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

Citation
Korczynska, M., M. J. Clark, C. Valant, J. Xu, E. V. Moo, S. Albold, D. R. Weiss, et al. 2018. “Structure-based discovery of selective positive allosteric modulators of antagonists for the M2 muscarinic acetylcholine receptor.” Proceedings of the National Academy of Sciences of the United States of America 115 (10): E2419-E2428. doi:10.1073/pnas.1718037115. http://dx.doi.org/10.1073/pnas.1718037115.

Published Version
doi:10.1073/pnas.1718037115

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Accessibility
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 M2 mAChR, seeking molecules that specifically stabilized antagonist binding. This led us to identify a positive allosteric modulator (PAM) that potentiated the antagonist N-methylscopolamine (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 Kp 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]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.

Author contributions: M.K., M.J.C., and C.V. designed research; M.K., M.J.C., C.V., J.X., Patrick M. Sexton, Brian K. Kobilka, Arthur Christopoulos, Brian K. Shoichet, and Roger K. Sunahara contributed equally to this work.

Published online February 16, 2018.

www.pnas.org/cgi/doi/10.1073/pnas.1718037115

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.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1718037115/-/DCSupplemental.
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 \( M_2 \) 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 \( M_2 \) mAChR, we sought such cooperative modulators for \( M_2 \) antagonists. Emerging from this screen was a unique family of triazolo-quinazolinones 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 \( M_2 \) 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 \( M_2 \) QNB (19) complex (PDB ID code 3UON). This site lies largely above the plane of the membrane, and three tyrosine residues, Tyr403, Tyr426, and Tyr427 (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 \( M_2 \) mAChR by a single residue [Leu220\(^{220}\)L2(M3) → Phe181\(^{181}\)L2(M2), substitutions 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 10\(^{10}\) 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 \( M_2 \) 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 vestibule of the \( M_2 \) mAChR (SI Appendix, Table S2), most making unique combinations of interactions with the site (Fig. 1B–D). What turned out to be the three active molecules exemplified the different docked geometries and interactions. ZINC00008573 stacks with Trp427\(^{35}\), 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 \( \beta 573 \) 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, ZINC00051029 engages the same Tyr177\(^{177}\)ECL2 (Fig. 1B) but does not engage Trp422\(^{35}\). Additionally, \( \beta 29 \) made unique interactions with Asn410\(^{410}\). Finally, ZINC05277389 docks directly above the three-conserved tyrosines that form a “septom” between the orthosteric and allosteric sites (Fig. 1A). The triazolo-quinazolinone scaffold of \( \beta 89 \) orients to \( \pi \)–stack with Tyr403\(^{403}\) or Tyr426\(^{426}\) (Fig. 1D), while hydrogen-bonding with the backbone of Ile178, potentially stabilizing the position of extracellular loop 2. In addition, the ester moiety of \( \beta 89 \) forms a hydrogen bond with the side chain of Asn419\(^{419}\)ECL2. As shown below, \( \beta 89 \) 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 \( M_2 \) mAChR, we assessed the effect of 10 \( \mu \)M 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 \( [\text{H}] \)NMS (antagonist) or 0.05 nM \( [\text{H}] \)IXO (agonist). Compound \( \beta 89 \) displayed a PAM effect with the antagonist radioligand, but a NAM effect with the agonist radioligand (G) in a G-protein-mediated \( [\text{H}] \)GTP\( \gamma \)S binding assay, increasing concentrations of \( \beta 89 \) promoted a concentration-dependent, but saturable, reduction in agonist potency, consistent with a NAM effect on the agonist.

**Fig. 1.** A structure-based docking screen for allosteric modulators of human \( M_2 \) mAChR antagonists. (A) The initial docking approach. Seven representative high-ranking docking hits illustrate tiling of the allosteric site (cyan). The orthosteric site is colored red, while residues separating the two sites are presented as purple spheres. Docking poses of (B) the NAM \( \beta 29 \), (C) the NAM \( \beta 573 \), (D) and the PAM \( \beta 89 \) for NMS. Modeled hydrogen bonds and hydrophobic interactions are indicated as dashed lines. (E) The effects on \( [\text{H}] \)NMS binding of 10 \( \mu \)M of the 13 initial docking hits. The structures of three active modulators are shown (docking ranks in SI Appendix, Table S2). (F) Equilibrium binding between 0.2 nM \( [\text{H}] \)NMS (antagonist) or 0.05 nM \( [\text{H}] \)IXO (agonist). Compound \( \beta 89 \) displayed a PAM effect with the antagonist radioligand, but a NAM effect with the agonist radioligand (G) in a G-protein-mediated \( [\text{H}] \)GTP\( \gamma \)S binding assay, increasing concentrations of \( \beta 89 \) promoted a concentration-dependent, but saturable, reduction in agonist potency, consistent with a NAM effect on the agonist.
More interesting was the activity of '589, which increased the binding of the radioligand, consistent with its activity as a PAM of the labeled antagonist.

To quantify the effects of '589 at the M₂ mACHR, we performed equilibrium binding assays with increasing concentrations (0.3–100 μM) of '589 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, '589 increased antagonist binding by ~20%. Using an allosteric ternary complex model (ATCM), we quantified the affinity (pKᵢ) of '589 for the allosteric site on the free receptor and its cooperativity (α) with [³H]NMS: pKᵢ = 5.35 ± 0.27 and LogαNMS = 0.20 ± 0.03 (αNMS = 1.6). Strikingly, when switching the orthosteric probe from antagonist to agonist, '589 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 '589 on cellular function, we examined its effects on the promotion of [³S]GTPᵢS binding to activated G proteins by the agonist carbachol (CCh); this is a prototypical effect mediated by Gαᵩ-coupled receptors such as the M₂ mACHR. Compound '589 caused a saturable inhibition in CCh's promotion of [³S]GTPᵢS 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, '589 was tested for colloidal aggregation (38, 39). Whereas particles were seen at 100 μM '589, these did not inhibit a classic countere screening enzyme AmpC β-lactamase, nor was scattering sensitive to detergent, suggesting that the compound was not an aggregator at relevant concentrations.

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

| ZINC ID     | % [³H]NMS binding | EC₅₀ (μM) | pKᵢ | LogαNMS (αNMS) | ZINC ID     | % [³H]NMS binding | EC₅₀ (μM) | pKᵢ | LogαNMS (αNMS) |
|-------------|-------------------|-----------|-----|----------------|-------------|-------------------|-----------|-----|----------------|
| 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) | 02395621    | 125 ± 6           | >50 ND    | 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  | ND            |
| 25339904    | 146 ± 5           | 25.0 ± 3.7 | 4.97 ± 0.14 | 0.23 ± 0.03 (1.7) | 03572779    | 105 ± 6           | ND  ND     | ND  | ND            |
| 03320344    | 118 ± 2           | 6.7 ± 0.2 | ND  | ND            | 03297234    | 107 ± 3           | 107 ± 3   | ND  | ND            |
| 05277589    | 122 ± 3           | 21 ± 7    | 5.35 ± 0.27 | 0.20 ± 0.03 (1.6) |             |                   |           |     |               |

Expansion of the scaffold toward Asn419⁻F42 in the allosteric pocket led to the discovery of several unique PAMs on [³H]NMS binding. Particularly, '628, '563, '768, '907, 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 '589 row is in bold as it was the initial docking hit.

**Structure-Guided Optimization.** Using the modeled pose of '589, 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 Asn419ECL1. 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 Asn419ECL1, (R2) compounds that test the docking pose of '589 by clashing with Tyr83211 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): this was an “analogue-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 (pKᵢ = 5.85 ± 0.31) while retaining robust positive cooperativity with the antagonist, that is, LogαNMS = 0.73 ± 0.16 (αNMS = 5.4) (Fig. 2A and B and Table 1). Conversely, compounds that eliminate the ester R1-moiety of '589, 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 '589, partly reflecting our use of the smaller vestibule present in the 4MQT structure that was used for docking at this stage (Fig. 2C and D). In the docked pose, the carbonyl oxygen of the R1 moiety appears to bridge Tyr80261 and Thr42536, while the amide nitrogen hydrogen bonds with...
Asn410ECL3/Glu175ECL2. The bulkier phenyl ring of 628 is modeled to be perpendicular to Tyr63D1 and the terminal amide substituent, hydrogen bonds with the backbone oxygen of Thr84ECL3 that caps the TM2 helix. In this optimized docking pose, the five-membered ring of the triazole–quinazolinone scaffold stacks with Trp427–236, while the cyclohexane ring is sandwiched between Leu100Cterminus 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 Phe181A2 and Tyr177ECL2, which in the docking pose of 628 make interaction with the alkenic 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 koff 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 dissociation affinity (pKoff) of the antagonist increased with increasing concentrations of modulator, allowing for the determination of a cooperativity factor of Log(pKoff) = 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 (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]GTPyS binding, LogGTPyS = 0.73 ± 0.19 (αNMS = 5.4); ERK1/2 phosphorylation, LogERKyS = 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 [3H]NMS radioligand titration assays, with increasing concentrations of '628 tested against an EC50 concentration of the orthosteric ligand in the presence of [3H]NMS (Fig. 5 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-desmethyleclozapine (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 trotopium 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 NAM, or at least nonaffecting, properties for agonists irrespective of the ligand [we infer that '628 is a NAM 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]GTPyS 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 [3H]NMS binding assay. Fig. 5D summarizes the 17 ligands investigated, their structures, and the type of modulatory effect displayed by '628.

Table 2. [3H]NMS Kd and dissociation half-life with addition of the allosteric ligand ‘628 at the five mACHR subtypes

| Human mACHR | Kd of [3H]NMS 2-h incubation | [3H]NMS dissociation half-life, min |
|-------------|-----------------------------|-----------------------------------|
|             | Control                     | +10 μM ‘628                       | Control                     | +10 μM ‘628 | Fold increase |
| M1          | 0.042 ± 0.010               | 0.027 ± 0.003                     | 36 ± 4                     | 56 ± 8     | 1.6           |
| M2          | 0.25 ± 0.02                 | 0.084 ± 0.020*                    | 8.2 ± 0.2                  | 415 ± 123**| 51            |
| M3          | 0.040 ± 0.009               | 0.038 ± 0.009                     | 147 ± 22                   | 239 ± 70   | 1.6           |
| M4          | 0.026 ± 0.003               | 0.018 ± 0.005                     | 68 ± 3                     | 250 ± 110  | 3.7           |
| M5          | 0.089 ± 0.004               | 0.11 ± 0.01                       | 195 ± 30                   | 157 ± 14   | 0.8           |

*P < 0.01, Student’s t test; **P < 0.0001, Student’s t test.
Met77<sup>2-58</sup> and Met406<sup>5-54</sup> are located on the extracellular side of the receptor on TM2 and TM6 (Fig. 6F). The change in the environment of the Met406<sup>5-54</sup> is likely due to its interaction with the side chain of Trp242<sup>2-35</sup>, which is predicted to stack with the triazoloquinazolinone moiety of 628 (Fig. 6G). Furthermore, the coinubcation of NMS with 628 induces a strong and well-defined Met77<sup>2-58</sup> peak compared with the antagonist alone (Fig. 6E). The shift of Met77<sup>2-58</sup> may reflect changes of the environment of Tyr80<sup>6-41</sup> and Tyr83<sup>5-54</sup> that are located on the same face of TM3. The methionine rod, in the docking pose, is predicted to interact with 628 (SI Appendix, Fig. S6). Importantly, Met77<sup>2-58</sup> 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, Meta12<sup>1-24</sup> and Meta202<sup>2-54</sup> (Fig. 6F). 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 Met12<sup>1-24</sup> peak, indicating a more uniform conformation of TM3, compared with NMS bound alone (Fig. 6E). Although 628 displays little influence on Met202<sup>2-54</sup> when coadministered with the potent inverse agonist tiotropium (Fig. 6B), the PAM significantly shifts the Met202<sup>2-54</sup> peak (Fig. 6E), coincidently 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.

### Table 3. Affinity estimates (pA<sub>2</sub> values) of NMS in functional assays in absence or presence of 628 at the human M<sub>2</sub> mAChR

| Modulator concentration | [35S]GTP<sup>γ</sup>S binding | ERK1/2 phosphorylation |
|-------------------------|-------------------------------|------------------------|
| NMS alone               | 9.47 ± 0.16                   | 10.24 ± 0.16           |
| -1 μM 628               | 9.51 ± 0.14                   | 10.03 ± 0.15           |
| +1 μM 628               | 9.69 ± 0.14                   | 10.51 ± 0.15           |
| +3 μM 628               | 10.01 ± 0.19                  | 10.76 ± 0.18           |
| +10 μM 628              | 10.22 ± 0.16                  | 10.81 ± 0.29           |

pA<sub>2</sub> values: Negative logarithm of the antagonist potency value for inhibiting 50% of the response to an E<sub>0</sub> concentration of Ixo.

### PAM Effect of 628 on Native Tissue Membranes

To determine the utility of 628 as a probe in physiological systems, we examined the effect of 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 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<sub>1</sub>-5 mAChRs, and no significant effects were observed at any of the receptors. These findings suggest either a lack of interaction of 628, or a NAL effect on endogenous signaling at all of the mAChR subtypes, making 628 an excellent tool compound to probe antagonist action in physiological systems.

To investigate the potential physiological relevance of the PAM effects of 628 on M<sub>2</sub> mAChR antagonists, we determined the effects of the modulator in the absence or presence of NMS agonist-mediated [35S]GTP<sup>γ</sup>S binding using membranes derived from rat hypothalamus and neonatal rat ventricular cardiomyocytes, which both natively express high levels of M<sub>2</sub> mAChRs (46, 47). We investigated the potentiation of NMS antagonism by 628 using both the potent agonist IXO in rat hypothalamic membranes (Fig. 8A) and on 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, 628 potentiated NMS potency with a cooperativity of Log<sub>2</sub> <sub>NMS</sub> = 1.10 ± 0.31, while in cardiomyocytes the cooperativity was Log<sub>2</sub> <sub>NMS</sub> = 0.56 ± 0.42.

Fig. 5. Probe dependence of 628 with a panel of antagonists and agonists. (A) Cooperativity estimates of 628 with each indicated ligand determined using [35S]NMS equilibrium binding assays (complete dataset shown in SI Appendix, Fig. S4). Functional cooperativity estimates of 628 with selected antagonists determined in (B) [35S]GTP<sup>γ</sup>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 628 at the M<sub>2</sub> mAChR.

### Subtype Selectivity of 628 for the M<sub>2</sub> mAChR

A motivation of this study was the discovery of selective allosteric modulators of the M<sub>2</sub> subtype of the mAChR; thus, we investigated the selectivity profile of 628 across all five mAChRs. In [35S]NMS equilibrium binding assays, 628 retained its strong PAM effect against the M<sub>2</sub> subtype, with slight PAM (M<sub>1,4</sub> mAChR) or even a slight NAM effect (M<sub>1,5</sub> mAChRs) for high concentrations of 628 at the other subtypes (Fig. 7 and SI Appendix, Table S5). This observation of differential allosterity 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 [35S]NMS at the non-M<sub>2</sub> mAChRs (Table 2 and SI Appendix, Fig. S7 A–D). Furthermore, the dissociation rate of [35S]NMS from the different mAChR subtypes was measured. Unlike the M<sub>2</sub> subtype, where 628 reduced the K<sub>off</sub> by 50-fold, a high concentration of 628 had no substantial effect on [35S]NMS dissociation, determined using isotopic dilution with atropine, at any of the non-M<sub>2</sub> mAChRs (Table 2 and SI Appendix, Fig. S7 E–H). A possible exception may be the M<sub>2</sub> mAChR, where radioligand dissociation was detectably slower—although even here, the effect was only fourfold—much less than with the M<sub>2</sub> subtype (Table 2 and Fig. S4 vs. SI Appendix, Fig. S7G). Perhaps this is not surprising, since the M<sub>2</sub> mAChR shows the highest sequence homology with the M<sub>2</sub> mAChR. Our results suggest that 628 is a selective modulator for NMS at the M<sub>2</sub> mAChR, and either inactive or weakly active at the remaining mAChR subtypes.
Encouragingly, and despite species effects that are common for allosteric ligands, no substantial difference was observed in the cooperativity between human and rat M$_2$ mAChR when coincubated with NMS [active (blue), inactive (orange)], Met$_{77}$NMS and Met$_{202}$NMS [active (blue), inactive (orange)], (B) Met$_{77}$NMS alone (green), (C) NMS alone (green), (D) 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 IDs codes 4mqa, 4mqt, and 3uon, respectively) with close-up for (G) Met$_{406}$ and (D) Met$_{202}$ provided.

**Discussion**

Two key observations emerge from this study. First, allosteric sites in GPCRs can be targeted by structure-based, large library screens. This was far from certain to us at the outset of this project. Unlike orthosteric sites, whose relatively constrained structures have proven amenable to docking screens (48–56), the mAChR allosteric sites are less defined sterically, are open to bulk solvent, and are more conformationally labile in response to orthosteric ligand binding than are the orthosteric sites themselves. Nonetheless, 3 of 13 docking-prioritized molecules from the initial screen acted as modulators of antagonist (hit rate of 23%). While the potencies and PAM efficacies of the initial docked compounds were modest, the optimized PAM has an EC$_{50}$ value and an $\alpha$-factor that are not far removed from widely used reagents like BPQA and LY2033298, and even medicines like cinacalcet (57, 58). Second, antagonist PAMs can confer specificity on orthosteric drugs that would otherwise lack it (7). Thus, by itself, scopolamine binds with similar affinity to all five receptor subtypes ($K_D$: 0.4–2.1 mmol/L) (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 M$_2$ mACh over the other receptor subtypes. This suggests a general strategy to confer specificity 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 allosteric 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 most often lack PAMs. Nonetheless, the ability to discover effective modulators for 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 M$_2$ 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 M$_1$ 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 M$_2$ mAChR.

Certain caveats bear mentioning. First, our SAR studies around certain caveats bear mentioning. First, our SAR studies around the optimized PAM 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 most often lack PAMs. Nonetheless, the ability to discover effective modulators for antagonists, and to optimize them without new synthesis, suggests that these sites remain accessible to structure-based discovery.

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we do not claim to have fully explored the mAChR in mAChRs. was able to increase the affinity of in the extracellular residues (residues 20 (Enamine; 'represents a fully optimized probe and interaction with Thr187 ii | Ex vivo validation of Korczynska et al. www.pnas.org/cgi/doi/10.1073/pnas.1718037115 all of these screens can help reduce the likelihood that and Ala184 is used to confer specifici β GPCR orthosteric sites, the extracellular vestibules of mAChRs re-tructure-based discovery. In large library docking 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 are main 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 (classically 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 antag-onist 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

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.

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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 Screes. 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 hysyme used to facilitated 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 π-stacking distance (parallel face-centered and perpendicular y-shaped) from Tyr17 and (ii) placing a naphthalene structure parallel to Trp422 and a phenyl ring in perpendicular t-shaped stacking conformation, and (iii) placing one phenyl ring in π-π interaction with Thr187 and an α-alkyl interaction with Val408 and Ala184. 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 were 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 generated 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 docking 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 ligand-fragment docking that is composed of two electrostatics orbitals, 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 large diversity, were removed. Finally, 16 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 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 returned as a coligand during docking and was prepared using PRODRG server (77), while the allosteric ligand 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. STX816972), while the PAM was acquired from Enamine (catalog no. Z324823878). The purity of the most efficacious PAMs, '563 (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 used 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 '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]GTP•S 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.
NRN Methods. The human MmAChR construct M2R5SM was expressed, labeled, and purified. Briefly, the receptor was expressed in Sf9 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. 152h-methionine was added into the medium during infection for specific labeling. The M2 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% (v/v) tert-butyl) lauryl maltose neopentyl glycol (Anatrace), and 0.003% (v/v) cholesterol hemisuccinate (Sigma), and was concentrated to around 100 μM at a volume of ∼250 μL. The NMR data collection and assignment of methionine methyl [1H,13C] resonances of M2 mAChR5SM were conducted. All NMR experiments were performed at 25 °C on a Bruker Avance 800-MHz spectrometer equipped with a cryogenic probe.

The spectra of M2 mAChR bound to different antagonist and [35S]GTPγS were acquired by the following procedure. All ligands were dissolved in perdeuterated dimethyl d6-sulfoxide (DMSO-d6). NMS or totiropium 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 totiropium-bound state, [35S]GTPγS spectra were further collected. The total collection time for each single experiment was around 10 h. All NMR spectra were processed using the software package NMRPipe (80) and visualized using the program NMRView.

Radioligand Kinetic Dissociation 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, in binding assay buffer containing 10 mM Hepes, pH 7.4, 10 mM NaCl, and 0.5 mM MgCl₂. Further characterization of 589 and its analog-by-catalog series was performed under identical conditions, but with increasing concentrations of each putative 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-w plate reader (PerkinElmer) using standard AlphaScreen settings.

ACKNOWLEDGMENTS. We thank Dr. Anat Levit for fruitful discussions and for comments about this manuscript. We also thank the NMR facility support at the Beijing NMR Center and the NMR facility of National Center for Protein Sciences at Peking University. This work was supported by Grant GM106990 (to B.K.K., R.K.S., and B.K.S.) and by Program Grant APP1055134 of the National Health and Medical Research Council (NHMRC) of Australia (to A.C. and P.M.S.), NHMRC Project Grant APP1082318 (to C.V.), and Australian Research Council Future Fellowship FT140100114 (to C.V.). A.C. is a Senior Principal, and P.M.S., a Principal, Research Fellow of the NHMRC. C.V. is an Australian Research Council Future Fellow.

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