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Muscarinic receptors as model targets and antitargets for structure-based ligand discovery

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Running Title: Structure-based ligand discovery for muscarinic receptors

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Abbreviations: GPCR: G protein-coupled receptors; NMS: N-methyl scopolamine; OXO-M: oxotremorine-M; QNB: quinuclidinyl benzilate; cAMP: cyclic AMP; LE: Ligand efficiency; T_c: Tanimoto coefficient;

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Abstract

G protein-coupled receptors (GPCRs) regulate virtually all aspects of human physiology and represent an important class of therapeutic drug targets. Many GPCR-targeted drugs resemble endogenous agonists, often resulting in poor selectivity among receptor subtypes and restricted pharmacological profiles. The muscarinic acetylcholine receptor family exemplifies these problems; thousands of ligands are known, but few are receptor subtype-selective and almost all are cationic in nature. Using structure-based docking against the M_2 and M_3 muscarinic receptors, we screened 3.1 million molecules for ligands with new physical properties, chemotypes, and receptor subtype-selectivities. Of 19 docking-prioritized molecules tested against the M_2 subtype, 11 had substantial activity and 8 represented new chemotypes. Intriguingly, two were uncharged ligands with low micromolar to high nanomolar K_i values, an observation with few precedents among aminergic GPCRs. To exploit a single amino-acid substitution among the binding pockets between the M_2 and M_3 receptors, we selected molecules predicted by docking to bind to the M_3 and but not the M_2 receptor. Of 16 molecules tested, eight bound to the M_3 receptor. Whereas selectivity remained modest for most of these, one was a partial agonist at the M_3 receptor without measurable M_2 agonism. Consistent with this activity, this compound stimulated insulin release from a mouse b-cell line. These results support the ability of structure-based discovery to identify new ligands with unexplored chemotypes and physical properties, leading to new biological functions, even in an area as heavily explored as muscarinic pharmacology.

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Introduction

G protein-coupled receptors (GPCRs) are integral transmembrane proteins that transduce extracellular signals from neurotransmitters, hormones, odorants, and many other signals across cellular membranes. The muscarinic acetylcholine receptors (M_1-M_5) are a subfamily of GPCRs recognizing the neurotransmitter acetylcholine and signaling through G proteins of the $G_{q/11}$ class (M_1, M_3, and M_5 subtypes), and the $G_{i/o}$ class (M_2 and M_4 subtypes). These receptors are targets for the treatment of many illnesses, including chronic obstructive pulmonary disease, urinary incontinence, and diabetes (Wess et al., 2007), and have been implicated in treatment of cognitive disorders such as Alzheimer's disease (Messer, 2002), and schizophrenia (Chan et al., 2008).

Tool and drug development at muscarinic receptors has been complicated by difficulties in finding subtype-selective ligands. None of the muscarinic agonists and antagonists currently used in the clinic are selective for a particular muscarinic receptor subtype. This reflects the high sequence identities among the orthosteric sites of the M_1-M_5 receptors, differing, for instance, between the M_2 and M_3 subtypes by only a single residue. Muscarinic receptors can also mediate various side effects (*e.g.*, adverse effects on heart rate, salivary secretion, and smooth muscle contractility). For instance, whereas recent evidence suggests that an M_3 agonist would promote insulin release in type 2 diabetes (Wess et al., 2007), M_2 agonism would have substantial cardiac effects that would complicate clinical use. Similarly, M_1 agonists have shown promise for treatment of Alzheimer's disease, but dose-limiting side effects have precluded clinical use (Caccamo et al., 2009). Consequently, recent attempts to develop selective muscarinic drugs have focused on ligands targeting either an allosteric site (Conn et al., 2009b), or both orthosteric and allosteric sites simultaneously (Mohr et al., 2010).

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The recent determination of the crystal structures for the M_2 and M_3 muscarinic receptor subtypes (Haga et al., 2012b; Kruse et al., 2012) enables a structure-based discovery program for novel muscarinic ligands. To discover new chemotypes and compounds with novel physical and pharmacological properties, we initially docked large compound libraries against the M_2 muscarinic structure. A high discovery rate of new chemotypes and new physical properties inspired us to seek M_3-selective molecules by exploiting the small region of sequence difference between the two receptor subtypes. Whereas most molecules displayed some selectivity for the M_3 subtype, as designed in the docking screen, this selectivity was modest, illustrating the challenges of discovering subtype-selective orthosteric muscarinic ligands. However, the discovery of a partial M_3 agonist that had no agonist activity at the M_2 receptor, and its efficacy in a cell-based model to promote insulin release in β-cells, also illustrates the potential of this approach.

Materials and Methods

Materials.

Compounds were obtained from the vendors Molport, Chembridge, Enamine, Scientific Exchange, Princeton Biomolecular Research and Asinex, as well as from the Developmental Therapeutics Program at the National Cancer Institute. All compounds were sourced at 95% or greater purity as described by the vendors. All active compounds were further tested for purity by LC/MS at UCSF, and were found to be at least 95% pure as judged by peak height and identity. For compounds 11 and 12, LC/MS was inconclusive and purity was confirmed by ^{1}H NMR spectroscopy at the Stanford Magnetic Resonance Laboratory using a Varian Inova 600 mHz spectrometer. Compound 5 was not commercially available in sufficient purity, and details regarding its preparation are given below.

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Chemistry.

Compound 5 (pyridin-3-ylmethyl 2-hydroxy-2,2-diphenylacetate) was not commercially available in sufficient purity, and was synthesized as follows. After stirring a suspension of 3-(hydroxymethyl)pyridine (30 µL, 0.31 mmol) and K_2CO_3 (100 mg, 0.72 mmol) in anhydrous DMF (12 mL) at room temperature for 1 h, a solution of methyl benzilate (50 mg, 0.21 mmol) in anhydrous DMF (3 mL) was added. The mixture was stirred at 65 °C at 70-100 mbar for 6 h and allowed to cool to room temperature. After addition of CH_2Cl_2 and water, the organic layer was washed with a saturated aqueous solution of NaCl, dried (Na_2SO_4) and evaporated. The residue was purified by flash chromatography (CH_2Cl_2 – MeOH 60:1) to yield pure pyridin-3-ylmethyl 2-hydroxy-2,2-diphenylacetate (24.3 mg, 36 %) as a white solid (mp: 98-101 °C). ^{1}H NMR ($CDCl_3$, 600 MHz) δ from TMS (ppm): 8.58 (brs, 1H), 8.51 (brs, 1H), 7.52 (brd, J = 7.6 Hz, 1H), 7.37-7.41 (m, 4H), 7.30-7.34 (m, 6H), 7.26-7.27 (m, 1H), 5.31 (s, 2H), 4.15 (brs, 1H); ^{13}C NMR ($CDCl_3$, 150 MHz) d from TMS (ppm): 174.3, 149.3, 148.8, 141.7, 136.6, 131.1, 128.4, 128.3, 127.5, 123.9, 81.4, 65.7; IR (NaCl), n (cm^{-1}): 3150, 3060, 1740, 1600, 1580, 1450, 1220, 1060, 700; HPLC: t_R = 18.55 min (eluent 1), t_R = 16.41 min (eluent 2), purity > 95 %; HRMS *(m/z)*: $[M]^+$ calcd for $C_{20}H_{17}NO_3$ (M + Na^+) 342.1101, found 342.1111.

IR spectra were recorded on a JASCO model FTIR 410 instrument as a film on NaCl. ^{1}H NMR (600 MHz) and ^{13}C NMR (150 MHz) spectra were determined on a Bruker AVANCE 600 spectrometer. ESI-ToF high mass accuracy and resolution experiments were performed on a Bruker maXis MS in the laboratory of the Chair of Bioinorganic Chemistry, FAU. HPLC analysis was performed on an analytical system (Agilent 1100 analytical series, VWD detector, Zorbax Eclipse XDB-C8 analytical column, 4.6×150 mm, 5 µm, flow rate: 0.5 ml/min). Eluent 1: CH_3OH in H_2O + 0.1% HCO_2H (0-3 min 10 %, 3-18 min 10 – 100%, 18-24 min 100%); eluent 2: CH_3CN in H_2O + 0.1% HCO_2H (0-3 min 5 %, 3-18 min 5-85%, 18-24 min 85%). Flash chromatography

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was done using silica gel (40-63 µm) as stationary phase. The purity of the test compound was determined to be >95%.

Molecular docking.

To predict new muscarinic ligands, we used DOCK 3.6 (Lorber and Shoichet, 2005; Irwin et al., 2009; Mysinger and Shoichet, 2010) to virtually screen the approximately 3.1 million lead-like and fragment-like subsets of ZINC (Irwin and Shoichet, 2005; Irwin et al., 2012) against the M_2 or M_3 muscarinic receptor structure. Compounds were docked in multiple orientations and multiple conformations. Each geometry was scored for electrostatic and van der Waals complementarity, and corrected for desolvation using the solvent-excluded volume method, and the complex with the lowest energy was picked. Compounds were manually selected for experimental testing from the top-ranking 500 molecules based both on their physical complementarity and chemical novelty, using criteria previously described (Mysinger et al., 2012).

To identify compounds that selectively bind to the M_3 receptor, a similar method was first employed to score lead-like and fragment-like subsets of ZINC against both receptors. The top 5,000 ranked molecules against the M_3 receptor were selected for further consideration. Each of these molecules was then ranked according to the difference in energy score between docking at M_3 and M_2. The 500 molecules with the largest energy score difference in favor of the M_3 receptor were then inspected and 16 were chosen for experimental testing on the basis of high physical and chemical complementarity to M_3, poor complementarity to M_2, and novelty.

Receptor expression and membrane preparation.

Human M_2 and rat M_3 muscarinic receptors were expressed with an amino-terminal FLAG epitope tag in Sf9 insect cells using the BestBac system (Expression Systems). Membranes

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were prepared using a glass dounce tissue grinder to homogenize cells in 20 mM Tris pH 7.5 and 1 mM EDTA. Homogenized cell material was then centrifuged at low speed (100 × G) for 5 min to remove debris. The supernatant was then centrifuged at 18,000 rpm in an SA-800 rotor for 15 min to pellet membranes. Membranes were resuspended in binding buffer (75 mM Tris, pH 7.4, 12.5 mM $MgCl_2$, 1 mM EDTA), aliquoted, and flash frozen in liquid nitrogen**.**

Radioligand binding assays.

Ligand affinities were measured by radioligand displacement binding assays. Binding assays were performed using ^{3}H-N-methyl scopolamine (NMS; Perkin Elmer) at 0.61 nM in all samples. Following mixing of membranes, cold ligand and NMS samples were shaken at 20 °C for two hours. Samples were then filtered on a glass fiber filter with a 48-well harvester (Brandel). Radioactivity was measured by liquid scintillation. Binding data are summarized in Tables 1 and 2, and representative binding curves are shown in figures S1, S2, and S3. Binding data analysis was performed using GraphPad Prism 4.0 software.

Calcium Mobilization Assay.

CHO cells stably expressing the human M_3 receptor or CHO cells stably co-expressing the human M_2 receptor and a hybrid G protein $G_{qi}5$ (Marlo et al., 2009) (a Ga_q subunit in which the last five amino acids were replaced with the corresponding Ga_i sequence) were incubated with increasing concentrations of ligands, and changes in intracellular calcium levels were determined using FLIPR technology (Molecular Devices, Sunnyvale, CA). All measurements were performed in 96-well plates, as described previously (Li et al., 2007; McMillin et al., 2011). Agonist concentration-response curves were analyzed using GraphPad Prism 4.0 software.

cAMP Assay.

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CHO cells stably expressing the human M_2 receptor were trypsinized, collected by centrifugation, and resuspended in phosphate-buffered saline containing glucose (1 mg/ml) and EDTA-free complete protease inhibitor (Roche Applied Science) at a density of 1×10^6 cells/ml. Subsequently, 20 µl aliquots were added to 200 µl PCR tubes and incubated with the same volume (20 µl) of increasing concentrations of ligands in the presence of 50 µM forskolin for 25 min at 37°C. The incubation mixtures were then transferred into white-bottom 384-well plates (□5000 cells/well), and cells were lysed to determine drug-dependent changes in cAMP levels using a fluorescence resonance energy transfer-based cAMP detection technique (cAMP dynamic 2 kit; Cisbio Bioassays, Bedford, MA) according to the manufacturer's protocol. Elevated 665 nm/620 nm ratio indicates decreased cAMP levels in this assay.

Insulin release assays (MIN6 cells).

MIN6 cells (a kind gift from Dr. Abner Notkins, NIDCR, NIH) were cultured as described previously (Ishihara et al., 1993). 60,000 cells were seeded into 96 well plates and cultured for 48 hr at 37 °C in 5% CO_2. After this time, MIN6 cells were washed with 3.3 mM glucose buffer (in Krebs-Ringer bicarbonate/HEPES buffer) and then incubated for 1 hr at 37 °C in 5% CO_2. After this step, MIN6 cells were incubated for another hour at 37 °C in 5% CO_2 with increasing concentrations of oxotremorine-M (OXO-M) or compound 16 in 16.7 mM glucose Krebs-Ringer buffer. Insulin release was determined by measuring insulin concentrations in the incubation medium using an insulin ELISA kit (Crystal Chem Inc., Downers Grove, IL, USA). To confirm that the observed responses were mediated by muscarinic receptors, some assays were carried out in the presence of atropine (10 µM). E_{max} and EC_{50} values were obtained from OXO-M and compound 16 concentration-response curves using GraphPad Prism 4.0 software.

Antagonism assay.

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To examine whether compounds 12, 13, and 20 were able to block M3 receptor-mediated responses, we determined their ability to inhibit OXO-M-induced increases in intracellular calcium levels via activation of M_3 receptors endogenously expressed by MIN6 cells. 50,000 cells were seeded into 96-well plates and FLIPR assays were carried out as described above (Calcium Mobilization Assay***)***. On the day of the assay, cells were pre-incubated with the calcium-chelating dye and the various compounds (atropine and compounds 12, 13, and 20) for 45 min, followed by the addition of the muscarinic receptor agonist OXO-M (1 μM). Compounds 12, 13, and 20 were used at a concentration of 10 μM (~10 times their K_i). Atropine was employed at a concentration of 10 nM.

Results

Identification of new muscarinic ligands.

To identify new muscarinic ligands and to assess the suitability of muscarinic receptor structures as templates for ligand discovery, we pursued a docking campaign against the M_2 muscarinic receptor structure. Like most GPCR structures available to date, the M_2 receptor was solved in an inactive conformation bound to a small molecule antagonist. It presents a deep, almost completely buried ligand binding site (Fig. 1A), covered by a layer of tyrosines long known to be critical for ligand binding. Such deeply buried cavities are well-suited to computational ligand discovery, and previous GPCR docking work has met with remarkable success (Sabio et al., 2008; Kolb et al., 2009; Katritch et al., 2010; Carlsson et al., 2010; de Graaf et al., 2011; Mysinger et al., 2012). Within the binding pocket, the crystallographic ligand quinuclidinyl benzilate (QNB) engages largely in hydrophobic interactions, while Asn404$^{6.52}$ forms a pair of hydrogen bonds and Asp103$^{3.32}$ serves as a counter ion to the positive charge of the ligand (Fig.

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1B; superscript numerals refer to the Ballesteros-Weinstein numbering system for GPCRs). (Ballesteros and Weinstein, 1995)

We screened 3.1 million fragments or "lead-like" molecules (Methods) from the ZINC database (Irwin and Shoichet, 2005; Irwin et al., 2012) against the structure of the M_2 receptor. Each fragment and "lead-like" molecule was sampled in an average of 222 and 274 orientations and 437 and 700 conformations, respectively, in the orthosteric site; overall, over 547 billion configurations of the 3.1 million molecules were sampled. Molecules were ranked based on van der Waals and electrostatic complementarity, corrected for ligand desolvation using a receptor volume-based implementation of the Generalized-Born equation (Mysinger and Shoichet, 2010). From among the top 500 ranked molecules, we selected 18 that interacted with key residues such as Asp103$^{3.32}$, Asn404$^{6.52}$, and Trp400$^{6.48}$, preferring molecules topologically or physically dissimilar to known muscarinic ligands. These 18 molecules were tested by single point competition binding against the high affinity antagonist ^{3}H-NMS (Supplementary table 1), and those with substantial inhibition at 20 μM were further tested in a competition binding assay. Of the 18 compounds tested, 11 had K_i values lower than 50 μM (Table 1; Supplementary figure S1; Supplementary table 2). The compound with the highest affinity (Compound **1**) displayed a K_i of 390 nM. Six of these compounds were fragments, with ligand efficiencies (LEs) ranging from 0.36 to 0.44 kcal/heavy-atom. Most of the 11 molecules were topologically dissimilar to known muscarinic agents. Using two-dimensional ECFP4 fingerprints and Tanimoto coefficients (T_c) (Hert et al., 2004) to all known muscarinic ligands in ChEMBL11 (Gaulton et al., 2011), 8 of the 11 compounds have a $T_c < 0.33$ to the closest muscarinic ligand of any class, a difference large enough to be typically considered a "scaffold hop" (Muchmore et al., 2008). Correspondingly, their binding poses differ substantially from that of the co-crystallized ligand (Fig. 1C).

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Intriguingly, two of the higher affinity ligands, compounds **5** and **11** (Table 1), lack the defining cationic amine that is ubiquitous among muscarinic ligands and other aminergic GPCRs (*e.g.*, histaminergic, adrenergic, dopaminergic, or serotonergic). Indeed, they were chosen for testing because of this unexpected physical property. Whereas in compound **5** the pyridine nitrogen might conceivably be cationic—though it would be expected to be neutral at physiological pH, and is docked in this form—compound **11** is constitutively neutral at all accessible pH values. Correspondingly, the phenyl analog of **5** and **11**, compound **12,** is also a ligand with low micromolar affinity. The loss of the Asp103$^{3.32}$ ion-pair with the ligand cation is a substantial insult, amounting to about 4 kcal/mol if one compares the affinity of compounds **11** and **12** to that of the analogous QNB, which binds with an affinity of 180 pM to the M_2 receptor (Heitz et al., 1999). However, the fact that such ligands can even bind to muscarinic receptors at meaningful reasonable concentrations has few precedents in the field (Barlow and Tubby, 1974). Indeed, No uncharged ligands of the M_2 or M_3 receptors are reported in the ChEMBL database (*i.e.*, all are expected to be ionized at physiological pH values), of the over 5000 ligands annotated, and while 4 neutral analogs of acetylcholine and other acetic-acid esters are reported to be active at acetylcholine receptors of the guinea-pig ileum (Barlow and Tubby, 1974), no further uncharged ligands have been reported subsequently, to the best of our knowledge.

***Docking for subtype selectivity*.**

Though the docking against the M_2 receptor had no selectivity goal—compounds were simply chosen based on complementarity to the M_2 receptor—we were interested to learn whether the unusual chemotypes and physical properties of the new ligands conferred selectivity. We thus tested those M_2 ligands with K_i values lower than 10 μM for binding to the M_3 receptor (Table 1; Supplementary figure S2) (those molecules with weaker affinity were not pursued). Intriguingly, all three uncharged ligands (**5, 11**, and **12**) bear some selectivity for the M_3 over the M_2 subtype.

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For example, compound **12** shows a 5-fold higher affinity for the M_3 subtype (K_i = 290 nM) as compared to the M_2 subtype. Prompted by this observation, we explicitly set out to exploit the few differences that do exist between the M_2 and M_3 orthosteric sites in docking screens for subtype-selective ligands, treating the M_2 subtype as a docking 'anti-target'. In the M_3 receptor, M_2 Phe181 is replaced by a leucine, creating an enlarged pocket that might be exploited to achieve binding selectivity (Fig. 2A, B). We again docked the fragment and "lead-like" subsets of the ZINC database against both the M_2 and M_3 receptors, this time selecting the top-ranked 5,000 molecules against the M_3 receptor. From these compounds, we chose 500 molecules with the largest rank difference between subtypes (Fig. 2C; Table 2; Supplementary figure S3; Supplementary table 3). For instance, compound **13** ranks 2496 out of 3.1 million (top 0.1%) docked against the M_3 receptor, but ranks only 1,238,745 out of 3.1 million (top 40%) against the M_2 receptor, suggesting much better complementarity to the M_3 subtype. From these 500 molecules, 16 candidates were selected for testing, again weighing key interactions and chemical novelty (Table 2). Of these, 8 compounds showed detectable binding to the M_3 receptor. We then tested each of these molecules for affinity against both receptor subtypes. Although most compounds showed detectably higher affinity for the M_3 receptor, the selectivity ratios were typically modest, reaching at best 6-fold **(**Table 2). The one exception was compound **16**, a ligand with an unprecedented sulfonamide core and a ECFP4-based T_c value of only 0.3 to the closest known muscarinic ligand in ChEMBL (Gaulton et al., 2011). This molecule proved to be a partial agonist at the M_3 receptor in a cell-based functional assay (5 μM EC_{50} value) without detectable activity at the M_2 receptor (see below).

Efficacy of new ligands.

Most docking screens against inactive GPCR structures have discovered only antagonists (Kolb et al., 2009; Carlsson et al., 2010; Katritch et al., 2010; Carlsson et al., 2011; de Graaf et al., 2011), while a docking screening against the activated state of the β_2-adrenergic receptor

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discovered only agonists (Weiss et al., 2013). Thus far, the only exception to this pattern is the κ opioid receptor, where an inactive state was used as a template for the docking-based discovery of specific agonists (Negri et al., 2013). We therefore investigated the efficacy of the new ligands against both M_2 and M_3 receptors, using a calcium mobilization assay to test for G protein activation. The M_2 receptor couples primarily to the G_i class of G proteins, which mediate inhibition of adenylyl cyclase, while the M_3 receptor preferentially couples to $G_{q/}$, mediating hydrolysis of phospoinositide lipids and consequent elevation of intracellular calcium. For these assays, we used CHO cells stably expressing the human M_3 receptor or CHO cells stably co-expressing the human M_2 receptor and a hybrid G protein $G_{qi}5$, which comprises of a $G\alpha_q$ subunit in which the last five amino acids were replaced with the corresponding $G\alpha_i$ sequence, allowing coupling to the M_2 receptor (Wess et al., 1997). Almost all compounds tested were devoid of agonist activity on either receptor. Additional functional studies with representative compounds showed that the uncharged compound **12** antagonized oxotremorine-M-induced activation of M_3 receptors in cultured MIN6 cells, as did compounds **13** and **20** (Supplementary figure S4).

The only agent that showed agonist activity at the M_3 receptor was compound **16.** This molecule was a partial agonist at the M_3 receptor, with an EC_{50} of 5.2 μM an E_{max} of 65%, but lacked detectable efficacy at the M_2 subtype (Fig. 3). The lack of agonist activity of **16** at the M_2 receptor was confirmed in both calcium mobilization (Fig. 3A) and adenylate cyclase inhibition (Fig. 3B) assays. To our knowledge, compound **16** represents the first pharmacological agent that can activate M_3 but not M_2 receptors. This novel activity profile mirrors its unusual chemotype: unlike most muscarinic ligands, compound **16** cannot form a paired hydrogen bond with $Asn^{6.52}$, as seen in the M_3 cocrystal structure with tiotropium, and instead may hydrogen bond through its unique sulfonamide to $Tyr^{3.33}$ (Fig. 3C). Whether this configuration is

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conserved in the activated M_3 structure to which it must bind is uncertain at this time; we cannot now rule out the possibility that compound **16** may even bind in a completely unexpected manner, including even to allosteric pockets that may initiate activation in their own right (Bluml et al., 1994; Avlani et al., 2010; Gregory et al., 2010). Further studies will be required to definitively establish the binding site for compound **16**. For now, it is the novelty of this chemotype to which we attribute its unexpected activity and selectivity.

Compound 16 stimulates insulin release in pancreatic β-cells.

The M_3 receptor is a critical regulator of acetylcholine-mediated glucose-dependent insulin release from pancreatic β-cells, and recent studies indicate that increasing M_3 receptor signaling would be useful in the treatment of type 2 diabetes (Gautam et al., 2010; Ruiz de Azua et al., 2010). However, further study of this concept has been stymied by the lack of selective M_3 agonists. We therefore tested the ability of compound **16**, a selective M_3 agonist**,** to stimulate insulin release from pancreatic β-cells. Specifically, we incubated MIN6 insulinoma cells, a mouse β-cell line expressing endogenous M_3 receptors, with increasing amounts of OXO-M, a potent muscarinic agonist, or compound **16**. Both compounds evoked a dose-dependent increase in insulin secretion, with a pEC_{50} of -4.21 and -5.75, respectively, for compound **16** and OXO-M. Compound **16** induced insulin secretion with an E_{max} 58% that of OXO-M (Fig. 4). Insulin release could be blocked by 10 μM atropine (Fig. 4B), confirming the involvement of M_3 receptors.

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Discussion

Four major observations emerge from this study. First, docking to the M_2 and M_3 muscarinic receptors led to the identification of multiple compounds with new physical properties and new chemical scaffolds. Second, as observed for other GPCRs, the docking hit-rates were high, between 50 and 60% of the compounds tested were active, with lead-like molecules often having affinities in the 0.1 to 1 μM range and with fragments with ligand efficiencies often above 0.4 kcal/heavy-atom (de Graaf et al., 2011). Third, an effort to explicitly dock for molecules specific for the M_3 over the M_2 subtype largely failed to successfully exploit the admittedly small difference between the two orthosteric sites, likely reflecting weaknesses in our current rigid-receptor docking models. Fourth, whereas it is not clear that the discovery of compound **16** reflects on our ability to select against binding to the M_2 subtype—it may simply reflect the unexplored functionality of this compound—compound **16** represents an important novel pharmacologic tool in that it can activate M_3 but not M_2 receptors. These findings hint at the potential of a structure-based program to discover compounds with new chemistry and correspondingly new pharmacology.

A promise of structure-based discovery is the identification of molecules that physically complement a binding site but escape from trends emerging from classic structure-activity relationships. The muscarinic ligands are a good example of how a few key chemotypes and physical properties have come to dominate an area of pharmacology. Of over 5000 M_2 or M_3 receptor ligands annotated in ChEMBL, all bear at least a single cationic nitrogen. The discovery of ligands that are constitutively uncharged demonstrates that orthosteric site binding in muscarinic receptors is not contingent on the presence of such a cationic group. Since both cationic and uncharged ligands were found in our screen, and ranked about equally in the

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docking screen, this discovery also attests to the ability of a physics-based docking scoring function to balance high-magnitude ionic interactions (favoring charged ligands) and desolvation (favoring uncharged ligands) to arrive at a list of uncharged and cationic candidates. The uncharged ligands may balance the loss of the energy contributed by the $Asp^{3.32}$ ion pair by hydrogen bonds with $Asn^{6.52}$ and quadrupolar stacking with $Tyr^{6.51}$ and $Trp^{6.48}$, as observed in the docked poses (Fig. 1). These interactions are less common among cationic docking hits, which tend to be dominated by the $Asp^{3.32}$ interaction (Fig. 1). Whereas the uncharged ligands bind as well as the new cationic ligands discovered here, they do lose about 4.5 kcal/mol in affinity compared to a structurally similar cationic ligand like QNB, attesting to the importance of the ion-pair in contributing to high affinity ligand binding. Still, as uncharged ligands will typically exhibit much greater membrane permeability than charged counterparts, such agents may show unique properties *in vivo* and may merit further exploration.

While the promise of discovering ligands with new chemotypes and new physical properties was realized in the docking screens, that of targeting particular differences between the M_2 and M_3 receptors to identify subtype-selective ligands was not. Though docking found molecules that fit much better against the rigid M_3 than the M_2 receptor structure owing to clashes with the larger Phe181 of the M_2 site, these apparent structural specificities largely disappeared on pharmacologic testing. Despite much more favorable M_3 docking ranks and scores (Table 2), experimental preference for the M_3 subtype never rose above six-fold in binding affinity. Thus, the steric clashes with Phe181 in the M_2 site were not realized, or only to a small degree, presumably reflecting conformational flexibility in the site. This has largely been true of other recent efforts to find molecules selective among different GPCR subtypes: where selective molecules have been found directly from docking, they may reflect more on the chemical novelty of the compounds than on specific interactions captured by the modeling (de Graaf et al., 2011; Carlsson et al., 2011; Kolb et al., 2012). The exception to this is where chemical

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synthesis of multiple analogs, guided by structure, has followed initial hit-discovery by virtual screening (Langmead et al., 2012). Whereas there are now several methods that allow one to model local receptor flexibility in docking (Durrant and McCammon, 2010; Henzler and Rarey, 2011), implementing these prospectively in a way that does not lead to the appearance or even dominance of non-binding decoys remains an ongoing challenge (Wei et al., 2004; Totrov and Abagyan, 2008). As the structures of more receptor subtypes are being solved or become amenable to homology modeling**,** the call for reliable methods that can exploit small differences in receptor structure among closely related subtypes will become increasingly pressing. Correspondingly, the call for strategies that exploit differences among allosteric sites, which are often substantially greater than those between the orthosteric sites of receptor subtypes (Conn et al., 2009a; May et al., 2007), is also supported by this study. In all such efforts, a close collaboration with medicinal chemistry will be crucial, as one cannot usually expect that just the right, specific molecule will be present even as large a library as ZINC represents, even though a lead chemotype might be.

Though we were unable to reliably exploit the subtle differences between the M_2 and M_3 orthosteric sites to identify M_3 selective antagonists, the discovery of a selective M_3 receptor agonist (compound **16**) hints at the promise of a structure-based discovery program. Whereas the unusual pharmacology of compound **16** may owe as much to its chemical novelty as to the differential docking, the exploration of new chemotypes is something that has been often realized in docking campaigns against GPCRs (Evers and Klebe, 2004; de Graaf et al., 2011; Langmead et al., 2012) and that can be relied on. The observation that this agent can induce insulin release from pancreatic b-cells in culture supports its status as a lead compound for chemical tool development, and this finding may have important therapeutic implications for the treatment of type 2 diabetes if selective M_3 receptor agonists endowed with higher affinity can be developed. More broadly, a structure-based program of ligand discovery against the M_3

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receptor and related GPCRs holds out the promise of identifying new chemotypes with new physical properties and correspondingly new specificities and pharmacological properties, with important implications for the discovery of new probes and therapeutic leads.

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Authorship contributions

Participated in research design: Kruse, Weiss, Wess, Kobilka, and Shoichet.

Conducted experiments: Kruse, Weiss, K. Hu, J. Hu, Rossi.

Contributed new reagents or analytic tools: Eitel, Gmeiner.

Performed data analysis: Kruse, Weiss, Wess, Kobilka, and Shoichet.

Wrote or contributed to the writing of the manuscript: Kruse, Weiss, Wess, Kobilka, and Shoichet.

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Footnotes

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Figure 1. Docking poses for selected M_2 muscarinic receptor hits. (a) The overall structure of the M_2 receptor (Haga et al., 2012) with the orthosteric site outlined. (b) The chemical structure of the co-crystallized antagonist QNB, its crystallographic geometry and key interactions (dashed lines). (c). Docking-discovered ligands (carbons in cyan) are superimposed in their docked poses on the crystallographic structure of QNB (carbons in yellow).

Figure 2. Docking for selective M_3 receptor ligands. (a) The M_3 (green) and M_2 receptor (orange) binding pockets are superimposed, and rendered as solvent-accessible surfaces, highlighting the enlarged binding pocket in the M_3 subtype (Kruse et al., 2012). (b) specific interactions with the co-crystallized M_3 antagonist tiotropium are shown. (c) Docking poses for select new ligands.

Figure 3. Compound 16 activates M_3 but not M_2 receptors. (a) Compound **16** showed partial agonism at the M_3 subtype, but not at the M_2 receptor in a calcium mobilization assay using CHO cells stably expressing M_2 or M_3 receptors (see Materials and Methods for details). This effect was blocked by the muscarinic antagonist atropine, consistent with direct activity at the M_3 receptor. (b) In a FRET-based cAMP assay (see Material and Methods for details), compound **16** did not lead to changes in intracellular cAMP levels in CHO-M_2 cells, confirming that this agent lacks efficacy at M_2 receptors. In this assay, an elevated 665 nm/620 nm ratio corresponds to decreased cAMP levels. The curves shown in panels (a) and (b) are representative of three independent experiments. (c)The unique structure and predicted binding mode of compound **16** may account for its novel activity profile.

Figure 4. Ligand-stimulated insulin release in MIN6 cells. (a) MIN6 cells, which express endogenous M_3 receptors, were incubated with increasing concentrations of OXO-M and

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compound **16**, and ligand-induced insulin release was measured. (b) The responses to both agonists were sensitive to blockade by atropine, indicating that the observed effects result from direct M_3 receptor activation. Data (means ± SE) are from three independent experiments: OXO-M $pEC_{50} = 5.75 \pm 0.17$, $E_{max} = 453 \pm 21$; Compound **16** $pEC_{50} = 4.21 \pm 0.18$, $E_{max} = 261 \pm 21$.

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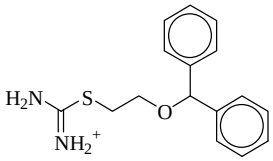
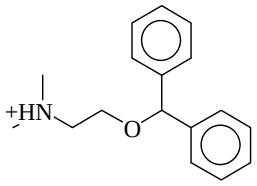
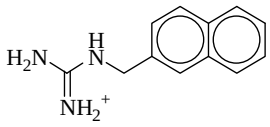
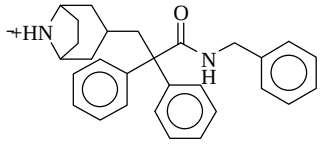
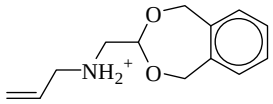
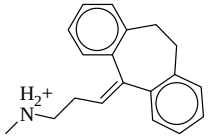
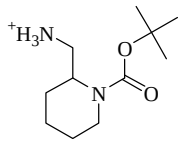
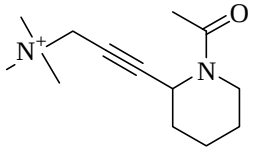
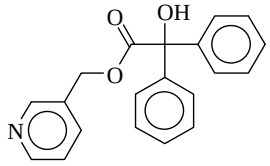
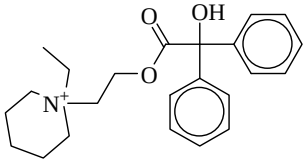
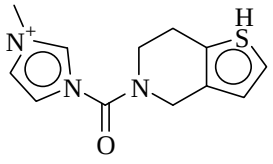
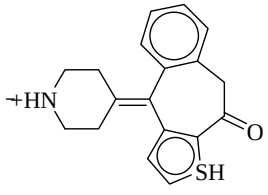
Table 1. Compounds identified by docking to M_2 receptor.

Compound ID (ZINC ID)[a]	Structure	Docking Rank[b]	M_2 K_i ± SEM[c]	M_3 K_i ± SEM	Tc[d]	Closest analog[e]
1 (C30009023)		241[f]	390 ± 32 nM	130 ± 3 nM	0.47	
2 (C01571130)		337[g]	17 ± 2 μM	N.D.[h]	0.25	
3 (C02293082)		379[g]	38 ± 6 μM	N.D.	0.23	
4 (C04202452)		89[g]	39 ± 3 μM	N.D.	0.30	
5 (C13283175)		198[f]	1.2 ± 0.2 μM	360 ± 65 nM	0.42	
6 (C32628700)		100[g]	33 ± 8 μM	N.D.	0.23	

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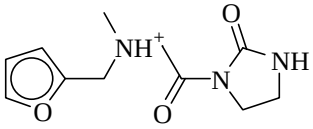
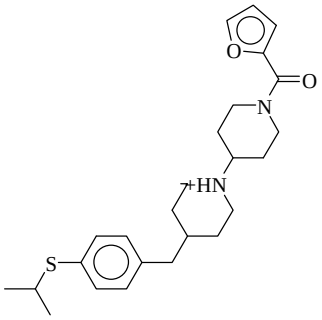
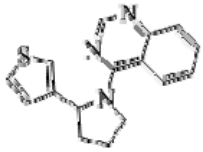
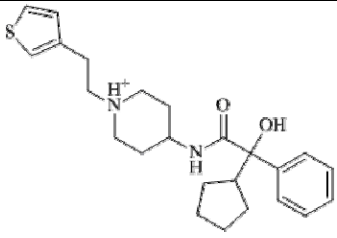
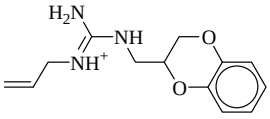
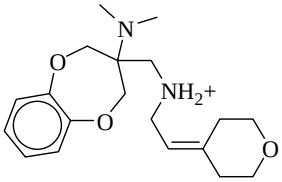
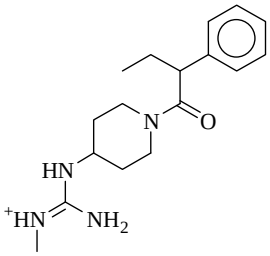
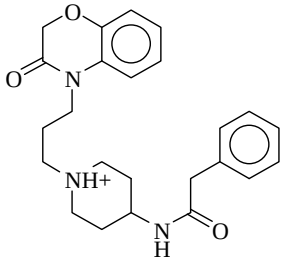
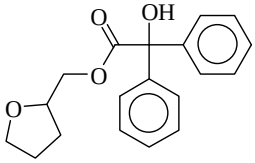
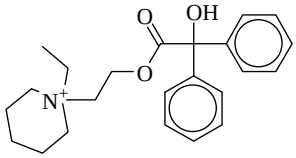
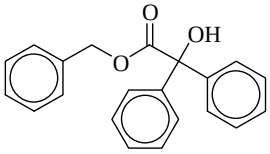
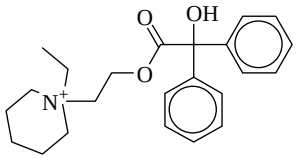
7 (C32810363)		299[g]	21 ± 5 μM	N.D.	0.24	
8 (C48231657)		58[f]	6.6 ± 1.4 μM	1.8 ± 1.3 μM	0.26	
9 (C58162941)		186[g]	22 ± 4 μM	N.D.	0.24	
10 (C58406123)		46[f]	4.7 ± 0.8 μM	5.8 ± 0.5 μM	0.32	
11 (C58857984)		370[f]	2.0 ± 0.1 μM	1.2 ± 0.4 μM	0.46	
12 (C04547851)		Analog of Cmpd. 5	1.6 ± 0.1 μM	290 ± 48 nM	0.49	

[a]From http://zinc.docking.org

[b]Out of 3.1 million fragments and "lead-like" molecules docked to the M^2 receptor.

[c]Values are from a minimum of two independent measurements performed in triplicate

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[d]ECFP4-based Tanimoto coefficient to the most similar muscarinic ligand in ChEMBL.

[e]Most similar molecule in ChEMBL.

[f]Rank among to 2662342 lead-like compounds

[g]Rank among 357594 fragments

[h]N.D. Not determined.

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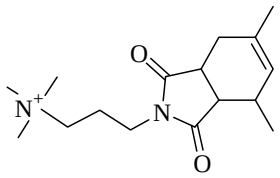
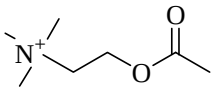
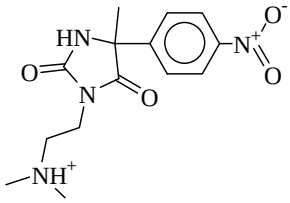
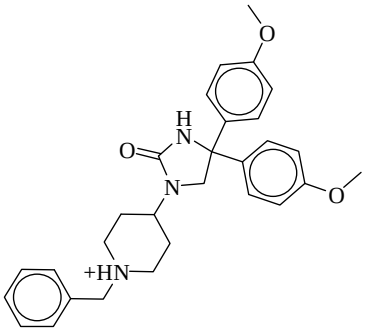
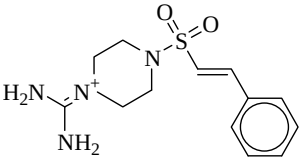
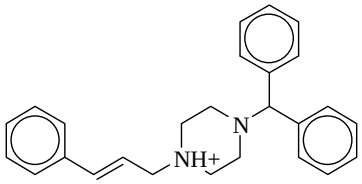
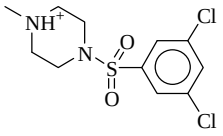
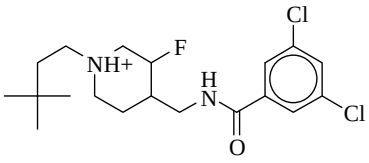
Table 2. Compounds docking well to M_3 receptor and poorly to M_2 receptor.

Compound ID (ZINC ID)	Structure	M_2 K_i ± SEM[a]	M_3 K_i ± SEM[a]	M_3/M_2	M_3 rank/ M_2 rank	T_c	Closest analog
13 (C18061786)		8.2 ± 0.8 μM	1.3 ± 0.1 μM	6.3 fold	2496 / 1238745	0.21	
14 (C00181425)		16 ± 1.6 μM	10 ± 2.9 μM	1.1 fold	3278 / 1018801	0.24	
15 (C06850766)		89 ± 7.7 μM	64 ± 14 μM	1.4 fold	3728 / 1157022	0.29	
16 (C21270353)		>100 μM	>100 μM (K_i) 5.4 ± 2.8 μM (EC_{50})[b]	N/A	4528 / 984037	0.30	

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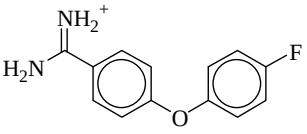
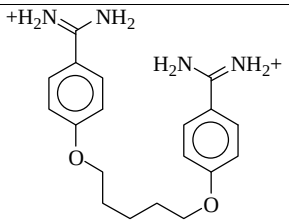
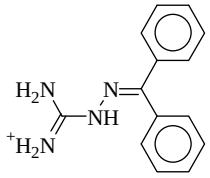
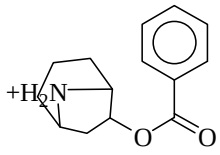
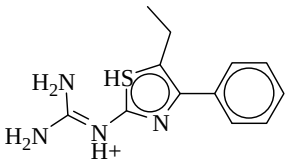
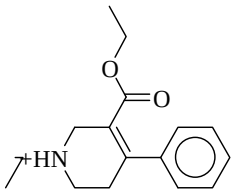
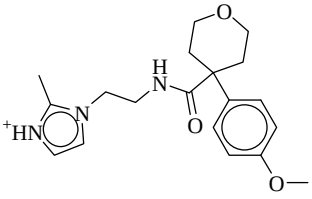
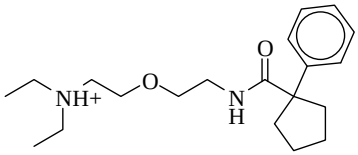
17 (C19866069)		24 ± 2.7 μM	5.1 ± 0.7 μM	4.8 fold	828/ 67487	0.48	
18 (C01694229)		18 ± 1.8 μM	8.8 ± 1.3 μM	2.0 fold	294 / 14466	0.23	
19 (C48433680)		740 ± 37 nM	780 ± 390 nM	1.0 fold	14 / 722	0.25	
20 (C49524426)		1.9 ± 0.5 μM	1.4 ± 0.4 μM	1.4 fold	369 / 471031	0.33	

[a]Values are from a minimum of two independent measurements performed in triplicate

[b]EC_{50} in a cell-based agonism assay (see Methods and Materials).

Figure 1

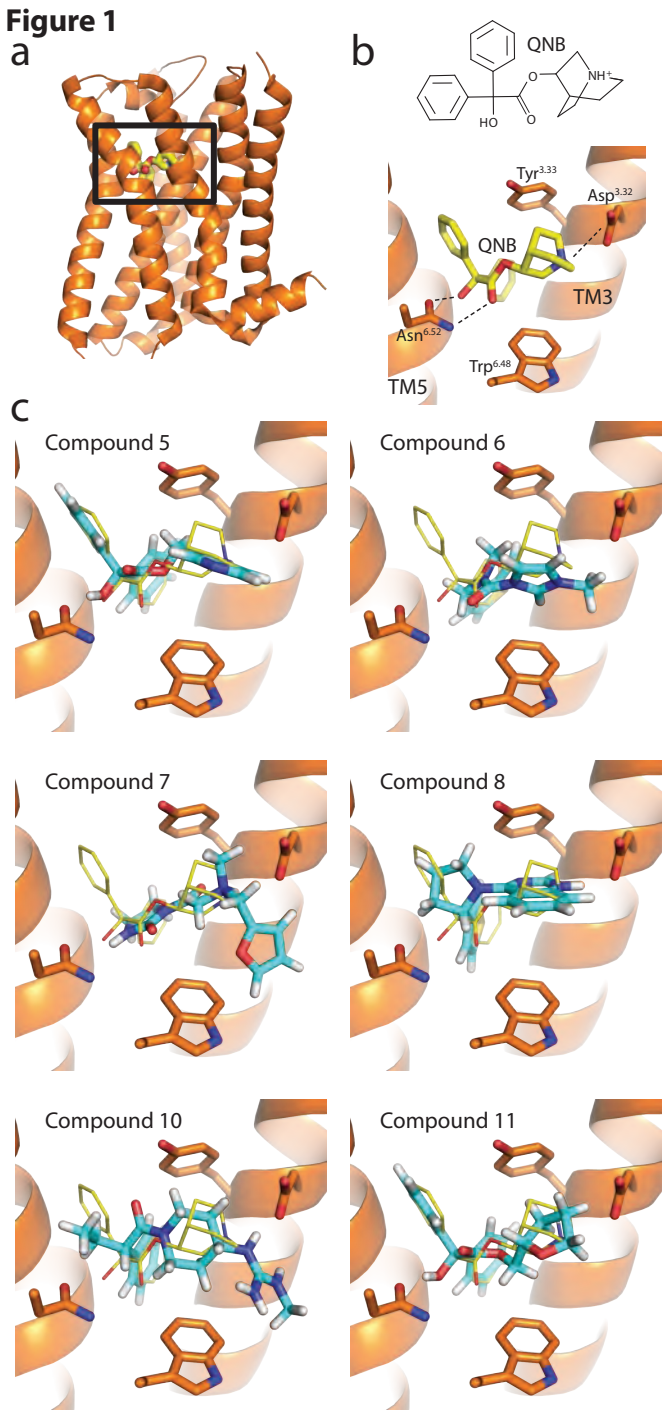

a
b
QNB
NH+
O
HO
O
Tyr3.33
Asp3.32
QNB
TM3
Asn6.52
Trp6.48
TM5
c
Compound 5
Compound 6
Compound 7
Compound 8
Compound 10
Compound 11

Figure 2

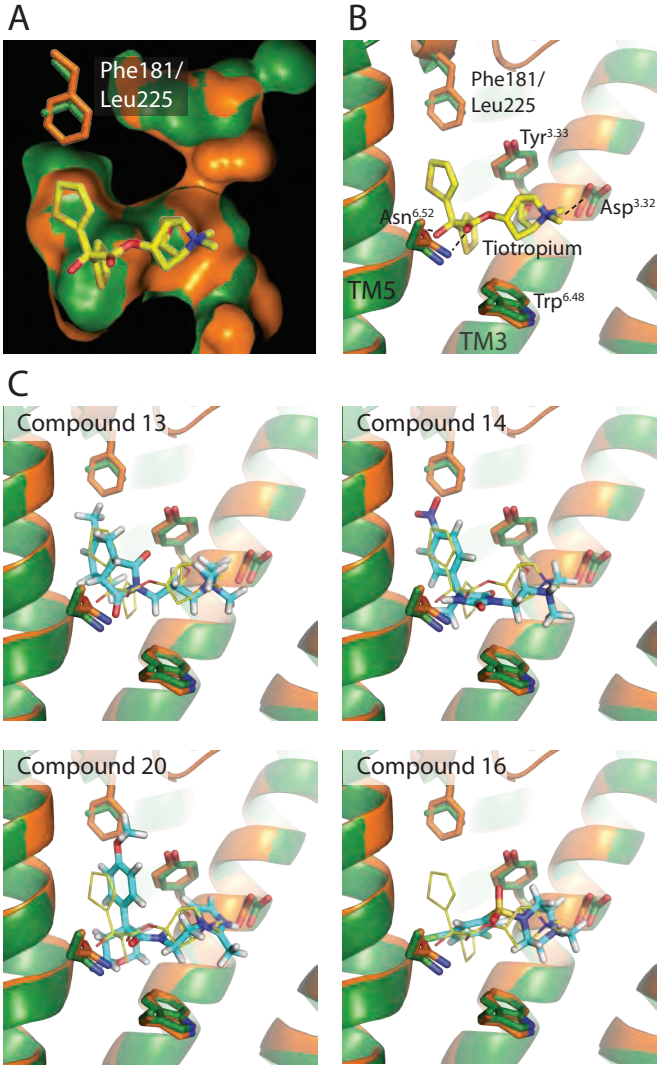

A
Phe181/
Leu225
B
Phe181/
Leu225
Tyr3.33
Asp3.32
Asn6.52
Tiotropium
TM5
Trp6.48
TM3
C
Compound 13
Compound 14
Compound 20
Compound 16

Figure 3

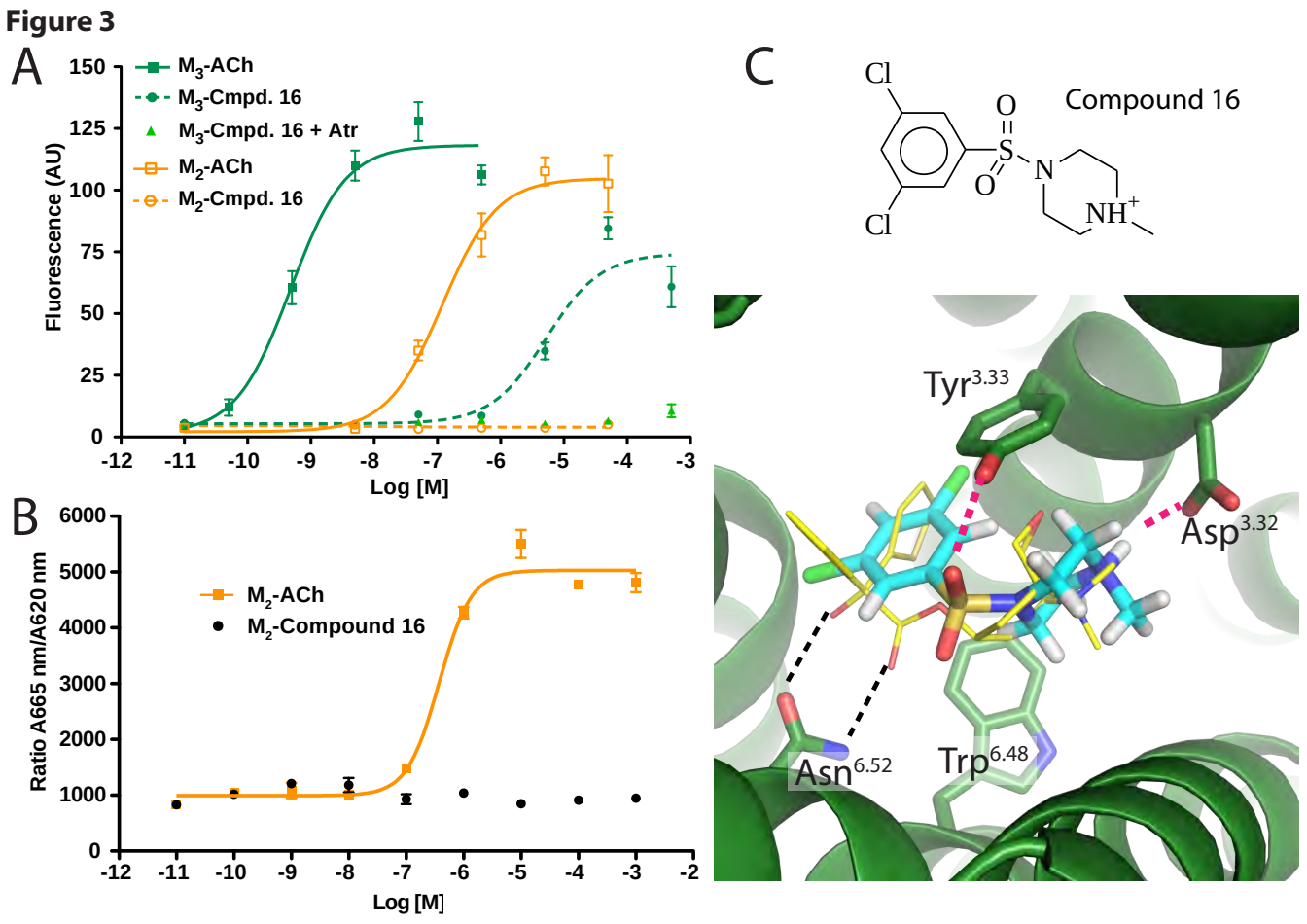
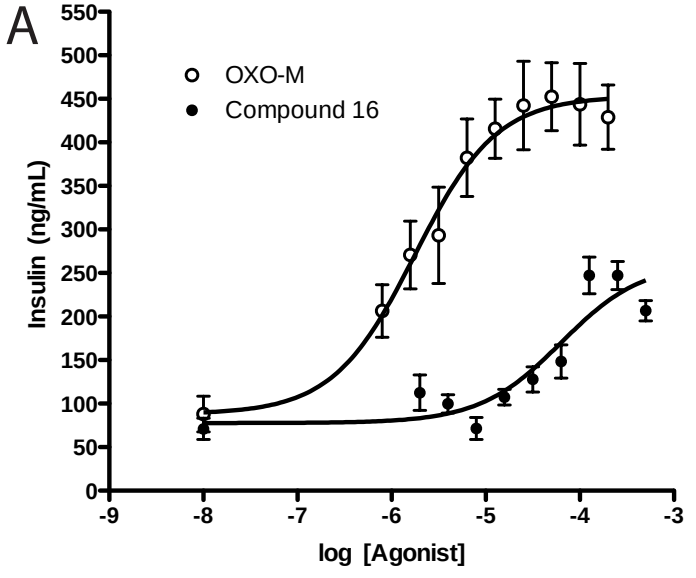
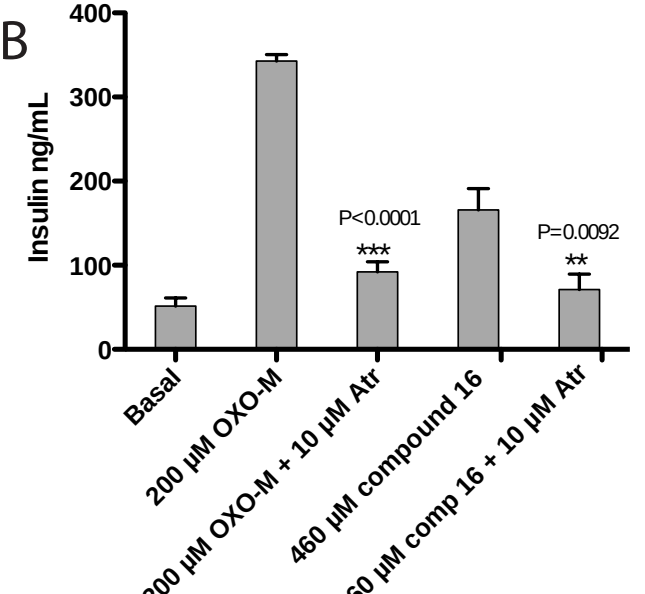

A
M3-ACh
M3-Cmpd. 16
M3-Cmpd. 16 + Atr
M2-ACh
M2-Cmpd. 16
Fluorescence (AU)
Log [M]
B
M2-ACh
M2-Compound 16
Ratio A665 nm/A620 nm
Log [M]
C
Compound 16
Cl
Cl
O
S
O
N
NH+
Tyr3.33
Asp3.32
Asn6.52
Trp6.48

Figure 4


A
OXO-M
Compound 16
Insulin (ng/mL)
550
500
450
400
350
300
250
200
150
100
50
0
-9
-8
-7
-6
-5
-4
-3
log [Agonist]


B
Insulin ng/mL
400
300
200
100
0
P<0.0001
***
P=0.0092
**
Basal
200 µM OXO-M
200 µM OXO-M + 10 µM Atr
460 µM compound 16
460 µM comp 16 + 10 µM Atr