Structure-based discovery of selective positive allosteric modulators of antagonists for the M<sub>2</sub> muscarinic acetylcholine receptor

Magdalena Korczynska<sup>a,1</sup>, Mary J. Clark<sup>b,1</sup>, Celine Valant<sup>c,1</sup>, Jun Xu<sup>d</sup>, Ee Von Moo<sup>e</sup>, Sabine Albold<sup>f</sup>, Dahlia R. Weiss<sup>f</sup>, Hayarpi Torosyan<sup>g</sup>, Weijiao Huang<sup>h</sup>, Andrew C. Kruse<sup>i</sup>, Brent R. Lyda<sup>j</sup>, Lauren T. May<sup>k</sup>, Jo-Anne Baltos<sup>d</sup>, Patrick M. Sexton<sup>b</sup>, Brian K. Kobilka<sup>d,e</sup>, Arthur Christopoulos<sup>c,2</sup>, Brian K. Shoichet<sup>h,k</sup>, and Roger K. Sunahara<sup>b,2</sup>

<sup>a</sup>Department of Pharmaceutical Chemistry, University of California, San Francisco, CA 94158; <sup>b</sup>Department of Pharmacology, University of California San Diego School of Medicine, La Jolla, CA 92039; <sup>c</sup>Drug Discovery Biology, Monash Institute of Pharmaceutical Sciences, Monash University, Parkville, VIC 3052, Australia; <sup>d</sup>Beijing Advanced Innovation Center for Structural Biology, School of Medicine, Tsinghua University, 100084 Beijing, China; <sup>e</sup>Department of Molecular and Cellular Physiology, Stanford University School of Medicine, Stanford, CA 94305; and <sup>f</sup>Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA 02115

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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 M<sub>2</sub> 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 K<sub>θ</sub> 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 M<sub>2</sub> 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]GTPγS 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.

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 M<sub>2</sub> 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.


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1. M.K., M.I.C., and C.V. contributed equally to this work.
2. To whom correspondence may be addressed. Email: arthur.christopoulos@monash.edu, bschoi@gmail.com, or rsunahara@ucsd.edu.

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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-quinazoliones 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. Selecting 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 QNZ (19) complex (PDB ID code 3U0N). This site lies largely above the plane of the membrane, and three tyrosine residues, Tyr104D, Tyr403F, and Tyr426H (superscripts indicate Ballesteros–Weinstein numbering), separate it from the orthosteric site (Fig. 1A and SI Appendix, Fig. S1 A and B). Unlike the orthosteric site, which only differs from the orthologous site of the M3 mAChR by a single residue [Leu226C → Phe181C, L226C], substrates 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 192 molecules–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 allostic site densely (Fig. L4).

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 makes unique combinations of interactions with the site (Fig. 1B–D). What turned out to be the three active molecules exemplify the different docked geometries and interactions. ZINC00085733 stacks with Trp4227.35, a residue that changes rotamers between the agonist versus the PAM/agonists (LY2119620) or antagonist-bound receptor structures (17), and on the other side of the vestibule the 5733 compound stacks with Tyr177 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, ZINC00350290 engages the same Tyr177ECL2 (Fig. 1B) but does not engage Trp4227.35. Additionally, 029 made unique interactions with Asn410ECL3. Finally, ZINC05775899 docks directly above the three-conserved tyrosines that form a “septum” between the orthosteric and allostic sites (Fig. 1A). The triazolo-quinazoline scaffold of 589 orients to π-stack with Tyr403D or Tyr426H (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 Asn419ECL3. 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 [3H]NMS, comparing their effects to that of the 13 docking hits (Fig. 1E). Consistent with its known NAM activity, gallamine substantially reduced the specific binding of [3H]NMS, whereas LY2033298 had a small NAM effect on the radioligand. Of the 13 docking hits, 10 did not alter the specific binding of [3H]NMS and were not further considered. Conversely, three of them, 029, 573, and 589 modulated [3H]NMS binding (Fig. 1B–E). Both 029 and 573 reduced [3H]NMS binding, suggesting that these were [3H]NMS NAMs.

![Image](https://www.pnas.org/cgi/doi/10.1073/pnas.1718037115)
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 0.05 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 (pKd) of °S89 for the allosteric site on the free receptor and its cooperativity (a) with [3H]NMS: pKd = 5.35 ± 0.27 and LogKd = 0.20 ± 0.03 (a = 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 Gbγ2,-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, Loga = 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 counter-structure 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 Asn419EC1. 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 Asn419EC1, (R2) compounds that test the docking pose of °S89 by clashing with Tyr83Z64, and (R3) variations of the hydrophobic group near the Phe181EC1. 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 “analog-by-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 (pKd = 5.85 ± 0.31) while retaining robust positive cooperativity with the antagonist, that is, LogKd = 0.73 ± 0.16 (a = 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 Tyr80, Thr425, and Thr425, while the amide nitrogen hydrogen bonds with

| Table 1. Allosteric effects of triazolo-quinazolinone analogs of [3H]NMS-specific binding at the M2 mAChR |
|-----------------------------------|--------|------------|--------------|--------|------------|--------|------------|--------|
| ZINC ID | % [3H]NMS binding | EC50 μM | pKd | LogKd | ZINC ID | % [3H]NMS binding | EC50 μM | pKd | LogKd |
| 12427628 | 163 ± 11 | 1.1 ± 0.4 | 5.85 ± 0.31 | 0.73 ± 0.16 (5.4) | 09635472 | 133 ± 5 | 11 ± 3 | ND | ND |
| 03590563 | 138 ± 4 | 2.0 ± 0.7 | 4.76 ± 0.09 | 0.59 ± 0.15 (3.8) | 03444509 | 139 ± 11 | 26 ± 10 | ND | ND |
| 02653768 | 146 ± 1 | 4.8 ± 0.8 | 5.03 ± 0.18 | 0.23 ± 0.02 (1.7) | 03295621 | 125 ± 6 | >50 | ND | ND |
| 03245507 | 141 ± 3 | 7.1 ± 1.5 | 5.19 ± 0.15 | 0.21 ± 0.02 (1.6) | 03597405 | 111 ± 4 | >50 | ND | ND |
| 25339004 | 146 ± 5 | 25.0 ± 3.7 | 4.97 ± 0.14 | 0.23 ± 0.03 (1.7) | 03572779 | 105 ± 6 | ND | ND | ND |
| 03320344 | 118 ± 2 | 6.7 ± 0.2 | ND | ND | 03292724 | 107 ± 3 | ND | ND | ND |
| 05277589 | 122 ± 3 | 21 ± 7 | 5.35 ± 0.27 | 0.20 ± 0.03 (1.6) | 111 ± 4 | >50 | ND | ND |

Expansion of the scaffold toward Asn419EC1 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.
Asn410ECL3/Glu175ECL2. The bulkier phenyl ring of 628 is modeled to be perpendicular to Tyr226ECL2 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 Trp4227.36, 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 R2 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 904, 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 koff of [3H]NMS from the M2 mAChR decreased very substantially (~30-fold), so that by 10 μM 628 the t1/2 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 (pKd) of the antagonist increased with increasing concentrations of modulator, allowing for the determination of a cooperativity factor of Logit(NMS) = 0.73 ± 0.06 (Fig. 3 B and Table 2). In contrast, no substantial effect was observed on the affinity of the agonist, [3H]IXO in analogous saturation binding experiments (Fig. 3 C), 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]GTPγS 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. 3 C), 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 (EC50) concentration of the agonist IXO in the absence or presence of increasing concentrations of 628, and effects on [35S]GTPγS binding were assessed (Fig. 4 C and D).

Fig. 3. Characterization of allosteric activity of 628 at M2 mAChR. (A) Dissociation of 0.2 nM [3H]NMS was initiated following 1-h incubation by adding 10 μM atropine with varying concentrations of 628 or DMSO. The half-life was determined by fitting with a one-phase exponential decay analysis using GraphPad Prism. Saturation binding of (B) [3H]NMS or (C) [3H]IXO with varying concentrations of 628 incubated for 2 h at room temperature with membranes from CHO cells stably expressing M2 mAChR. The binding curves were fit by the allosteric modulator shift analysis using GraphPad Prism.
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 to the ATCM allowed for the determination of the functional cooperativity between NMS and '628, which was essentially identical between the two pathways: [³⁵S]GTPγS binding, Logₐ₀[NMS] = 0.73 ± 0.19 (pA₂ [NMS] = 5.4); ERK1/2 phosphorylation, Logₐ₀[NMS] = 0.67 ± 0.20 (pA₂ [NMS] = 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 [³⁵S]NMS radioligand titration assays, with increasing concentrations of '628 tested against an EC₅₀ concentration of the orthosteric ligand in the presence of [³⁵S]NMS (Fig. 5A and SI Appendix, 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-desmethylclozapine (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 nonaffacting, 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, QNB, 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 [³⁵S]GTPγS binding and ERK1/2 phosphorylation (Fig. 5B 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 [³⁵S]NMS binding assay. Fig. 5D summarizes the 17 ligands investigated, their structures, and the type of modulatory effect displayed by '628.

Table 2. [³⁵S]NMS Kₐ and dissociation half-life with addition of the allosteric ligand '628 at the five mAChR subtypes

<table>
<thead>
<tr>
<th>Human mAChR</th>
<th>Control</th>
<th>+10 μM '628</th>
<th>[³⁵S]NMS dissociation half-life, min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.042 ± 0.010</td>
<td>0.027 ± 0.003</td>
<td></td>
</tr>
<tr>
<td>M₁</td>
<td>0.27 ± 0.03</td>
<td>0.084 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>M₃</td>
<td>0.044 ± 0.01</td>
<td>0.038 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>M₄</td>
<td>0.026 ± 0.01</td>
<td>0.019 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>M₅</td>
<td>0.089 ± 0.04</td>
<td>0.11 ± 0.01</td>
<td></td>
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</table>

*P < 0.01, Student’s t test; **P < 0.0001, Student’s t test.
Met77,25 and Met406,54 are located on the extracellular side of the receptor on TM2 and TM6 (Fig. 6f). The change in the environment of the Met406 is likely due to its interaction with the side chain of Trp422,8,35, which is predicted to stack with the triazolo-quinazolinone moiety of 628 (Fig. 6g). Furthermore, the coinubcation of NMS with 628 induces a strong and well-defined Met77 peak compared with the antagonist alone (Fig. 6e). The shift of Met77 may reflect changes of the environment of Tyr80,61 and Tyr83,54 that are located on the same face of the methionine rod, in the docking post, and are predicted to interact with 628 (SI Appendix, Fig. S6). Importantly, Met77 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 628 additionally stabilizes changes in two methionine residues toward the intracellular part of the receptor, Met112 and Met202,54 (Fig. 6j). Here, 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 peak, indicating a more uniform conformation of TM3, compared with NMS bound alone (Fig. 6e). Although 628 displays little influence on Met202 when coadministered with the potent inverse agonist tiotropium (Fig. 6b), the PAM significantly shifts the Met202 NMS peak (Fig. 6e), coincidentally toward the position of tiotropium-bound state. It is possible that these spectral changes reflect the capacity of 628 to enhance NMS-mediated stabilization of the inactive conformation of the receptor (Fig. 6f). Together, these data suggest that the spectral modification of the methionines by 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 628 has on NMS.

Subtype Selectivity of 628 for the M2 mAChR. A motivation of this study was the discovery of selective allosteric modulators of the M2 subtype of the mAChR; thus, we investigated the selectivity profile of 628 across all five mAChRs. In [3H]NMS equilibrium binding assays, 628 retained its strong PAM effect against the M2 subtype, with slight PAM (M1,4 mAChR) or even a slight NAM effect (M1,3 mAChRs) for high concentrations of 628 at the other subtypes (Fig. 7 and SI Appendix, Table S5). This observation of differential allosteric between the PAM and the antagonist at the various mAChRs is further supported by kinetic studies. In saturation binding studies, no significant effect of 10 μM 628 was observed on [3H]NMS at the non-M2 mAChRs (Table 2 and SI Appendix, Fig. 7A–D). Furthermore, the dissociation rate of [3H]NMS from the different mAChRs subtypes was measured. Unlike the M2 subtype, where 628 reduced the Kd by 50-fold, a high concentration of 628 had no substantial effect on [3H]NMS dissociation, determined using isotopic dilution with atropine, at any of the non-M2 mAChRs (Table 2 and SI Appendix, Fig. S7E–I). A possible exception may be the M4 mAChR, where radioligand dissociation was detectably slowed—although even here, the effect was only fourfold—much less than with the M2 subtype (Table 2 and Fig. 3d vs. SI Appendix, Fig. S7G). This perhaps is not surprising, since the M4 mAChR shows the highest sequence homology with the M2 mAChR. Our results suggest that 628 is a selective modulator for NMS at the M2 mAChR, and either inactive or weakly active at the remaining mAChR subtypes.

Table 3. Affinity estimates (pA2 values) of NMS in functional assays in absence or presence of 628 at the human M2 mAChR

<table>
<thead>
<tr>
<th>Modulator concentration</th>
<th>[35S]GTPγS binding</th>
<th>ERK1/2 phosphorylation</th>
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<tr>
<td>NMS alone</td>
<td>9.47 ± 0.16</td>
<td>10.24 ± 0.16</td>
</tr>
<tr>
<td>+0 μM 628</td>
<td>9.51 ± 0.14</td>
<td>10.03 ± 0.15</td>
</tr>
<tr>
<td>+1 μM 628</td>
<td>9.60 ± 0.14</td>
<td>10.51 ± 0.15</td>
</tr>
<tr>
<td>+3 μM 628</td>
<td>10.01 ± 0.19</td>
<td>10.76 ± 0.18</td>
</tr>
<tr>
<td>+10 μM 628</td>
<td>10.22 ± 0.16</td>
<td>10.81 ± 0.29</td>
</tr>
</tbody>
</table>

pA2 values: Negative logarithm of the antagonist potency value for inhibiting 50% of the response to an EC50 concentration of I XO.

Fig. 5. Probe dependence of 628 with a panel of antagonists and agonists. (A) Cooperativity estimates of 628 with each indicated ligand determined using [3H]NMS equilibrium binding assays (complete dataset shown in SI Appendix, Fig. S4). Functional cooperativity estimates of 628 with selected antagonists determined in (B) [35S]GTPγS 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 628 at the M2 mAChR.
Encouragingly, and despite species effects that are common for allosteric ligands, no substantial difference was observed in the spectral shifts for tiotropium or tiotropium analogues, no matter whether they were coincubated with NMS or not. This was far from certain to us at the outset of this project. Unlike orthosteric sites,得以发现合子配体的筛选结果往往局限于少数几个受体亚型，而非整个GPCR家族。这为我们提供了宝贵的洞见，即可以针对特定受体亚型开发特定的合子配体。这不仅有助于我们更好地理解合子作用机理，也为药物开发提供了新的方向。
available from vendors—we do not claim to have fully explored the SAR of this series, nor that 628 represents a fully optimized probe or lead. Thus, while the affinity and cooperativity of this molecule are within range of optimized FAMs from other series, on mAChRs 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 (67) 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 628 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 628 is used to confer specificity on a second, orthosteric antagonist 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 628 in the extracellular vestibular allosteric site of the M2 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 nonsel ective) orthosteric antagonists, these PAMs can confer selectivity on otherwise potent and highly efficacious drugs. Importantly, the optimized modulator, 628, consistently acted as an antagonist PAM while an agonist NAL at human and rodent M2 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 Screens. We used the inactive state structure of M2 mAChR in complex with QNB (PDB ID code 3UON). The receptor was prepared for docking by keeping just the M2 residues (residues 20-48, 56-124, 135-210, and 384-444), while removing residues in the intracellular section that encompass the T4 helix 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 (i) placing two phenyl rings in perfect π-stacking distance (parallel face-centered and perpendicular y-shaped) from Tyr172Cl52, (ii) placing a naphthylene structure parallel to Trp42735 and a phenyl ring in perpendicular t-shaped stacking conformation, and (iii) placing one phenyl ring in π−π interaction with Thr18246 and π−alkyl interaction with Val40857 and Ala18457. 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); QNIFIT (35) was used, which implements the Poisson–Boltzmann equation to generate electrostatics 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 lowest root mean square deviation (RMSD) from the docked orthosteric ligand. This ligand was then used as the input for a second docking screen to select for 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 ligand 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 M2 mAChR. The M2 mAChR inactive structure was prepared for docking as previously described; however, the matching sphere set was used as the xtal-ligand input. The active M2 mAChR structure complexed with I XO and LY2119620 was prepared using residues 20–214 and 379–456 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 ligand was prepared 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) (70) and from Vitas-M (catalog no. STK816972), while the PAM 589 was acquired from Enamine (catalog no. Z16439559) and 628 (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 concentrations above 25 μM 628 in 10 mM Hepes, pH 7.5, and 1% DMSO, the solutions had to be heated to 42 °C for 628 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%. Fig. 8. Ex vivo validation of 628 as a PAM of NMS in native rat tissues expressing the M2 mAChR. [35]GTPyS binding was determined (A) in rat hypothalamus membranes, where 628 was able to increase the affinity of NMS when tested against an EC50 concentration of I XO, or (B) where similar experiments were performed in rat neonatal cardiomyocytes membranes, ACh as the agonist. (C) Statistical comparison of the cooperativity estimates of 628 as a PAM of NMS determined in five different experimental paradigms, using both human and native rat M2 mAChRs.
NMR Methods. The human M3 mAChR construct M2RΔSM was expressed, labeled, and purified. Briefly, the receptor was expressed in S9 cells using Bac-to-Bac baculovirus system. Cells were grown in methionine-deficient medium (Expression System) and infected at a density of 4 x 10^6 Ml^-1. 13C,15N-methionine was added into the medium during infection for specific labeling. The M3 mAChR receptor was purified by Ni-NTA chromatography, Flag affinity chromatography, and size exclusion chromatography sequentially. The final NMR sample was prepared in a buffer prepared in D_2O containing 20 mM Hepes, 100 mM NaCl, 0.01% (wt/vol) lauryl maltose neopentyl glycol (Anatrace), and 0.001% (wt/vol) cholesterol hemisuccinate (Sigma), and was concentrated to around 100 μM at a volume of ∼250 μL. The NMR data collection and assignment of methionyl methyl 13C,15N resonances of M3 mAChRs were conducted. All NMR experiments were performed at 25 °C on aBruker Avance 800-MHz spectrometer equipped with a cryogenic probe.

The spectra of M3 mAChR bound to different antagonist and ‘628 were acquired following the following procedure. All ligands were dissolved in perdeuterated dimethyl d6-sulfoxide (DMSO-d6). NMS or trotopiotoxin was added to the receptor at a saturation concentration of 1 mM. The 13C,15N heteronuclear single-quantum coherence (HSQC) spectra of M3 mAChRs bound to either antagonist were collected. After the NMR experiments in scopolamine- or tiotropium-bound states, ‘628 was added to the antagonist-bound sample at a final concentration of 250 μM, and the 13C,15N HSQC spectra were further collected. The total collection time for each NMR experiment was around 10 h. All NMR spectra were processed using the software package NMRPipe (80) and visualized using the program NMRView.

Radioligand Binding Assays. In our original biological screen to validate our VLS method, cell membranes from CHO cells expressing M3 mAChR were incubated for 1.5 h at 25 °C with 0.2 nM [3H]NMS, in absence or presence of either a fixed concentration of our VLS selected hits, LY2119620 or gallamine at 10 μM. Further characterization of ‘589 and its analog-by-catalog series was performed under identical conditions, but with increasing concentrations of each putative modulator, ranging from 0 to 100 μM. For the probe dependence study, radioligand binding was performed with identical concentration of [3H]NMS as described above, but on intact CHO cells expressing the M2 mAChR, and incubated for 6 h at 21 °C.

For saturation binding assays, cell membranes from CHO cells expressing either M1–M3 human AChR (for M3, an M3RΔCL3 construct was used) were incubated for 2 h at 25 °C with 0–2.5 nM [3H]NMS or 0–0.25 nM [3H]IXO, and 0–100 μM test compound or 10 μM atropine (to determine non-specific binding) in binding assay buffer. Samples were harvested on GF/C filter plates, quickly washed with cold assay buffer, and dried, and liquid scintillation mixture was added to determine radioactivity retained on the filters.

Radioligand Kinetic Dissociation Binding Assays. Cell membranes from CHO cells expressing either M1–M3 mAChR (for M3, an M3RΔCL3 construct was used) were incubated for 60 min at 25 °C with 0.2 nM [3H]NMS in binding buffer. Atropine (20 μM) with 0–100 μM test compound was added to determine dissociation for the indicated times. Samples were harvested, washed, and counted. Shown are combined results from three separate experiments.


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[13S]GTPγS Binding Assay Following Overnight Pretreatment. 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 the 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 100:60:3 (vol/vol/mol) and added to the lysate/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-α plate reader (PerkinElmer) using standard AlphaScreen settings.


