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Structure-based discovery of selective positive allosteric modulators of antagonists for the M₂ muscarinic acetylcholine receptor

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Subtype-selective antagonists for muscarinic acetylcholine receptors (mACHRs) have long been elusive, owing to the highly conserved orthosteric binding site. However, allosteric sites of these receptors are less conserved, motivating the search for allosteric ligands that modulate agonists or antagonists to confer subtype selectivity. Accordingly, a 4.6 million-molecule library was docked against the structure of the prototypical M₁ mACHR, seeking molecules that specifically stabilized antagonist binding. This led us to identify a positive allosteric modulator (PAM) that potentiated the antagonist N-methyl scopolamine (NMS). Structure-based optimization led to compound 628, which enhanced binding of NMS, and the drug scopolamine itself, with a cooperativity factor (α) of 5.5 and a Kᵦ of 1.1 μM, while sparing the endogenous agonist acetylcholine. NMR spectral changes determined for methionine residues reflected changes in the allosteric network. Moreover, 628 slowed the dissociation rate of NMS from the M₂ mACHR by 50-fold, an effect not observed at the other four mACHR subtypes. The specific PAM effect of 628 on NMS antagonism was conserved in functional assays, including agonist stimulation of [³⁵S]GTP⁰S binding and ERK 1/2 phosphorylation. Importantly, the selective allosterity between 628 and NMS was retained in membranes from adult rat hypothalamus and in neonatal rat cardiomyocytes, supporting the physiological relevance of this PAM/antagonist approach. This study supports the feasibility of discovering PAMs that confer subtype selectivity to antagonists; molecules like 628 can convert an armamentarium of potent but nonselective GPCR antagonist drugs into subtype-selective reagents, thus reducing their off-target effects.

G-protein–coupled receptors (GPCRs) are the largest family of cell surface receptors and the target of ~27% of marketed drugs (1). The five subtypes (M₁–M₅) of the muscarinic acetylcholine receptor (mACHR) family exemplify this, modulating physiology relevant to mental health, motion perception, salivation, respiration, and excretion. The mACHRs are the targets of approved and investigational drugs for several debilitating conditions such as psychosis (2), Alzheimer’s disease (3), motion sickness (4), asthma (5), and incontinence (6), among others. Ideally, medicines for treating these diseases should be devoid of adverse effects mediated by interactions with one or more subtypes of mACHR that are not involved in the targeted disorder. Unfortunately, all five mACHRs share high amino acid sequence identities in their orthosteric sites, and current drugs that act at one subtype frequently interact with others (7). As a consequence, off-target but “on-family” effects are a major cause of adverse drug reactions among the mACHRs. For instance, mAChR antagonists such as darifenacin and tolterodine that treat incontinence mediated via the M₃ mAChR often lead to dry mouth due to effects at glandular M₁ and M₃ mAChRs, increase heart rate via the M₂ mAChR, or increase drowsiness (6, 8, 9). Such intrafamily off-target effects for the mAChRs have reduced the usefulness of what are otherwise highly effective medicines.

To overcome intrafamily promiscuity of mAChR orthosteric drugs, investigators have begun to target the allosteric sites of these receptors (10, 11). The best-established of these sites, first identified by functional pharmacology (12–16), have recently been structurally characterized by crystallography (17) and are now known to atomic resolution for most mAChR subtypes (M₁–M₄) (17–19). This classical muscarinic allosteric pocket (17) is located just above the orthosteric hormone binding site and is partially formed by extracellular loops, which show greater sequence variation among the mAChR subtypes than is observed for the orthosteric sites, and thus have become the focus for the discovery of subtype-selective allosteric ligands. Positive allosteric modulators (PAMs) could provide a solution to the promiscuity inherent in mAChR orthosteric antagonists, reducing off-target effects. Accordingly, a large library docking campaign was prosecuted seeking unique positive allosteric modulators (PAMs) for antagonists, ultimately revealing a PAM that substantially potentiates antagonist binding leading to subtype selectivity at the M₂ mAChR. This study supports the feasibility of discovering PAMs that can convert an armamentarium of potent but nonselective G-protein–coupled receptor (GPCR) antagonist drugs into subtype-selective reagents.

Significance

The orthosteric binding sites of the five muscarinic acetylcholine receptor (mACHR) subtypes are highly conserved, making the development of selective antagonists challenging. The allosteric sites of these receptors are more variable, allowing one to imagine allosteric modulators that confer subtype selectivity, which would reduce the major off-target effects of muscarinic antagonists. Accordingly, a large library docking campaign was prosecuted seeking unique positive allosteric modulators (PAMs) for antagonists, ultimately revealing a PAM that substantially potentiates antagonist binding leading to subtype selectivity at the M₂ mAChR. This study supports the feasibility of discovering PAMs that can convert an armamentarium of potent but nonselective G-protein–coupled receptor (GPCR) antagonist drugs into subtype-selective reagents.


Supporting Information

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The authors declare no conflict of interest.

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Conflict of interest.

www.pnas.org/cgi/doi/10.1073/pnas.1718037115
be specific for one subtype over the other four family members and can convert nonselective but otherwise potent orthosteric agonists and antagonists into selective ligands for a particular receptor subtype (13, 20–22).

Here, we investigated the ability of a structure-based approach to discover allosteric molecules that are cooperative with the binding and activity of M2 mAChR antagonists. Antagonists, such as scopolamine and atropine, have long been investigated for the treatment of diseases like motion sickness, depression, and blocking cholinergic bradycardia (4, 23–26), but have been limited by intrafamily off-target adverse reactions. By screening a library of 4.6 million compounds for complementarity to the inactive state of the M2 mAChR, we sought such cooperative modulators for M2 antagonists. Emerging from this screen was a unique family of triazolo-quinazolinolines unrelated to previously investigated chemotypes for this target. The ability of these unique antagonist PAMs to confer target selectivity, probe specificity, and activity in native tissues was investigated.

Results

Structure-Based Docking at the M2 mAChR. Seeking selective PAMs of mAChR antagonists, we docked the 4.6 million-molecule lead-like (27) subset of the ZINC database (28, 29) against the allosteric site observed in the antagonist-bound inactive structure of the M2 QNB (19) complex (PDB ID code 3U0N). This site lies largely above the plane of the membrane, and three tyrosine residues, Tyr104(33), Tyr403(58), and Tyr426(39) (superscripts indicate Ballesteros–Weinstein numbering), separate it from the orthosteric site (Fig. 1A and SI Appendix, Fig. S1A and B). Unlike the orthosteric site, which only differs from the orthologous site of the M3 mAChR by a single residue [Leu220(32)→Phe181(32)], substituents in the vestibule are more common, where two receptors can differ by up to 11 substitutions among the 24 residues that define the site (18, 19, 30, 31) (SI Appendix, Fig. S1 C, D, and G and Table S1). Each ZINC molecule was docked in multiple orientations and conformations to the vestibule; overall, about 1012 molecule–receptor complexes were sampled. Each was scored using the physics-based scoring function in DOCK3.6 (32, 33) that calculates van der Waals (34) and electrostatic complementarity (35–37); the latter is corrected for context-dependent ligand desolvation (30, 32). The best-scoring configuration of each molecule in the library was retained, and the library was ranked from best to worst scoring. The docked molecules tiled the vestibular M2 mAChR allosteric site densely (Fig. 1A).

The top 2,000 docking-ranked compounds (top 0.04% of the docked library) were visually inspected and prioritized, based on features not captured by the DOCK3.6 scoring function (38), such as chemical diversity in addition to their docking rank. Ultimately, 13 compounds were picked as potential ligands for the extracellular loop 2. In addition, the ester moiety of Ile178, potentially stabilizing the position of extracellular loop 2. This creates a four-layered aromatic stacking system that differs from the orthologous site of the M3 mAChR, with its known NAM activity, gallamine substantially reduced the NAM effect on the radioligand. Of the 13 docking hits, 10 did not engage the vestibule the 573 compound stack with Trp177 from extracellular loop 2. This creates a four-layered aromatic stacking system that would wedge the vestibule into an open and inactive conformation (Fig. 1C). Meanwhile, ZINC00350029 engages the same Tyr177ECL2 (Fig. 1B) but does not engage Trp427(35). Additionally, 029 made unique interactions with Asn410(19). Finally, ZINC05277859 docks directly above the three-conserved tyrosines that form a “septum” between the orthosteric and allosteric sites (Fig. 1A). The triazolo-quinazolinolone scaffold of 589 orients to π-stacking with Tyr403(58) or Tyr426(39) (Fig. 1D), while hydrogen-bonding with the backbone of Ile178, potentially stabilizing the position of extracellular loop 2. In addition, the ester moiety of 589 forms a hydrogen bond with the side chain of Asn419ECL2. As shown below, 589 proved to be a PAM for antagonists and was the focus for subsequent structure–activity relationship (SAR) studies.

Receptor Binding of the Initial Docking Hits. The 13 docking hits were purchased for initial experimental testing. Using membranes of CHO cells stably expressing the human M2 mAChR, we assessed the effect of 10 μM concentrations of two well-characterized allosteric modulators, the strong negative allosteric modulator (NAM) of both agonists and antagonists, gallamine, and the weak NAM of antagonists, LY2033298, on the specific binding of 0.2 nM [3H]NMS (antagonist) or 0.05 nM [3H]NMS (agonist) to NMS. Modeled hydrogen bonds and hydrophobic interactions are indicated as dashed lines. The effects on [3H]NMS binding of 10 μM of the 13 initial docking hits. The structures of three active modulators are shown (docking ranks in SI Appendix, Table S2).
More interesting was the activity of ‘S89’, which increased the binding of the radioligand, consistent with its activity as a PAM of the labeled antagonist.

To quantify the effects of ‘S89’ at the M2 mAChR, we performed equilibrium binding assays with increasing concentrations (0.3–100 μM) of ‘S89’ against two orthosteric radioligands that stabilize distinct receptor conformations; 0.2 nM [3H]NMS, an antagonist/inverse agonist favoring the inactive state, and 0.015 nM [3H]peroxo ([3H]IXO), an agonist stabilizing the active state (Fig. 1F). Consistent with the single concentration screen, ‘S89’ increased antagonist binding by ~20%. Using an allosteric ternary complex model (ATCM), we quantified the affinity (pKAI) of ‘S89’ for the allosteric site on the free receptor and its cooperativity (α) with [3H]NMS: pKAI = 5.35 ± 0.27 and LogαNMS = 0.20 ± 0.03 (nNMS = 1.6). Strikingly, when switching the orthosteric probe from antagonist to agonist, ‘S89’ reduced [3H]IXO binding, indicating NAM activity (~50% decrease in binding at the highest concentration tested; Fig. 1F). To investigate this agonist NAM activity of ‘S89’ on cellular function, we examined its effects on the promotion of [35S]GTPγS binding to activated G proteins by the agonist carbachol (CCh); this is a prototypical effect mediated by Gαo-coupled receptors such as the M2 mAChR. Compound ‘S89’ caused a saturable inhibition in CCh’s promotion of [35S]GTPγS binding, a hallmark of a NAM with limited negative cooperativity, that is, Logα ≈ 0.92 ± 0.07 (Fig. 1G). To ensure the effect observed was the direct consequence of a drug–receptor interaction, ‘S89’ was tested for colloidal aggregation (38, 39). Whereas particles were seen at 100 μM ‘S89’, these did not inhibit a classic counterscreening enzyme AmpC β-lactamase, nor was scattering sensitive to detergent, suggesting that the compound was not an aggregator at relevant concentrations.

Structure-Guided Optimization. Using the modeled pose of ‘S89’, we sought to optimize its affinity by substitutions to the triazolo-quinazolinone scaffold, focusing on groups that could potentially interact with the rim of the allosteric site near Asn419ECL3. This region has been implicated by both mutagenesis (40) and by molecular dynamics simulations (17, 41) as important for allosteric modulator binding. Compounds with three different substitutions were picked: (R1) compounds that interacted with the rim of the allosteric site near Asn419ECL3, (R2) compounds that test the docking pose of ‘S89’ by clashing with Tyr85S3, and (R3) variations of the hydrophobic group near the Phe181ECL2. Sixteen triazolo-quinazolinone analogs that docked well or, in the case of the R2 substitutions, docked informatively, were purchased and tested (Table 1 and SI Appendix, Table S3); because this was an “analogy-catalog” exercise, we were not always able to test compounds that measured the effect of one side chain in isolation, as might ordinarily be done in an SAR campaign.

Broadly consistent with these expectations, compounds with larger R1 groups often increased the potency of the PAMS (Table 1). For instance, ZINC12427628 had one of the largest R1 substitutions and displayed the highest affinity (pKAI = 5.85 ± 0.31) while retaining robust positive cooperativity with the antagonist, that is, LogαNMS = 0.73 ± 0.16 (nNMS = 5.4) (Fig. 2A and B and Table 1). Conversely, compounds that eliminate the ester R1-moiety of ‘S89’, such as ZINC6367722, lost most binding cooperativity (SI Appendix, Table S3). Switching from an ester to an amide had little effect on total antagonist binding, as observed with the PAM, ‘621’ (Table 1).

The pose of ‘628’ changed slightly versus ‘S89’, partly reflecting our use of the smaller vestibule present in the 4MQT structure that was used for docking at this stage (Fig. 2 C and D). In the docked pose, the carbonyl oxygen of the R1 moiety appears to bridge Tyr80S3 and Thr425S3, while the amide nitrogen hydrogen bonds with

Table 1. Allosteric effects of triazolo-quinazolinone analogs of [3H]NMS-specific binding at the M2 mAChR

<table>
<thead>
<tr>
<th>ZINC ID</th>
<th>% [3H]NMS binding</th>
<th>ECsp μM</th>
<th>pKα</th>
<th>LogαNMS</th>
<th>ZINC ID</th>
<th>% [3H]NMS binding</th>
<th>ECsp μM</th>
<th>pKα</th>
<th>LogαNMS</th>
</tr>
</thead>
<tbody>
<tr>
<td>12427628</td>
<td>163 ± 11</td>
<td>1.1 ± 0.4</td>
<td>5.85 ± 0.31</td>
<td>0.73 ± 0.16 (5.4)</td>
<td>09635472</td>
<td>133 ± 5</td>
<td>11 ± 3</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>03590563</td>
<td>138 ± 4</td>
<td>2.0 ± 0.7</td>
<td>4.76 ± 0.09</td>
<td>0.59 ± 0.15 (3.8)</td>
<td>03444509</td>
<td>139 ± 11</td>
<td>26 ± 10</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>02651768</td>
<td>146 ± 1</td>
<td>4.8 ± 0.8</td>
<td>5.03 ± 0.18</td>
<td>0.23 ± 0.02 (1.7)</td>
<td>03295621</td>
<td>125 ± 6</td>
<td>&gt;50</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>03245507</td>
<td>141 ± 3</td>
<td>7.1 ± 1.5</td>
<td>5.19 ± 0.15</td>
<td>0.21 ± 0.02 (1.6)</td>
<td>03597405</td>
<td>111 ± 4</td>
<td>&gt;50</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>25339904</td>
<td>146 ± 5</td>
<td>25.0 ± 3.7</td>
<td>4.97 ± 0.14</td>
<td>0.23 ± 0.03 (1.7)</td>
<td>03572779</td>
<td>105 ± 6</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>03320344</td>
<td>118 ± 2</td>
<td>6.7 ± 0.2</td>
<td>ND</td>
<td>ND</td>
<td>03297234</td>
<td>107 ± 3</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>05277589</td>
<td>122 ± 3</td>
<td>21 ± 7</td>
<td>5.35 ± 0.27</td>
<td>0.20 ± 0.03 (1.6)</td>
<td></td>
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</table>

Expansion of the scaffold toward Asn419ECL3 in the allosteric pocket led to the discovery of several unique PAMS on [3H]NMS binding. Particularly, ‘628’, ‘563’, ‘768’, ‘507’, and ‘904’, with 50-100% increase in receptors bound by 0.2 nM [3H]NMS and affinity estimate in the micromolar range. Two-hour radioligand incubation; ND, inactive up to 10 μM. Values represent the mean ± SEM from at least three experiments performed in duplicate. Bold highlight of ZINC ID indicates shorthand used to refer to compounds. The ‘S89’ row is in bold as it was the initial docking hit.
Asn410ECL1/Glu175ECL2. The bulkier phenyl ring of '628 is modeled to be perpendicular to Tyr63ECL2 and the terminal amide substituent, hydrogen bonds with the backbone oxygen of Thr84ECL2 that caps the TM2 helix. In this optimized docking pose, the five-membered ring of the triazolo-quinazolinon scaffold stacks with Trp422ECL3, while the cyclohexane ring is sandwiched between Leu100ECL2 and Tyr226ECL2. Consistent with the steric constraints of the modeled pose, bulky substitutions on the cyclohexane ring at the R3 position result in loss of activity, as with compounds '570 and '567 (SI Appendix, Table S3). Similarly, diminished activity is observed for hydrophobic substitutions that are larger than the original hit at the R3 position, as with compound '094, perhaps caused by steric clashes with the hydrophobic pocket formed by Phe181TM2 and Tyr177ECL2, which in the docking pose of '628 make interaction with the alkene moiety at R3 (Fig. 2 C and D). Mass spectrometry analysis was performed on the purchased '628 compound, indicating that it was pure (SI Appendix, Fig. S2), and subsequent analysis was carried out with this compound.

**The Effect on Orthosteric Inverse-Agonist Kinetics and Function of '628.** A hallmark of allosteric affinity modulators is their ability to change the association or dissociation rates of orthosteric ligands (42). Since '628 increased the affinity of [3H]NMS for the M2 mACHr in equilibrium binding assays, we expected it to alter the dissociation rate of the orthosteric ligands that it modulates. We thus determined the rate of [3H]NMS dissociation, using isotopic dilution with atropine, in the absence or presence of increasing concentrations of '628. As the concentration of '628 was increased, the k<sub>off</sub> of [3H]NMS from the M2 mACHr decreased very substantially (~30-fold), so that by 10 μM '628 the k<sub>off</sub> was increased to 415 min, compared with 8.2 min without the PAM (Fig. 3 A and Table 2). Similarly, in saturation binding assays with [3H]NMS, the affinity (pK<sub>a</sub>) of the antagonist increased with increasing concentrations of modulator, allowing for the determination of a cooperativity factor of Log(1/ν) = 0.73 ± 0.06 (Fig. 3B and Table 2). In contrast, no substantial effect was observed on the affinity of the agonist, [3H]IXO in analogous saturation binding experiments (Fig. 3C), which was observed for the parent compound '589. This identifies '628 as a neutral allosteric ligand (NAL) of IXO, in contrast to its strong PAM activity against the antagonist NMS.

To assess the allosteric effects of '628 on M2 mACHr receptor function, we investigated two distinct signaling pathways: [35S]GTPyS binding as a direct measure of proximal receptor activation, and ERK1/2 phosphorylation as a measure of downstream and convergent activation. Consistent with the observations from the [3H]IXO saturation experiments (Fig. 3C), '628 had no appreciable effect on responses to the endogenous agonist, ACh (Fig. 4 A and B), or to the high efficacy agonist, IXO (SI Appendix, Fig. S3 A and B), confirming its status as a NAL of both agonist function and of agonist binding. This afforded us a rare opportunity to probe allosteric effects on antagonist function without the confounds from agonist modulation. Accordingly, NMS was titrated against a fixed (EC<sub>50</sub>) concentration of the agonist IXO in the absence or presence of increasing concentrations of '628, and effects on [35S]GTPyS binding.
binding (Fig. 4C, Left) and ERK1/2 phosphorylation (Fig. 4D, Left) were measured. The neutral cooperativity between ’628 and IXO meant that any shift in the antagonist (NMS) inhibition curve solely reflected the functional PAM effect of the modulator on NMS. The resulting antagonist potency estimates (pA₂ values) are shown in Table 3; absolute differences between the two pathways most likely reflect differences in the assay conditions. Irrespective, and most importantly, a plot of each NMS pA₂ estimate as a function of ’628 concentration (Fig. 4C and D, Right) fitted for the ATCM allowed for the determination of the functional cooperativity between NMS and ’628, which was essentially identical between the two pathways: [³²S]GTPγS binding, Log₈₀NMS = 0.73 ± 0.19 (αNMS = 5.4); ERK1/2 phosphorylation, Log₈₀NMS = 0.67 ± 0.20 (αNMS = 4.8).

**Probe Dependence of ’628**. A common observation with many GPCR allosteric modulators is their “probe dependence,” where the magnitude and even direction of the allosteric effect can change dramatically for the same modulator/GPCR pair depending on the orthosteric ligand (43). To determine the differential modulation effects on different orthosteric ligands, that is, the “probe specificity” of ’628, we determined its effects on a panel of 17 different orthosteric ligands, including 11 structurally distinct mAChR antagonists, and 6 mAChR agonists of varying degrees of efficacy. All 17 orthosteric ligands were initially assessed in [³²H]NMS radioligand titration assays, with increasing concentrations of ’628 tested against an EC₅₀ concentration of the orthosteric ligand in the presence of [³²H]NMS (Fig. S4 and Table S4).

From these probe dependence experiments, three observations seem noteworthy. First, in addition to NMS, ’628 was a PAM of two other antagonists, atropine and N-desmethyloxpipatropine (NDMC). The effect on atropine is perhaps unsurprising as it closely resembles NMS. Conversely, several profound functional effects from small chemical changes in the orthosteric probe molecules were unanticipated: thus, ’628 is a NAM for clozapine itself, and for tiotropium or ipratropium, for which ’628 has negligible binding effects, notwithstanding its strong effects on the related NMS and atropine (Fig. 5A). A second important point is that ’628 retained its NAL, or at least nonaffecting, properties for agonists irrespective of the ligand [we infer that ’628 is a NAL for agonist as is precursor, ’589, inhibited agonist radioligand binding affinity as a NAM (Fig. 1F), although we cannot fully discount the possibility that ’628 simply does not bind to receptors in the activated state for most agonists]. Third, ’628 was a NAL for most of the other antagonists tested, such as 4-DAMP, ONB, pirenzepine, tiotropium, glycopyrrolate, and ipratropium, most of which are structurally distinct. Intriguingly, ’628 had profound NAM activity against himbacine or clozapine. Indeed, the negative cooperativity with himbacine was so pronounced that the interaction was indistinguishable from competition (SI Appendix, Table S4). This observation may be reconciled with himbacine’s ability to bind to both the allosteric and orthosteric sites (44). For three of the antagonists—atropine, for which ’628 acted as a PAM, and himbacine or clozapine, for which ’628 acted as a strong NAM—probe dependence was further tested in functional titration assays, again using [³²S]GTPγS binding and ERK1/2 phosphorylation (Fig. 5 B and C and SI Appendix, Fig. S5). Here, the type and magnitude of the functional cooperativity for the three antagonists reflect the observations made in the initial characterizations of the probes in the [³²H]NMS binding assay. Fig. 5D summarizes the 17 ligands investigated, their structures, and the type of modulatory effect displayed by ’628.

**NMR Spectra Support ’628s Probe-Dependent Allosteric Function**. Solution NMR spectroscopy, using methionine residues as conformational probes, is used to identify structural changes in the M₂ mAChR that may be used to understand the probe dependence via differential ligand coupling (Fig. 6A). For example, the NMR spectra reveal that tiotropium (Fig. 6B) and NMS (Fig. 6C) stabilize distinct conformations, in agreement with their different functional responses to ’628. The incubation of ’628 together with NMS caused chemical shifts in spectra for four M₂ mAChR methionine residues: Met72¹⁻, Met114³⁻, Met202⁵⁻, and Met406⁶⁻ (Fig. 6 D and E). Two of these methionines,
Met77\textsuperscript{2-58} and Met406\textsuperscript{6-54} are located on the extracellular side of the receptor on TM2 and TM6 (Fig. 6f). The change in the environment of the Met406\textsuperscript{6-54} is likely due to its interaction with the side chain of Trp22\textsuperscript{2-35}, which is predicted to stack with the triazole-quinoxaline moiety of NMS (Fig. 6g). Furthermore, the coinduction of NMS with NMS induces a strong and well-defined Met77\textsuperscript{2-58} peak compared with the antagonist alone (Fig. 6e). The shift of Met77\textsuperscript{2-58} may reflect changes of the environment of Tyr80\textsuperscript{2-61} and Tyr83\textsuperscript{2-64} that are located on the same face of the methionine rod, in the docking pose, are predicted to interact with NMS (SI Appendix, Fig. S6). Importantly, Met77\textsuperscript{2-58} is located at the interface of TM2/TM3/TM7, and mutagenesis of the tyrosine residues suggests that this network is key to the cooperativity between allosteric and orthosteric compounds (18). Compound \textsuperscript{628} additionally stabilizes changes in two methionine residues toward the intracellular part of the receptor, Met112\textsuperscript{2-41} and Met202\textsuperscript{3-54} (Fig. 6f). Here, \textsuperscript{628} appears to enhance the capacity of NMS to stabilize the conformational changes of the TM3 hinge (45). This is supported by the appearance of a single Met112\textsuperscript{2-41} peak, indicating a more uniform conformation of TM3, compared with NMS bound alone (Fig. 6e). Although \textsuperscript{628} displays little influence on Met202\textsuperscript{3-54} when coadministered with the potent inverse agonist tijotropium (Fig. 6b), the PAM significantly shifts the Met202\textsuperscript{3-54} NMS peak (Fig. 6e), coincidentally toward the position of tijotropium-bound state. It is possible that these spectral changes reflect the capacity of \textsuperscript{628} to enhance NMS-mediated stabilization of the inactive conformation of the receptor (Fig. 6h). Together, these data suggest that the spectral modification of the methionines by \textsuperscript{628} reflects changes in the structure and the dynamics of the allosteric network as well as the G-protein-coupling domain, which might account for the affinity and efficacy modulation of \textsuperscript{628} in NMS.

**Table 3. Affinity estimates (pA\textsubscript{2} values) of NMS in functional assays in absence or presence of \textsuperscript{628} at the human M\textsubscript{2} mAChR.**

<table>
<thead>
<tr>
<th>NMS alone</th>
<th>+0.2 μM \textsuperscript{628}</th>
<th>+0.5 μM \textsuperscript{628}</th>
<th>+1.0 μM \textsuperscript{628}</th>
<th>+3.0 μM \textsuperscript{628}</th>
<th>+10.0 μM \textsuperscript{628}</th>
</tr>
</thead>
<tbody>
<tr>
<td>pA\textsubscript{2}</td>
<td>9.47 ± 0.16</td>
<td>10.24 ± 0.16</td>
<td>10.43 ± 0.15</td>
<td>10.51 ± 0.15</td>
<td>10.76 ± 0.18</td>
</tr>
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\(\text{pA}_2\) values: Negative logarithm of the antagonist potency value for inhibiting 50\% of the response to an EC\textsubscript{50} concentration of IXO.

**PAM Effect of \textsuperscript{628} on Native Tissue Membranes.** To determine the utility of \textsuperscript{628} as a probe in physiological systems, we examined the effect of \textsuperscript{628} on an endogenous ligand (ACh) and a commonly used potent agonist (IXO) in functional assays. The effect of high concentrations (3 and 10 μM) of \textsuperscript{628} was tested on both ACH-mediated (Fig. 4b and SI Appendix, Fig. S3 C, E, G, and J) or IXO-mediated (SI Appendix, Fig. S3 B, D, F, H, and J) ERK1/2 phosphorylation at M\textsubscript{1-5} mAChRs, and no significant effects were observed at any of the receptors. These findings suggest either a lack of interaction of \textsuperscript{628}, or a NAM effect on endogenous signaling at all of the mAChR subtypes, making \textsuperscript{628} an excellent tool compound to probe antagonist action in physiological systems.

To investigate the potential physiological relevance of the PAM effects of \textsuperscript{628} on M\textsubscript{2} mAChR antagonists, we determined the effects of the modulator in the absence or presence of NMS on agonist-mediated \[^{[35]}\text{S}\]GTP\textsubscript{S} binding using membranes derived from rat hypothalamus and neonatal rat ventricular cardiomyocytes, which both natively express high levels of M\textsubscript{2} mAChRs (46, 47). We investigated the potentiation of NMS antagonism by \textsuperscript{628} using both the potent agonist IXO in rat hypothalamic membranes (Fig. 8a), and on \textsuperscript{628}’s potentiation of the same antagonist against the endogenous neurotransmitter, ACh, in neonatal rat ventricular cardiomyocytes (Fig. 8b). In the hypothalamic membranes with IXO, \textsuperscript{628} potentiated NMS potency with a cooperativity of Log\textsubscript{10}NMS = 1.10 ± 0.31, while in cardiomyocytes the cooperativity was Log\textsubscript{10}NMS = 0.56 ± 0.42.

**Fig. 5.** Probe dependence of \textsuperscript{628} with a panel of agonists and antagonists. (A) Cooperativity estimates of \textsuperscript{628} with each indicated ligand determined using \[^{[35]}\text{H}\]NMS equilibrium binding assays (complete dataset shown in SI Appendix, Fig. S4). Functional cooperativity estimates of \textsuperscript{628} with selected antagonists determined in (B) \[^{[35]}\text{S}\]GTP\textsubscript{S} binding assays or (C) ERK1/2 phosphorylation assays. Full dataset shown in SI Appendix, Fig. S5. (D) Chemical structures of all ligands investigated and their classification in terms of the allosteric effect induced by \textsuperscript{628} at the M\textsubscript{2} mAChR.
Encouragingly, and despite species effects that are common for allosteric ligands, no substantial difference was observed in the superposition of the different spectral shifts for tiotropium (cyan) or tiotropium incubated with '628 (green). Different spectral shifts of the apo spectra (black) with (C) NMS alone (green), (D) with NMS coincubated with allosteric compound '628 (purple), or (E) the latter two together. (F) The M2 mAChR indicating the location of the four methionines augmented by '628 when coincubated with NMS (active [blue]), inactive (orange) structure and agonist/PAM (yellow); PDB ID codes 4mq, 4mqt, and 3uon, respectively with close-up for (G) Met605, 606, 607, and Met202; and (D) Met202B/C provided.

**Discussion**

Two key observations emerge from this study. First, allosteric sites in GPCRs can be targeted by structure-based, large library screens. For example, a recent virtual screen of the National Cancer Institute diversity library against the M1 mAChR found two novel allosteric ligands, NSC-322661 and NSC-13316 (20), but these molecules are also active against nine other GPCRs (i.e., NPY-Y1, NPY-Y2, GPR7, OXTR, MOR, DOR, 5HT5A, D1DR, S1P4, and even the M1 mAChR). Conversely, not only are '589 and '628 dissimilar to other mAChR ligands, they have not been characterized as ligands for any other target in ZINC or ChEMBL. The antagonist PAM '628 thus has promise as a specific tool compound for the M1 mAChR antagonists that it potentiates: NMS, atropine, and NDMC.

Fig. 6. The coincubation of '628 with NMS resulted in spectral shift of four methionine residues of the M1 mAChR: Met112.58, Met113.41, Met202.54, and Met606.54. (A) Chemical shifts for five methionines of the apo M1 mAChR are shown (Met112.58, Met113.41, Met143.50, Met202.54, and Met606.54). (B) The superposition of the different spectral shifts for tiotropium (cyan) or tiotropium incubated with '628 (green). Different spectral shifts of the apo spectra (black) with (C) NMS alone (green), (D) with NMS coincubated with allosteric compound '628 (purple), or (E) the latter two together. (F) The M2 mAChR indicating the location of the four methionines augmented by '628 when coincubated with NMS (active [blue]), inactive (orange) structure and agonist/PAM (yellow); PDB ID codes 4mq, 4mqt, and 3uon, respectively with close-up for (G) Met605, 606, 607, and Met202; and (D) Met202B/C provided.

**Far removed from widely used reagents like BPQA and LY2033298, and even medicines like cinacalcet (57, 58). Second, antagonist PAMs can confer selectivity on orthosteric drugs that would otherwise lack it (7). Thus, by itself, scopolamine binds with similar affinity to all five receptor subtypes (K_i 0.4–2.1 nM) (24). Exploiting the specificity potential of the allosteric site, a PAM like '628, which on its own has no detectable signaling effect nor, crucially, does it modulate agonists, preferentially enhances antagonist binding at M1 mACh over the other receptor subtypes. This suggests a general strategy to confer selectivity onto potent but nonselective GPCR orthosteric drugs.

Although the sequence variability in the extracellular allosteric sites of the mAChRs makes them good targets for selective targeting in principle, the sites nonetheless present druggability challenges. In the inactive state, the allosteric sites are more open to solvent and less sterically defined than the orthosteric sites, as supported by the fact that prior, empirically discovered, inactive-state modulators, such as gallamine, alcuronium, and W-84 (41, 59), are often large and occasionally floppy. Even here, these challenges are reflected in the relatively high molecular weights of the antagonist PAMs that emerged, and their still modest affinities. We suspect that this will be often true for GPCR allosteric sites—both in the extracellular vestibule that we have targeted here (17), and in the sites emerging from new crystal structures (60–66). While GPCR allosteries presents genuine opportunities for conferring selectivity and for compounds that lack the tonic liabilities of orthosteric-active molecules, allosteric sites may often be more challenging for identifying ligands with good physical properties and affinities, antagonists, and to optimize them without new synthesis, suggests that these sites remain accessible to structure-based discovery.

An important feature of these allosteric modulators is their chemical novelty—they do not resemble any known mAChR ligand chemotype for any subtype of which we are aware. Neither the original lead '589, nor the optimized analog, '628, display more than 0.28 EFCP4 Tanimoto coefficient (Tc) similarity to any mAChR ligand in ChEMBL (6,780 compounds both active and inactive), supporting the novelty of the triazolo-quinazolinones. This reflects the value of large library screens, especially compared with smaller chemical library screens targeting the same well-studied family. For example, a recent virtual screen of the ~1,600 compound National Cancer Institute diversity library against the M1 mAChR found two novel allosteric ligands, NSC-322661 and NSC-13316 (20), but these molecules are also active against nine other GPCRs (i.e., NPY-Y1, NPY-Y2, GPR7, OXTR, MOR, DOR, 5HT5A, D1DR, S1P4, and even the M1 mAChR). Conversely, not only are '589 and '628 dissimilar to other mAChR ligands, they have not been characterized as ligands for any other target in ZINC or ChEMBL. The antagonist PAM '628 thus has promise as a specific tool compound for the M1 mAChR antagonists that it potentiates: NMS, atropine, and NDMC.

**Fig. 7.** Subtype selectivity of PAM '628 for [3H]NMS at the M1 mAChR over M2, M3, M4, and M5. Increasing concentrations of modulator '628 were incubated at room temperature for 16 h with membranes from CHO cells expressing M1–M3 mAChR subtypes at a single concentration of [3H]NMS at the K_D concentration for the receptor subtype. Specific binding was measured, and curves were fit using GraphPad Prism to determine the E_C50 and maximal stimulation values for '628.
We do not claim to have fully explored the M1 mAChR. The M1 mAChR structure complexed with IXO and on other receptors, its physical properties may not be optimal for use as an in vivo probe. Also, it would be important to counter-screen the molecule for off-target effects from outside the muscarinic GPCR family. This can be done by testing activity against GPCR panels (69), and kinase (68) panels, as well as against side-effect target panels (69). Even wider nets for off-targets may be cast computationally (70)—all of these screens can help reduce the likelihood that a biological effect of a compound like IXO is mediated by an unexpected target, which would reduce its reliability as a probe. Other than testing against muscarinic receptor subtypes, none of these off-target tests have been conducted here. A second caveat is that when a molecule like IXO is used to confer specificity on a second, orthosteric ligand like NMS that ordinarily would be nonspecific, concerns of differential metabolism of the two molecules can arise—this is most pressing for in vivo uses of the combination. Finally, whereas the methionine NMR supports the binding of IXO in the extracellular vestibular allosteric site of the M1 mAChR, the atomic resolution accuracy of the docking models remains to be fully tested.

These caveats should not obscure the main observations of this study. Despite sites that are admittedly more challenging than many GPCR orthosteric sites, the extracellular vestibules of mAChRs remain accessible to structure-based discovery. In large library docking screens it is possible to find unprecedented scaffolds for these sites that can be optimized to a level of subtype selectivity inaccessible to most orthosteric antagonists. Through cooperativity with such (clasically nonselective) orthosteric antagonists, these PAMs can confer selectivity on otherwise potent and highly efficacious drugs. Importantly, the optimized modulator, IXO, consistently acted as an antagonist PAM while an agonist NAL at human and rodent M1 mAChRs, in native tissues, and across multiple assays. Thus, the effect is robust to assay and to species variation, which has not always been true for allosteric modulators. This suggests a general strategy for conferring selectivity to orthosteric drugs of the family A GPCRs, especially those older therapeutics that often suffer from intrafamily off-target effects but are otherwise potent and efficacious therapeutics.

Materials and Methods

See the SI Appendix for data analysis.

Molecular Docking Screen. We used the inactive state structure of M1 mAChR in complex with QNB (PDB ID code 3UON). The receptor was prepared for docking by keeping just the M1 residues (residues 20-48, 56-124, 135-210, and 384-444), while removing residues in the intracellular section that encompass the T4 lysosome used to facilitate crystallization. All water molecules, ions, and the orthostatic ligand were removed. To indicate the position of the allosteric binding site, an input xtal-ligand was created by (i) placing two phenyl rings in perfect σ stacking distance and (parallel face-centered and perpendicular y-shaped) from Tyr17°C12, (ii) placing a naphthalene structure parallel to Trp422-C35 and a phenyl ring in perpendicular t-shaped stacking conformation, and (iii) placing one phenyl ring in π-π interaction with Thr182-C40 and α-alkyl interaction with Val408-C57 and Ala184-C37. These atoms were used as the input into the SPHGEN program (71) to calculate a 60 spheres set that represent the allosteric site. This matching sphere set was later used to superimpose compounds from the virtual screening library and generate ligand poses. Following this, the automatic target preparation script was run to prepare the receptor (72). More specifically, the receptor polar atoms were protonated using REDUCE (73); however, the side chains were restricted to the original rotamer orientations with flipping turned off. To calculate the grid maps for scoring, three programs were used: CHEMGRID (34) was used to generate the van der Waals complementarity maps using the united-atom AMBER force-field (74); QNIFT (35) was used, which implements the Poisson-Boltzmann equation to generate electrostatic grids; and SOLVMAP (32) was used to generate the ligand desolvation grid. Over 4.6 million commercially available lead-like molecules (xlogP ≤ 3.5; molecular weight ≤ 350 amu; and ≤ 7 rotatable bonds) (28) were docked using DOCK3.6 (32, 33, 75). Each compound was sourced from the ZINC database (76), which stores precalculated conformations and grids for flexible ligand docking. Ligands were matched in all orientations within the allosteric site that allow for four-point superposition of the rigid fragment onto the matching sphere set. For each compound, only a single top scoring pose was retained based on the following parameters that are considered optimally electrostatic and van der Waals complementarity, and corrected for ligand desolvation. The parameters used for docking were as follows: receptor and ligand bin sizes of 0.4 Å, an overlap of 0.1–0.2 Å, a bump allowance of 1, a distance tolerance of 1.5 Å, labeled matching turned on, and 250 cycles of rigid-body minimization. From the top 2,500 scoring molecules, any compounds extending beyond the allosteric vestibule was omitted (Fig. 1A, cyan surface). Next, all other compounds were visually inspected; molecules with unsatisfied polar interactions, or with less diversity, were removed. Finally, 38 compounds were chosen for the hit picking party, from which 13 compounds were purchased for testing.

For docking of the analog-by-catalog compounds, DOCK3.7 (37) was used with both the inactive (PDB ID code 3UON) and active structures (PDB ID code 4MQT) of M1 mAChR. The M1 mAChR inactive structure was prepared for docking as previously described; however, the matching sphere set was used as the xtal-ligand input. The active M1 mAChR structure complexed with IOX and LY2119620 was prepared using residues 20–214 and 378–456 for target. Furthermore, the orthosteric ligand (agonist), IXO, was retained as a coligand during docking and was prepared using PRODRG server (77), while the allosteric site was used as the xtal-ligand. Based on the docking poses of the available analogs in the ZINC database, 16 compounds were chosen for further investigation (Discussion, Table 1, and SI Appendix, Table S2).

The two NAM compounds were purchased from Specs (catalog no. AE-848/42025900) (7029) and from Vitas-M (catalog no. STK616972), while the PAM 589 was acquired from Enamine (catalog no. Z324823878). The purity of the most efficacious PAMs, 563 (Enamine; catalog no. Z16439559) and 589 (Enamine; catalog no. 16439767), was determined by mass spectroscopy (SI Appendix, Fig. S2), indicating that both compounds were >98% homogeneous by weight.

Colloidal Aggregation. Molecules were tested for colloidal aggregation by measuring scattering by dynamic light scattering (DLS) and by measuring nonspecific enzyme inhibition in an AmpC β-lactamase counterscreen (38, 39, 78, 79). Concentrations from 25 to 100 µM were tested for 589 and 628. At concentration above 25 µM IXO in 10 mM Hepes, pH 7.5, and 1% DMSO, the solutions had to be heated to 42 °C for IXO to dissolve the compound. Additives, such as PEG-300 and solutol, can be used to solubilize the compound above 100 µM. AmpC β-lactamase counterscreen with 589 and 628 concentrations of up to 100 µM retained enzyme activity of above 90%.
The human MmAChR construct M2R was designed to be used as a model for studying the interaction of muscarinic acetylcholine receptors (mAChRs) with allosteric ligands. We thank Dr. Anat Levit for fruitful discussions and support.

mAChRs bound to 3H[NMS] were used as a reference for the following procedure. All ligands were dissolved in purified dimethyl sulfoxide (DMSO). NMS or tiotropium was added to the receptor at a saturation concentration of 1 mM. The 1H-13C heteronuclear single quantum coherence (HSQC) spectra of M2 mAChRs bound to either antagonist were collected. After the NMR experiments in scopolamine- or tiotropium-bound states, the sample was added to the antagonist-bound state to a final concentration of 250 μM, and the H-13C HSQC spectra were further collected. The total collection time for each sample experiment was around 10 h. All NMR spectra were processed using the software package NMRPipe (80) and visualized using the program NMRView.

Radioiodination Protocols. In our original biological screen to validate our VLS method, cell membranes from CHO cells expressing M2 mAChR were labeled with 125I (3.7 mCi/mmol) and incubated for 1.5 h at 25 °C. The activity of the untreated control was used as a reference value for the following procedures. All ligands were dissolved in DMSO and incubated for 6 h at 25 °C with 0.2 nM [3H]NMS, atropine, himbacine, or tiotropium. Then, [3H]GTPγS and GDP were added to get final concentrations of 0.1 nM [3H]GTPγS and 30 μM GDP and then incubated for 1 h at 30 °C. Samples were washed, harvested, and counted. Figures show the combined results from three separate experiments, performed in duplicate.

Extracellular Signal-Regulated Kinase 1/2 Phosphorylation Assays. Initial ERK1/2 phosphorylation time course experiments were performed to determine the time at which ERK1/2 phosphorylation was maximal after stimulation by each ligand. Cells were seeded into transparent 96-well plates at 20,000 cells per well and grown for over 8 h. Cells were then washed once with PBS and incubated in serum-free DMEM at 37 °C overnight to allow FBS-stimulated phosphorylated ERK1/2 levels to subside. Cells were then stimulated for 25 min without or with antagonist, followed by a 5-min agonist incubation at 37 °C in 5% CO2. For all experiments, 10% (vol/vol) FBS was used as a positive control, and vehicle controls were also performed. The reaction was terminated by removal of drugs and lysis of cells with 100 μL of SureFire lysis buffer (TGR Biosciences), and 5 μL of this lysate was added in a 384-well white ProxiPlate (PerkinElmer). A mixture of SureFire activation buffer, SureFire reaction buffer, and AlphaScreen beads was prepared in a ratio of 100:600:3 (vol/vol/vol) and added to the lysate for a lysis/mixture ratio of 5:8 (vol/vol). Plates were incubated for 1–1.5 h at 37 °C before the fluorescence signal was measured on a Fusion-xl plate reader (PerkinElmer) using standard AlphaScreen settings.

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