαi antibodies were generated by Eli Lilly & Co. Scintillation proximity assay beads were purchased from GE Healthcare (Chalfont St. Giles, Buckinghamshire, UK). Fluo-4, probenecid, Opti-MEM I, and tissue culture reagents were purchased from Invitrogen (Carlsbad, CA) unless otherwise indicated. Ready Solv HB scintillation fluid was purchased from Beckman Coulter, Inc. (Brea, CA). Wild-type, M1AChR knockout, and M3AChR knockout 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. In brief, frozen membrane preparations were thawed and resuspended in assay buffer (20 mM HEPES, 100 mM NaCl, 10 mM MgCl2, pH 7.4). Twenty-five micrograms per well of protein 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) through GF/A filters that had been presoaked in 0.3% polyethyleneimine. The filters were washed with 5 ml of ice-cold 50 mM Tris buffer, pH 7.4, and air-dried overnight. The dried filters were treated with MultiLex A melt-on scintillator sheets, and the radioactivity retained on the filters was counted using a Wallac 1205 Betaplate scintillation counter (PerkinElmer Life and Analytical Sciences). IC50 values were determined from the Cheng and Prusoff (1973) relationship IC50 = IC0/g1 + [ligand]/Kd, where IC0 is determined from a four-parameter fit of displacement curves, [ligand] = 1 nM [3H]NMS, and Kd is the equilibrium dissociation constant of [3H]NMS for each receptor determined by saturation binding experiments carried out by the membrane supplier. Nonspecific binding was determined using 1 or 10 μM atropine, as indicated in the figure legend.

Cell-Based and Native Tissue GTPγ[S] Binding Assays. The GTPγ[S] binding assay measures the level of G protein activation after agonist treatment by determining the amount of nonhydrolyzable GTPγ[S] bound to Go subunit subtypes of interest (see Harrison and Traynor, 2003; for a thorough review). GTPγ[S] binding in cells was determined using the antibody capture technique in a 96-well plate format as described previously by DeLapp et al. (1999). In brief, 100 μl (20–40 fmol/well) of membrane preparation from CHO cells that overexpress 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–, M5–, or M3– membranes.) After the incubation period, 50 μl/well (500 pM) diluted GTPγ[S] was added to each well, and the mixture was allowed to incubate for 30 min. The labeled membranes were then solubilized with 0.27% Nonidet 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–, M2–, or M5AChR membranes, anti-Gαq antibody was used, whereas anti-Gαi3 antibody was used for the M3– and M4AChR membranes. After the 60-min incubation, 50 μl/well (1.25 mg/well) scintillation proximity assay beads were added, and the plates were incubated for an additional 3 h. The plates were centrifuged for 10 min at 1000 rpm using an Accuspin 1R centrifuge (Thermo Fisher Scientific, Waltham MA) and counted for 1 min/well using a Wallac MicroBeta TriLux scintillation counter (PerkinElmer Life and Analytical Sciences–Wallac, Gaithersburg, MD). All incubations took place at room temperature.
in GTP-binding assay buffer (20 mM HEPES, 100 mM NaCl, 5 mM MgCl\(_2\), pH 7.4). GTP\(^{[35]S}\) binding in native tissue was performed essentially as described previously (Overt et al., 2010).

**Calcium Mobilization Assay.** Calcium mobilization was measured using assay guidance according to the National Institutes of Health’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 overexpressing either M1, M3, or M5AChR were plated at 50,000 cells/well in poly-lysine-coated 96-well black, clear-bottom plates and grown overnight at 37°C with 5% CO\(_2\) and 95% humidity in growth media (Dulbecco’s modified Eagle’s medium/F-12 (3:1), 10% FBS, 20 mM HEPES, 2 mM L-glutamine (Glutamax), 40 μg/ml L-proline, 250 μg/ml G418 (Genetec), 0.5% penicillin-streptomycin). Twenty-four hours later, media were removed and cells were preloaded with 50 μl of Fluor-4 Dye (5 μg/μl) for 45 min before replacing with 100 μl of fresh assay buffer (1× Hanks’ balanced salt solution, 20 mM HEPES, 250 μM probenecid. Cells were allowed to equilibrate at room temperature in buffer for 6 min before the addition of compounds using the FLIPR instrument.

**β-arrestin Recruitment Assay.** β-arrestin 3 recruitment was measured using the PathHunter β-arrestin complementation assay system, including a β-arrestin-induced CHO-K1 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). In brief, 40,000 cells/well were treated in Opti-MEM I with ligand for 90 min at 37°C with 5% CO\(_2\). β-Arrestin 3 recruitment, as determined by β-galactosidase complementation, was measured after the addition of PathHunter detection reagent for 60 min at room temperature followed by luminescence signal detection using a GENiosPro (Tecan, Männedorf, Switzerland).

**Animal Experiments.** Rats used were maintained in facilities fully accredited by the Association for the Assesment 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 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). In brief, six colony-acclimated male Sprague-Dawley rats (95–110 g) were anesthetized and prepared for stereotaxic administration of 2 μCi of \[^{3}H\]myoinositol (18.6 Ci/mmol) in 8 μl of 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 s.c.). Vehicle (water + 10% capryloycaproyl macrogol-8 glycride (Labrasol)) or LY593093 (3 or 10 mg/kg p.o.) was administered 1 h 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 g for 10 min, the \[^{3}H\]inositol monophosphates in 1 ml of supernatant were determined by chromatography. Total radioactive 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 approximately 40% counting efficiency. Effects of in vivo phosphoinositide hydrolysis were calculated using a within-animal design by determining percentage conversion of \[^{3}H\] radioactivity to inositol monophosphates and treatment differences analyzed via analysis of variance.

**Radial Arm Maze Acquisition.** Six male Sprague-Dawley rats, approximately 200 to 225 g, were tested for eight-arm radial maze learning using food reward. Rats were single-housed and acclimated for at least 4 days at a 12:12 light/dark colony with onset of light at 6:00 AM. 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, Allentown, PA), 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 eight runway arms, and a single food pellet (45 mg; Bio-Serv, Frenchtown, NJ) was placed in each food hopper. Each daily session terminated when either all eight 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 and 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% caprylocaprylol macrogol-8 glycride within sterile water. Injections were administered orally 3 h before each daily session.

**Results**

**LY593093 Is Selective for the M1AChR**

The selectivity of LY593093 (Fig. 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 \(K_{i}\) determination following displacement of NMS binding.

**Ca\(^{2+}\) Mobilization**

Among the three \(G_{\alpha}\)-coupled subtypes (M1AChR, M3AChR, and M5AChR), LY593093 preferentially stimulated M1AChR-mediated calcium mobilization (85.3 ± 2.86% control) (Fig. 1C). LY593093 displayed 100- and 2000-fold better potency at M1AChR (EC\(_{50}\) = 49.9 ± 6.32 nM) versus either M3AChR (EC\(_{50}\) = 6053 ± 2490 nM) or M5AChR (EC\(_{50}\) = 100,000 nM), respectively, distinguishing itself from the pre-existing molecules in terms of M1AChR functional selectivity (Figs. 1C and 2; Table 1). Whereas 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 percentage of maximal efficacy (\(E_{\text{max}}\)) demonstrated by LY593093 (Fig. 2; Table 1). EC\(_{50}\) (log\(_{10}\) 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; subcomeline = −8.3/−8.4/−7.4; talsaclidine = −6.6/−6.0/−4.0; xenomeline = −7.2/−6.6/−5.9. The percentage of \(E_{\text{max}}\) 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 xenomeline) were observed as follows (%\(E_{\text{max}}\)): LY593093 = 85.3 ± 2.86/43.9 ± 0.98/not applicable; alvameline = 46.9 ± 7.72/44.0 ± 8.99/not applicable; and xenomeline = 115 ± 4.32/29.4 ± 9.20/64.2 ± 8.77. It is noteworthy that xenomeline behaved as a partial agonist at M5AChR (Fig. 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 (EC\(_{50}\) = 2.8 ± 1.1 nM) full agonist (118 ± 6.6% \(E_{\text{max}}\)) at M1AChR in terms of calcium mobilization (Jones et al., 2006, 2008) (Fig. 2). It is surprising that the M1AChR positive allosteric modulator (PAM) benzyl quinolone carboxylic acid (BQCA) also displayed weak partial agonist activity (EC\(_{50}\) =
1.8 ± 1.7 μM; 73 ± 7.2% E_max) with respect to M1AChR-mediated calcium mobilization (Fig. 2).

**GTPγ[S] Binding**

The concentration response curves for LY593093-stimulated GTPγ[S] 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 (Fig. 1B). In terms of G protein activation, at M1AChR, LY593093 (EC_50 = 219 ± 31.9 nM) displayed ~95% of the control E_max observed with the full orthosteric agonist oxotremorine M (oxo-m; 30 μM), whereas LY593093 displayed less than 25% E_max at both M2AChR and M4AChR, (EC_50 = 1.07 ± 0.17 μM and >10 μM, respectively) (Fig. 3; Table 2). These functional results contrast those of the M1AChR-preferred control agonists, which failed to exhibit strong functional selectivity for M1AChR over both M2AChR and M4AChR (Fig. 3; Table 2). Xanomeline displayed relatively no functional selectivity for the M1AChR subtype (EC_50 = 43.6 ± 7.33 nM) over either M2- or M4AChR (EC_50 = 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% E_max) compared with 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 (EC_50 = 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% E_max, respectively) (Table 2). The augmented M3AChR activity observed with the calcium mobilization assay (Figs. 1C and 2; Table 2), relative to the GTPγ[S] binding assay (Figs. 1B and 4), probably demonstrates the amplified downstream calcium mobilization response measured with the FLIPR assay, whereas the GTPγ[S] binding assay measures initial receptor activation.

Native GTPγ[S] 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 (Fig. 4). Frontal cortex membrane preparations from M3AChR knockout and wild-type animals treated with oxo-m displayed similar EC_50 and E_max values with respect to Goα activation (EC_50_FCTx-oxo(WT)) = 99.7 ± 23.8 nM, % E_max_FCTx-oxo(WT) = 102.4 ± 3.2; EC_50_FCTx-oxo(M3KO) = 23.8 nM, % E_max_FCTx-oxo(M3KO) = 99.7 ± 23.8 nM, % E_max_FCTx-oxo(M3KO) = 102.4 ± 3.2.

**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>
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<tr>
<td></td>
<td>EC_50</td>
<td>E_max</td>
<td>EC_50</td>
</tr>
<tr>
<td>LY593093</td>
<td>-7.30 ± 0.20</td>
<td>85.3 ± 2.86</td>
<td>-5.21 ± 5.60</td>
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<tr>
<td>Acetylcholine</td>
<td>-7.62 ± 0.64</td>
<td>130 ± 3.90</td>
<td>-8.77 ± 9.82</td>
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<td>46.9 ± 7.72</td>
<td>-5.82 ± 6.43</td>
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<td>Sabcomeline</td>
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<td>-8.39 ± 9.30</td>
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<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>
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* Indicates <20% efficacy at the highest doses tested (100 nM).
69.9 ± 22.1 nM, % $E_{\text{max}}$\textsubscript{FCTx-oxo(M3KO)} = 99.2 ± 5.2). G\textsubscript{q} activation in M3AChR knockout and wild-type hippocampal membrane preparations treated with oxo-m was relatively similar, both in terms of percentage of $E_{\text{max}}$ and potency (EC\textsubscript{50Hippo-oxo(WT)} = 70.5 ± 17.4 nM, % $E_{\text{max}}$\textsubscript{Hippo-oxo(WT)} = 102.8 ± 3.3; EC\textsubscript{50Hippo-oxo(M3KO)} = 66.8 ± 21.3 nM, % $E_{\text{max}}$\textsubscript{Hippo-oxo(M3KO)} = 101.7 ± 6.5) (Fig. 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) (Fig. 4, A and C). LY593093-mediated G\textsubscript{q} signaling was nearly identical in M3AChR and wild-type membrane preparations of both hippocampal and frontal cortex (EC\textsubscript{50FCTx-LY593093(WT)} = 3.6 ± 3.3, % $E_{\text{max}}$\textsubscript{FCTx-LY593093(WT)} = 92.9 ± 4.5; EC\textsubscript{50FCTx-LY593093(M3KO)} = 2.7 ± 1.6 nM, % $E_{\text{max}}$\textsubscript{FCTx-LY593093(M3KO)} = 90.9 ± 6.6; EC\textsubscript{50Hippo-LY593093(WT)} = 3.2 ± 1.6 nM, % $E_{\text{max}}$\textsubscript{Hippo-LY593093(WT)} = 93.5 ± 4.5; EC\textsubscript{50Hippo-LY593093(M3KO)} = 1.6 ± 0.2 nM, % $E_{\text{max}}$\textsubscript{Hippo-LY593093(M3KO)} = 95.0 ± 5.9) (Fig. 4, B and D).
TABLE 2
Summary of effects of LY593093 and reference muscarinic agonist compounds on stimulation of GTP\(\gamma\)\(^{35}\)S binding activity in membranes prepared from CHO cells stably expressing human M1, M2, or M4 muscarinic receptors.

Data were analyzed using nonlinear regression for a sigmoidal concentration-response curve (Prism version 4.0) and are expressed as mean ± S.E.M. from \(n \geq 3\) separate experiments. Potency data are expressed in log\(_{10}\) molar (M) units, and efficacy data are expressed as a percentage of response at 10 \(\mu M\) oxotremorine M.

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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>
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<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>
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<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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<td>Sabcomeline</td>
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<td>(-7.25 ± 8.18)</td>
<td>89.9 ± 2.85</td>
<td>(-7.17 ± 7.95)</td>
<td>20.0 ± 1.41</td>
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<td>Talsacidine</td>
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<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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<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>
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\(\* <20\%\) efficacy at the highest doses tested (10 \(\mu M\) for LY593093 and 30 \(\mu M\) for Alvameline).

LY593093-mediated G\(\alpha_q\) signaling in M1AChR knockout tissues was dramatically diminished in both frontal cortex and hippocampus, relative to wild type (Fig. 4, B and D).

\([\text{H}]\text{NMS Displacement}\)

LY593093 dose-dependently competed with the nonselective mAChR antagonist \([\text{H}]\text{NMS for binding at the M1AChR and M2AChR orthosteric sites (K}_i = 622 ± 23.2 and 890 ± 28.6 nM, respectively) (Fig. 5A). However, LY593093 displayed no appreciable affinity for receptor subtypes M3AChR-M5AChR, distinguishing itself from the other M1AChR-prefering agonists tested (Table 3). Whereas sabcomeline, talsacidine, and xanomeline all displayed relatively similar binding affinities for the different mAChR subtypes, only LY593093 exhibited no appreciable affinity for receptor subtypes 3 to 5.

Curve-shift analysis was performed to determine whether LY593093 had any modulatory role in ACh functional activity. Curve-shift analyses indicated that LY593093 does not potentiate ACh-mediated displacement of \([\text{H}]\text{NMS as does the prototypical M1AChR-selective PAM BQCA (Fig. 5, B and C) but rather competed with acetylcholine for binding to the M1AChR (Fig. 5B). It is noteworthy that BQCA did not modulate the competitive nature of LY593093 binding (Fig. 5D), indicating that the interaction between LY593093 and M1AChR is different from 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 (CEREP, Paris, France). At 10 \(\mu M\), LY593093 was relatively inefficient at displacing the binding of radioligand selective for any of the non-mAChR human proteins in the panel. LY593093 (10 \(\mu M\) inhibited the binding of radioligand by greater than 50% at only three of the >60 proteins tested: dopamine transporter (58.8% inhibition); 5-hydroxytryptamine\(_2\) receptor (53.99% inhibition); and glutamate receptor, chloride-dependent site (54.06% inhibition) (Supplemental Table 1).

LY593093 Stimulates \(\beta\)-Arrestin 3 Recruitment at M1AChR

Paralleling the effect on GTP\(\gamma\)\(^{35}\)S 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-prefer-
ing orthosteric agonist xanomeline (Fig. 6, A and B, and
data not shown). LY593093 and xanomeline partially stim-
ulated 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 (Fig. 6A) and confirming the observation
made previously by Ma et al. (2009). The EC20 concentra-
tion of ACh used when testing BQCA in potentiator mode
was determined based on previous experiments and not
immediately before the potentiator assay was run. Assay-
to-assay variability probably explains why, at low concen-
trations of BQCA, 20% Emax was observed in the pres-
cence of what we previously observed in this assay to be an
EC20 concentration of ACh.

Unlike xanomeline and BQCA, not every control com-

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TABLE 3
Summary of effects of LY593093 and reference muscarinic agonist compounds on displacement of N-methylscopolamine binding to membranes
prepared from CHO cells stably expressing human M1–M5 muscarinic receptors

Nonspecific binding was measured in the presence of 1 µM atropine. K_i values were determined from the Cheng-Prusoff relationship K_i = IC50/(1 + [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 = 3 separate experiments in log10 molar (M) units.
LY593093 Is Active at M1AChR In Vivo

LY593093 Effects on In Vivo Hippocampal Phosphoinositide Hydrolysis. Conversion of $^3$H radioactivity to $[^3$H]inositol monophosphates was increased in a dose-related manner with the 10 mg/kg dose of LY593093 producing a 165% increase compared with the lithium-alone group (Fig. 7; $P_{0.034}$ versus lithium alone control). These data suggest that LY593093 activates muscarinic M1 receptors and stimulates the formation of inositol triphosphate in a brain region rich in M1AChRs.

LY593093 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 two separate studies. In experiment 1 (Fig. 8, left), 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 (Fig. 8, right), the top dose of 3 mg/kg LY593093 significantly reduced error ($t(13) = 2.73, P < 0.01$). These data show consistent efficacy of LY593093 within a 3 to 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 (Rainer and Mucke, 1998; Raedler et al., 2007; Langmead et al., 2008). 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 (Buckley et al., 1988; Anagnostaras et al., 2003; 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-preferring muscarinic agonist xanomeline showed symptom reduction efficacy in patients with both Alzheimer’s disease and schizophrenia; however, parasympathetic side effects, particularly in elderly patients, have limited its therapeutic potential (Bodick et al., 1997; Shekhar et al., 2008; Heinrich et al., 2009). 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 probably essential in the successful development of a tolerable M1ACHR orthosteric agonist (Heinrich et al., 2009). Although 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. 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 (Fig. 1C). By use of the GTPγS 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) (Fig. 1B). These results were further supported by a decrease in LY593093-stimulated GTPγS Gαq activation in M1ACHR knockout, but not M3ACHR or wild-type, hippocampal and frontal cortex membrane preparations (Fig. 4). Similar results were shown previously for the nonselective 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. Although the results clearly show that genetic ablation of M1ACHR drastically affects LY593093-mediated Gαq activation, other nonobvious signaling pathways may also be modulated by LY593093 treatment in the absence of the M1ACHR that affect 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. (2009). In an initial pharmacological characterization of LY593093 functional selectivity, Heinrich et al. (2009) observed LY593093 affinity at M2ACHR (Kᵢ = 0.79 µM) to be similar to that which we report here (0.62 µM). However, in terms of functional selectivity, they reported (Heinrich et al., 2009) modest LY593093 activity (>35-fold less potent compared with M1ACHR) at the M2- and M4ACHRs, which conflicts with our observations reported here. Once again, these differences may be explained by the assay method chosen to assess LY593093 activity. For their experiments, Heinrich et al. (2009) used 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 GTPγS binding assay. Assaying calcium mobilization, Heinrich et al. (2009) also reported modest activation of M3ACHR by LY593093 (EC₅₀ = 1.42 µM; 102% Eₘₐₓ), which is similar to our results (6.05 ± 2.5 µM; Table 1), despite the fact that in our hands, the maximal agonist effect of LY593093 (43.9% Eₘₐₓ, Table 1) was 2-fold less (102% Eₘₐₓ) than that observed by Heinrich et al. (2009). Although 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 et al. 2009).

A CEREP 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 [³⁵³H]NMS at M1ACHR with no effect on [³⁵³H]NMS binding at the M3-M5ACHRs (Fig. 5, A and B; 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 (Fig. 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 $E_{\text{max}}$ values at M1AChR versus any of the other subtypes, including M2AChR (Tables 1 and 2).

Ma et al. (2009) recently suggested that ligand-stimulated β-arrestin recruitment at M1AChR may be an important indicator of pharmacological efficacy in improved cognition. The requirement for this pathway in memory and learning was further supported in studies with wild-type and mutant M2AChRs 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 after 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 Gox$_3$-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 PAMs have shown promise as a means of selectively modulating M1AChR signaling (Spalding et al., 2002; Ma et al., 2009; Shirey et al., 2009). 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 with orthosteric agonists and exhibits a decreased likelihood to induce receptor desensitization and internalization (Thomas et al., 2009). Furthermore, the M1AChR-selective PAM BQCA was shown to potentiate ACh-mediated β-arrestin recruitment, whereas the selective M1AChR allosteric agonist TBPA did not, despite similar stimulation of other M1AChR-mediated signaling pathways (Fig. 6, A and C) (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 (Fig. 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)-quinolinenone at M1AChR and Mcn-A-343, 4-[2-(3-chlorophenyl)carbamoyloxy]-2-butylnitrilmethylammonium chloride at M2AChR (Valant et al., 2008; Avlani et al., 2010). Such ligand-specific signaling bias at M1AChR may help to illustrate 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, in this study we have presented the pharmacological characterization of the partial M1AChR orthosteric agonist LY593093. In comparison with the other orthosteric agonists tested here, LY593093 displays selectivity for M1AChR versus the remaining four mAChR subtypes (Figs. 1–3, Tables 1 and 2). Furthermore, LY593093 stimulates β-arrestin recruitment at M1AChR and displays in vivo activity in pharmacological and cognition animal models (Figs. 6–8). We propose that LY593093 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. Furthermore, 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.

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Authorship Contributions
Participated in research design: Watt, Schober, Hitchcock, Chesterfield, McKinzie, and Felder.
Conducted experiments: Watt, Schober, Chesterfield, and McKinzie.
Contributed new reagents or analytic tools: Hitchcock, Liu, and Felder.
Performed data analysis: Watt, Schober, Chesterfield, McKinzie, and Felder.
Wrote or contributed to the writing of the manuscript: Watt, McKinzie, and Felder.
Other: Liu designed LY593093.

References
Cheng Y and Prusoff WH (1973) Relationship between the inhibition constant (K) and the concentration of inhibitor which causes 50 per cent inhibition (50%) of an enzymatic reaction. *Biochem Pharmacol* 22:3099–3108.
Conn PJ, Jones CK, and Lindsley CW (2009b) Subtype-selective allosteric modulation


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**Supplemental Table 1 Legend:** Summary of LY593093 activity in broad panel binding affinity screen. Binding affinities of LY593093 for various receptors, enzymes, transporters and channels were measured at CEREPOS (Paris, France).