Bitopic binding mode of an M₁ muscarinic acetylcholine receptor agonist associated with adverse clinical trial outcomes

Sophie J. Bradley, Colin Molloy, Christoffer Bundgaard, Adrian J. Mogg, Karen J. Thompson, Louis Dwomoh, Helen E. Sanger, Michael D. Crabtree, Simon M. Brooke, Patrick M. Sexton, Christian C. Felder, Arthur Christopoulos, Lisa M. Broad, Andrew B. Tobin and Christopher J. Langmead.

Institute of Molecular, Cell and Systems Biology, College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow, Scotland, G12 8QQ (S.J.B., C.M., K.J.T., L.D., S.M.B., A.B.T.); Eli Lilly & Co, Neuroscience, Erl Wood Manor, Windlesham, Surrey, GU20 6PH, UK (A.J.M., C.B., L.M.B.); Drug Discovery Biology, Monash Institute of Pharmaceutical Sciences, Monash University, Parkville, Victoria, 3052 Australia (P.M.S., A.C., C.J.L.); Eli Lilly & Co. Neuroscience, Lilly Corporate Center, Indianapolis, Indiana 46285, USA (C.C.F.)
Novel bitopic mode of M₁ agonist

*Corresponding authors:
Prof. Andrew B. Tobin, Institute of Molecular, Cell and Systems Biology, College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow, Scotland, G12 8QQ. Email: andrew.tobin@glasgow.ac.uk.

Dr. Christopher J. Langmead, Drug Discovery Biology, Monash Institute of Pharmaceutical Sciences, Monash University, Parkville, Victoria, 3052 Australia. Email: chris.langmead@monash.edu.

Number of text pages: 32
Number of tables: 5
Number of figures: 12
Number of references: 35
Abstract word count: 216
Introduction word count: 621
Discussion word count: 751

Nonstandard abbreviations
G protein-coupled receptor (GPCR); muscarinic acetylcholine receptor (mACHR); Alzheimer’s disease (AD); N-methylscopolamine (NMS); 1-[1'-(2-methylbenzyl)-1,4'-bipiperidin-4-yl]-1,3-dihydro-2H-benzimidazol-2-one (TBPB); benzyl quinolone carboxylic acid (BQCA), Designer receptor exclusively activated by designer drug (DREADD), extracellular signal-regulated kinases (ERK).
Abstract

The realisation of the therapeutic potential of targeting the M₁ muscarinic acetylcholine receptor (M₁ mAChR) for the treatment of cognitive decline in Alzheimer’s disease has prompted the discovery of M₁ mAChR ligands showing efficacy in alleviating cognitive dysfunction in both rodents and humans. Among these is GSK1034702, described previously as a potent M₁ receptor allosteric agonist, which showed pro-cognitive effects in rodents and improved immediate memory in a clinical nicotine withdrawal test but induced significant side-effects. Here we provide evidence using ligand binding, chemical biology and functional assays to establish that rather than the allosteric mechanism claimed, GSK1034702 interacts in a bitopic manner at the M₁ mAChR such that it can concomitantly span both the orthosteric and an allosteric binding site. The bitopic nature of GSK1034702 together with the intrinsic agonist activity and a lack of muscarinic receptor subtype selectivity reported here, all likely contribute to the adverse effects of this molecule in clinical trials. We conclude that these properties, whilst imparting beneficial effects on learning and memory, are undesirable in a clinical candidate due to the likelihood of adverse side effects. Rather, our data supports the notion that “pure” positive allosteric modulators showing selectivity for the M₁ mAChR with low levels of intrinsic activity would be preferable to provide clinical efficacy with low adverse responses.
**Introduction**

The M<sub>1</sub> muscarinic acetylcholine receptor (M<sub>1</sub> mAChR) has emerged as an attractive molecular target to overcome cognitive decline associated with cholinergic degeneration in Alzheimer’s disease (AD) (Anagnostaras et al., 2003). Activation of M<sub>1</sub> mAChRs, which are abundantly expressed in the amygdala, cerebral cortex, striatum and hippocampus (Buckley et al., 1988; Levey et al., 1995), has been reported to rescue learning and memory deficits associated with neurodegeneration in a number of mouse models (Bradley et al., 2017; Lange et al., 2015; Puri et al., 2015; Vardigan et al., 2015). Translating these promising findings to successful clinical candidates has however been challenging due to adverse effects associated with a lack of selectivity of orthosteric M<sub>1</sub> mAChR agonists. This is exemplified by the M<sub>1</sub>/M<sub>4</sub>-preferring mAChR agonist xanomeline, which significantly improved cognitive function in AD patients (Bodick et al., 1997) but ultimately failed due to adverse effects attributed to activation of peripheral cholinergic signalling likely through M<sub>2</sub> and M<sub>3</sub> mAChR receptors (Langmead et al., 2008).

There is, therefore, an urgent need to develop novel approaches to build selectivity into M<sub>1</sub> mAChR ligands. Two related approaches have been taken to this problem: (i) the development of positive allosteric modulators that bind to a site that is topographically distinct from that of the endogenous ligand acetylcholine (May et al., 2007) and (ii) development of a newer generation of more selective agonists (whose mechanism of action is not always well-defined (Langmead et al., 2008). Allosteric modulators enhance acetylcholine binding and/or signalling, the magnitude of which can vary with different degrees of positive cooperativity (Conn et al., 2009; Langmead and Christopoulos, 2006; May et al., 2007). They can also possess direct allosteric agonist activity (intrinsic activity). M<sub>1</sub> mAChR allosteric modulators with high functional selectivity over other muscarinic receptor subtypes (i.e M<sub>2</sub>-M<sub>5</sub> mAChRs) have been reported to reverse phenotypes associated with neurodegenerative disease (Bradley et al., 2017; Ma et al., 2009; Puri et al., 2015; Vardigan et al., 2015) whilst showing no adverse side effects in animal models (Bradley et al., 2017).

Less well described in the literature is the development of a new generation of M<sub>1</sub> mAChR agonists, which have variously been described as “ectopic” (Spalding et al., 2002), “allosteric”(Budzik et al., 2010; Jones et al., 2008; Langmead and
Christopoulos, 2006), “atypical” (Lebon et al., 2009) and “bitopic” (Keov et al., 2011). With but a few exceptions (Keov et al., 2014) this broad nomenclature reflects a paucity in understanding of mechanism(s) of action; it is not clear whether some of these ligands exert true allosteric agonism (i.e. bind solely to an allosteric binding site to activate the receptor), are subtype-selective orthosteric agonists or a represent a hybrid of the two (with a pharmacophore that engages both orthosteric and allosteric binding pockets).

GSK1034702 has been described as an allosteric M₁ mAChR agonist; it was identified from a series of benzimidazolones and reversed scopolamine-induced amnesia in rodents (Budzik et al., 2010) and had positive effects on cognitive function in humans. Unfortunately it also induced gastrointestinal adverse effects, consistent with activation of peripheral mAChRs (Nathan et al., 2013).

Here we conduct a comprehensive pharmacological analysis of GSK1034702 and show that this molecule is not a purely allosteric ligand as previously suggested. Radioligand binding (using [³H]-N-methylscopolamine (NMS) and [³H]-GSK1034702) and functional IP accumulation studies at the muscarinic M₁ receptor reveal that GSK1034702 interacts with the orthosteric acetylcholine binding site and likely, concomitantly, with an allosteric binding site. Importantly, this bitopic mode of action is able to mediate beneficial effects on learning and memory but the lower degree of selectivity of GSK1034702 compared to ligands that engage solely with an allosteric binding site, together with the intrinsic agonist activity reported here, may account for the adverse effects observed with this molecule in the clinic.
Materials and Methods

Materials

GSK1034702 and xanomeline were synthesized by Eli Lilly (Erl Wood Manor, Windlesham, Surrey, UK). IP-one and ERK1/2 phosphorylation assay kits were purchased from Cisbio Assays (Codolet, France). TBPB was obtained from Tocris Bioscience (Bristol, UK). All other chemicals and reagents were purchased from Sigma-Aldrich Company Ltd. (Dorset, England).

Mouse maintenance and diet

All experiments were performed under a project licence from the British Home Office (United Kingdom) under the Animals (Scientific Procedures) Act of 1986. C57Bl/6J mice used in this study were purchased from Charles River (Margate, UK). Mice were fed ad libitum with a standard mouse chow, and maintained within the animal facility at least one week prior to experiments.

Fear conditioning

C57Bl/6J male mice (8-12 week old) were acclimatised to the behavioural testing suite at least 2 hours prior to the test. Mice were injected (i.p.) with vehicle (5% glucose) or scopolamine (1.5 mg/kg) alone or in combination with xanomeline, GSK1034702 or TBPB 30 minutes prior to training. Mice were placed in the conditioning chamber (Stoelting ANY-maze Fear Conditioning System) and, after a 2-minute adaptation period, received 3 tone/foot shock pairings where the foot shock (unconditioned stimulus [US]; 2 seconds; 0.4 mA) always co-terminated with a tone (conditioned stimulus [CS]; 2.8 kH; 85 dB; 30 seconds). The CS-US pairings were separated by 1-minute intervals. After completion of training, the mice remained in the conditioning chamber for 1 minute and were then returned to their home cages. The next day, mice were placed back in the conditioning chamber, and time spent immobile was recorded for 3 minutes to assess context-dependent learning. Data were analyzed using ANY-maze software.

Mouse pharmacokinetics

Pharmacokinetic analyses were conducted as previously described (Witkin et al., 2017). Compounds were administered via i.p. injection (in 5% glucose) 30 minutes prior to blood collection. Mice were anaesthetised with 3% isoflurane (2 l/min), and
blood was collected by cardiac puncture of the left ventricle. Blood was immediately transferred to EDTA tubes and centrifuged at 1,000 g for 10 minutes at 4°C; supernatant was collected and frozen. Brains from each mouse were also dissected and snap-frozen on dry ice.

Brain samples were homogenized in 3 volumes of methanol/water (1:4, v/v) by weight. A 25 µl aliquot of each study sample, calibration standard, and control sample were added to a 96-well plate and mixed with 180 µl of acetonitrile/methanol (1:1, v/v) containing internal standard. The samples were subsequently centrifuged, and the resulting supernatants were diluted 12.5-fold with methanol/water (1:1, v/v) prior to analyzing 10 µl aliquots by liquid chromatography-MS/MS (LC-MS/MS) as previously described (Bradley, Bourgognon et al. 2017).

**Equilibrium [³H]-NMS binding**

CHO-FlpIn cells expressing wild-type M₁ mAChR ($B_{\text{max}} = 870$ fmol/mg) or M₁ DREADD ($B_{\text{max}} = 2400$ fmol/mg) were plated at 7,500 cells/well in clear 96-well plates and grown to confluence. Prior to the experiment, cells were washed with 100 µl phosphate-buffered saline. Increasing concentrations of test compounds and an approximate equilibrium dissociation constant ($K_D$) concentration of [³H]-NMS ($K_D$ (nM) for [³H]-NMS binding to CHO-FlpIn cells expressing wild-type M₁ mAChR or M₁ DREADD were $0.37 \pm 0.10$ and $18.70 \pm 3.49$ (n=3) were incubated with cells overnight at room temperature in a final volume of 100 µl binding buffer of the following composition: 110 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl₂, 1 mM MgSO₄, 25 mM glucose, 20 mM HEPEs, 58 mM sucrose, pH 7.4. Binding was terminated by rapid aspiration followed by 2 washes with 200 µl ice-cold 0.9% NaCl. Bound radioactivity was determined by liquid scintillation (Ultima Gold, Perkin Elmer, Boston, MA, USA) counting. Non-specific binding was determined in the presence of 10 µM atropine.

For competition binding experiments at M₁-, M₂-, M₃-, M₄- and M₅ mAChRs, CHO membranes were purchased from Perkin Elmer (Boston, MA, USA). All experiments were performed in assay buffer of the following composition: 20 mM HEPES, 100 mM NaCl and 10 mM MgCl₂, pH 7.5, and used 10 µg of protein/well in a total assay volume of 1 ml using deep well blocks. CHO cell membranes over-expressing human mAChR M₁-M₅ subtypes were incubated with a concentration of [³H]-NMS that was
close to the calculated $K_D$ for each receptor ($M_1$ 200 pM, $K_D = 196$ pM; $M_2$ 700 pM, $K_D = 769$ pM; $M_3$ 700 pM, $K_D = 642$ pM; $M_4$ 200 pM, $K_D = 143$ pM; $M_5$ 400 pM, $K_D = 410$ pM), in the presence or absence of 11 different concentrations of compound. Non-specific binding was determined in the presence of 10 µM atropine. All assay incubations were initiated by the addition of membrane suspensions and deep well blocks were shaken for 5 min to ensure complete mixing. Incubation was then carried out for 2 h at 21°C. Binding reactions were terminated by rapid filtration through GF/A filters (Perkin Elmer, Boston, MA, USA) pre-soaked with 0.5% w/v PEI for 1 h. Filters were then washed 3 times with 1 ml ice-cold assay buffer. Dried filters were counted with Meltilex A scintillant using a Trilux 1450 scintillation counter (Perkin Elmer, Boston, MA, USA). The specific bound counts (d.p.m.) were expressed as a percentage of the maximal binding observed in the absence of test compound (total) and non-specific binding determined in the presence of 10 µM atropine.

**Kinetic $[^3]H$-NMS binding**

For determination of $[^3]H$-NMS dissociation kinetics, membranes (5 µg/tube) expressing the $M_1$ mAChR were pre-incubated with $[^3]H$-NMS for 1 hour at 37°C in binding buffer containing: 100 mM NaCl, 10 mM MgCl$_2$, 20 mM HEPEs, pH 7.4. Dissociation of the bound radioligand was initiated by addition of atropine (10 µM) alone or atropine (10 µM) plus100 µM GSK1034702 added in a reverse time course protocol. Reactions were terminated by rapid filtration onto GF/B filter paper (Whatman, Maidstone, UK) and three washes with 3 ml ice-cold 0.9% NaCl using a Brandel harvester. Membrane bound radioactivity was determined by liquid scintillation (Ultima Gold, Perkin Elmer, Boston, MA, USA) counting.

**IP-one accumulation assay**

Stimulation of inositol phosphate accumulation was determined using the Cisbio IP-One Gq assay kit as per manufacturer instructions. For agonist concentration response curves, agonists (2X concentrated) were added to 384-well white ProxiPlates (Perkin Elmer, Boston, MA, USA) in 7µl stimulation buffer. CHO-FlpIn cells stably expressing the human $M_1$ mAChR were grown to confluence in T75 cell culture flasks at 37°C. Cells were washed with warm phosphate-buffered saline and detached using phosphate-buffered saline with 0.1M EDTA. Detached cells were centrifuged at 1000 x g and the cells were resuspended in stimulation buffer. 7 µl of this cell
suspension (1.43 x 10^6 cells/ml) was added to each well, and cells were stimulated for 45 minutes at 37°C.

For functional interaction studies, CHO-FlpIn cells stably expressing the human M1 mAChR were seeded at 5,000 cells/well in 384-well white ProxiPlates. Experiments were conducted 48 hours later. Cells were washed once with 50 µl phosphate-buffered saline, and then incubated in F12 media containing phenoxybenzamine (where applicable) at 37°C for 30 minutes. Cells were washed with 50 µl phosphate-buffered saline and incubated in stimulation buffer containing agonists in a final volume of 14 µl for 45 minutes at 37°C.

All IP-one stimulations were terminated by the addition of 3 µl/well of IP1-d2 solution, followed by 3 µl/well anti IP1-Cryptate solution and incubation for 1 hour at room temperature with shaking. Fluorescence emission at two different wavelengths (665 nm and 620 nm) was measured with a PHERAstar plate reader (BMG Labtech).

**ERK1/2 phosphorylation**

Stimulation of phosphor-ERK1/2 (Thr 202/Tyr 204) was determined using the Cisbio Phospho-ERK Cellular Assay Kit. CHO-FlpIn cells stably expressing the human M1 receptor were seeded onto transparent 96 well plates at 20,000 cells/well and grown to confluence. Cells were serum starved overnight prior to the experiment. Prior to the stimulations, cells were washed with 100 µl of phosphate-buffered saline and then incubated in serum free F12 medium at 37°C. Cells were stimulated with ligands for 5 min at 37°C in a final volume of 200 µl. The stimulations were terminated by rapid aspiration and addition of 50 µl lysis buffer supplemented with blocking reagent as per manufacturer instructions, followed by gentle agitation at room temperature for 30 minutes. Subsequently, 16 µl of this lysate was transferred to a 384-well white ProxiPlate (Perkin Elmer, Boston, MA, USA) and incubated with 4 µl of premixed antibody solution for 2 hours at room temperature. Fluorescence emission at two different wavelengths (665 nm and 620 nm) was measured with a PHERAstar plate reader (BMG Labtech).

**Native tissue GTPγ[^35S] Binding Assays**

GTPγ[^35S] binding in rat membranes was determined in triplicate using an antibody capture technique in 96-well plate format (DeLapp et al., 1999). Native rat
membranes were prepared as follows. All procedures were performed at 4°C. 10-15ml of sucrose buffer (10 mM HEPES, 1 mM EGTA, 1 mM DTT, 10% sucrose and 1 tablet/50 ml Complete Protease Inhibitor Cocktail; pH 7.4) was added to each tissue sample and homogenized for 10 strokes using an electric IKA RW20 homogeniser (800 rpm) with glass homogenizer. The homogenate was centrifuged at 1000 x g for 10 min at 4°C and the supernatant collected. The pellet was re-homogenised and centrifuged again as above and the supernatant pooled and centrifuged at 11,000 x g for 20 min at 4°C. The resulting pellet was suspended in 40 ml of final storage buffer (10 mM HEPES, 1 mM EGTA, 1 mM MgCl₂, 1 mM DTT; pH 7.4) and centrifuged at 27,000 x g for 20 min at 4°C. The supernatant was removed and the final pellet was suspended in 2ml of final storage buffer. The protein concentration was measured using the Bradford method (Coomassie Plus, Bio-Rad protein assay kit) with BGG standards. Samples were aliquoted and stored at -80°C. Membrane aliquots (15 µg/well) were then incubated with test compound and GTPγ[³⁵S] (500 pM/well) for 30 mins. Labelled membranes were then solubilised with 0.27 % Nonidet P-40 plus Gqα antibody (E17, Santa Cruz) at a final dilution of 1:200 and 1.25 mg/well of anti-rabbit scintillation proximity beads. Plates were left to incubate for 3 hours and then centrifuged for 10 mins at 2000 r.p.m. Plates were counted for 1 min/well using a Wallac MicroBeta Trilux scintillation counter (PerkinElmer). All incubations took place at room temperature in GTP-binding assay buffer of the following composition: 20 mM HEPES, 100 mM NaCl and 5 mM MgCl₂, pH 7.5. Data were converted to % response compared to oxotremorine M (100 µM) or % over basal and EC₅₀ values generated (4 parameter-logistic curve) using GraphPad Prism 6.

[³H]-GSK1034702 binding

[³H]-GSK1034702 (specific activity 3.65 TBq/mmol) was synthesized by direct titration on Pd Black (performed by Quotient Bioresarch). All experiments were performed in assay buffer of the following composition: 20 mM HEPES, 100 mM NaCl and 10 mM MgCl₂, pH 7.5, and used 50 µg of protein/well in a total assay volume of 250 µl.

CHO cell membranes over-expressing human M₁ mAChR (Perkin Elmer, Boston, MA, USA) were incubated with [³H]-GSK1034702 (20 nM) in the presence of 11 concentrations of test compound. All assay incubations were initiated by the addition
of membrane suspensions. Incubation was then carried out for 4 hours at 21°C. Binding reactions were terminated by rapid filtration through GF/A filters (Perkin Elmer, Boston, MA, USA) pre-soaked with 0.5% (w/v) PEI for 1 hour. Filters were then washed 6 times with 1 ml ice-cold assay buffer. Dried filters were counted using Meltilex A scintillant using a Trilux 1450 scintillation counter (Perkin Elmer, Boston, MA, USA). The specific bound counts (d.p.m.) were expressed as a percentage of the maximal binding observed in the absence of test compound (total) and non-specific binding determined in the presence of 100 µM non-radiolabelled GSK1034702.

**Isolated rat atria and ileum contraction experiments**

Adult Wistar rats were humanely killed and left atria and ileum were placed in an organ bath containing oxygenated Tyrode solution. Measurement of negative inotropic responses in atria or contraction of ileum was performed as described by Lambrecht et al., 1989. For atrial responses, ligands were administered for 5 minutes at 32°C in McEwens buffer (pH 7.4) in a bath volume of 10ml. Agonist responses were measured as negative inotropy relative to 1µM methacholine, and antagonism was measured as inhibition of methacholine (1µM)-induced negative ionotropic response. For rat ileum experiments, ligands were administered to for 5 minutes at 32°C in Kreb’s buffer (pH 7.4) in a bath volume of 10ml. Contraction of ileum as a percentage of methacholine-induced contraction were measured, and antagonistic effects were measured by inhibition of methacholine-induced responses.

**Data analysis**

Inhibition binding data were curve-fit using GraphPad Prism 6 to derive the potency (IC\textsubscript{50}) of the test compound. The equilibrium dissociation constant (K\textsubscript{D}) of the test compound was then calculated by the Cheng–Prusoff equation: 
\[ K_D = \frac{IC_{50}}{1 + ([L]/K_D)} \]
using the K\textsubscript{D} value derived separately from saturation binding studies.

Functional concentration-response curves were fitted according to a four-parameter logistic equation (to determine minimum and maximum asymptotes, LogEC\textsubscript{50} and slope; GraphPad Prism 6). For ACh curves in the presence of multiple concentrations of GSK1034702 or TBPB (after phenoxybenzamine treatment), the following form of the Gaddum and Schild equations was applied globally to the datasets:
\[ Y = Bottom + \frac{(Top - Bottom)}{1 + \left(10^{\log EC_{50} \left(1 + \frac{[B]}{10^{-pA_2}}\right)^{n_H}}\right) + \frac{[A]}{[B]}} \]

where Top represents the maximal asymptote of the curves, Bottom represents the minimum asymptote of the curves, \( \log EC_{50} \) represents the logarithm of the ACh EC\(_{50}\) in the absence of GSK1034702 or TBPB, \([A]\) represents the concentration of ACh, \([B]\) represents the concentration of GSK1034702 or TBPB, \(n_H\) represents the Hill slope of the agonist curve, \(s\) represents the Schild slope for the antagonist, and \(pA_2\) represents the negative logarithm of the concentration of antagonist that shifts the agonist EC\(_{50}\) by a factor of 2. In the absence of antagonist ([B] = 0), this equation becomes the standard four-parameter logistic equation for fitting agonist concentration-response data.

\[^{3}H\]-NMS binding interaction studies with BQCA were fitted to an allosteric ternary complex model (Leach et al., 2010):

\[ Y = \frac{B_{max}[A]}{[A] + \left(\frac{K_{A}K_{B}}{\alpha'[B] + K_{B}}\right) \left(1 + \frac{[I]}{K_{i}} + \frac{[B]}{K_{B}} + \frac{\alpha'[I][B]}{K_{i}K_{B}}\right)} \]

where \(B_{max}\) represents the total number of receptors; \([A]\), \([B]\) and \([I]\) are concentrations of radioligand, allosteric modulator and orthosteric ligand, respectively; \(K_{A}\), \(K_{B}\), and \(K_{i}\) represent equilibrium dissociation constants of radioligand, allosteric modulator and orthosteric ligand, respectively. \(\alpha'\) and \(\alpha\) are the binding cooperativities between the allosteric modulator and radioligand and the allosteric modulator and the orthosteric ligand, respectively. An \(\alpha\) value of >1 denotes positive cooperativity, <1 denotes negative cooperativity, and a value of 1 denotes neutral cooperativity of binding.
To assess agonist bias, the same concentration-response curves were analyzed according to a modified form of the operational model of agonism, re-cast to directly yield a transduction ratio (Log[\tau/K_A]; (van der Westhuizen et al., 2014)):

\[
Y = \text{Basal} + \frac{(E_m - \text{Basal})}{1 + \left(10^{\log_{10} K_A} + 1\right)^n} \left(\frac{[A]}{10^{\log_{10} (\tau/K_A)} \cdot [A]}\right)
\]

where Basal represents the response in the absence of agonist, \(E_m\) represents the maximal response of the assay system, \(K_A\) represents the equilibrium dissociation constant of the agonist, \([A]\) represents the concentration of agonist, \(\tau\) is an index of the coupling efficiency (or efficacy) of the agonist and \(n\) is the slope of the transducer function linking agonist occupancy to response. For the analysis, all families of agonist curves at each pathway were globally fitted to the model with the parameters, Basal, \(E_m\), and \(n\) shared between all agonists. For full agonists, the Log\(K_A\) was constrained to a value of zero, whereas for partial agonists this was directly estimated by the curve fitting procedure; the Log(\(\tau/K_A\)) parameter was estimated as a unique measure of activity for each agonist. Agonist bias factors (10^\(\Delta \Delta \log[\tau/K_A]\)) were calculated as described in (van der Westhuizen et al., 2014)).
Results

GSK1034702, TBPB and xanomeline reverse scopolamine-induced deficits in fear conditioning

It is well established that muscarinic receptor agonists and positive allosteric modulators can reverse deficits in learning and memory induced by the administration of a broad spectrum muscarinic antagonist such as scopolamine (Ma et al., 2009; Young et al., 1995). Here, doses of scopolamine above 1 mg/kg administered to mice 30 minutes prior to fear conditioning training were sufficient to induce a significant reduction in contextual fear conditioning learning and memory (Supplementary Fig. 1). The effects of muscarinic receptor agonists on this deficit were tested by the co-administration of scopolamine (1.5 mg/kg) with escalating intraperitoneal doses of xanomeline (Fig. 1A), GSK1034702 (Fig. 1B) or TBPB (Fig. 1C). All three agents significantly improved learning and memory compared with vehicle controls (5% glucose solution in ddH2O) (P<0.05 versus administration of 1.5 mg/kg scopolamine alone; one-way ANOVA with Tukey’s multiple comparisons test). Free brain concentrations of xanomeline and GSK1034702 determined 30 minutes after administration were seen to increase linearly with escalating doses (Fig. 1D, 1E). In contrast, the effects of these compounds on learning and memory were bell shaped with lower doses improving learning and memory and high doses showing reduced effect (Fig. 1A, 1B). This bell-shaped response is characteristic of pro-cognitive agents. Interestingly, brain exposure of TBPB could not be increased beyond that observed at 10 mg/kg, remaining relatively low even after intraperitoneal injection of higher doses (Fig. 1F). This resulted in TBPB effects on learning and memory being similar at both low and high-administered doses with no evidence of a bell-shaped dose response (Fig. 1C).

GSK1034702 and TBPB interact competitively with [3H]-NMS at M1 mAChRs

GSK1034702 and TBPB have previously been described as allosteric agonists of the M1 mAChR (Budzik et al., 2010; Jones et al., 2008; Nathan et al., 2013). To test this assertion, [3H]-NMS binding studies were conducted on monolayers of CHO Flp-In cells expressing the human M1 mAChR to determine the nature of their interaction at the receptor. Both GSK1034702 and TBPB fully inhibited binding of [3H]-NMS (0.5
nM) to M₁ mAChRs, with estimated pKᵢ values of 6.5 ± 0.2 and 6.8 ± 0.1, respectively and in a similar manner to the orthosteric agonist, xanomeline (Fig. 2; Table 1). These data suggest that GSK1034702, in contrast to the allosteric mechanism of action previously reported, binds instead in a competitive manner consistent with interaction at the orthosteric site of the M₁ mAChR.

GSK1034702 and TBPB do not alter [³H]-NMS dissociation kinetics

As allosteric ligands with high negative cooperativity can still fully inhibit orthosteric ligand binding, kinetic binding experiments were performed to probe any allosteric interactions of GSK1034702 or TBPB with the M₁ mAChR. Membranes of CHO Flp-In cells expressing the human M₁ mAChR were pre-equilibrated with [³H]-NMS, and bound radioligand dissociated from the receptor with atropine (10 µM) with a rate constant of k_{off} = 0.188 ± 0.009 min⁻¹. The presence of either GSK1034702 (10 µM) or TBPB (10 µM) had no effect on the [³H]-NMS dissociation rate (Fig. 3). These data further argue against an allosteric mode of action of GSK1034702 at the orthosteric site of M₁ mAChRs rather than an allosteric interaction as previously reported (Nathan et al., 2013).

Receptor alkylation studies establish orthosteric binding of GSK1034702

GSK1034702 activity in inositol phosphate accumulation (IP₁) assays was compared to ACh, xanomeline and TBPB (Fig. 4A; Table 2). GSK1034702 stimulated robust increases in IP₁ accumulation, reaching approximately 90% of the maximal response elicited by ACh, with nanomolar potency (pEC₅₀ = 7.1 ± 0.1; Fig. 4A). TBPB behaved as a partial agonist with a modest increase in potency relative to GSK1034702 (pEC₅₀ = 7.6 ± 0.2). In membranes prepared from rat cortex, GSK1034702 is a partial agonist with respect to Gαᵣ protein coupling, stimulating approximately 37% of the maximum [³⁵S]-GTPγS Gαᵣ binding elicited by the full agonist oxotremorine-M (pEC₅₀ = 6.7 ± 0.1; Fig. 4B).

In order to verify mechanism of action, we performed receptor alkylation experiments with the orthosteric site covalent binder, phenoxybenzamine, to deplete the level of available and functional muscarinic receptors. Phenoxybenzamine, at a concentration of 3 µM (for 30 min), reduced the functional human M₁ mAChR population in CHO
MOL #111872

Flp-In by ~80%, to an expression level where GSK1034702 had no agonist effect in an IP₁ accumulation assay, but where acetylcholine still yielded a response (Supplementary Fig. 2A-B). Under these conditions establishing if GSK1034702 and TBPB acted as competitive antagonists with respect to acetylcholine would verify the interaction of these compounds with the orthosteric site.

In phenoxybenzamine-treated cells, GSK1034702 caused a concentration-dependent, parallel rightward shift in ACh-stimulated IP₁ accumulation (Fig. 5A) consistent with a competitive antagonist. This effect was similar to that of TBPB (Fig. 5B), which was reported previously to act as a competitive antagonist in a similar preparation (Keov et al., 2013). Analysis of these data using a modified form of the Gaddum and Schild equations yielded Schild slopes approximating to unity and pA₂ values of 6.2 ± 0.2 and 7.0 ± 0.1 for GSK1034702 and TBPB antagonism of ACh-stimulated responses, respectively (Table 3).

The prototypical PAM, BQCA, potentiates ACh, but not GSK1034702 affinity

Having established that GSK1034702 interacts competitively with ACh at the orthosteric site, radioligand binding experiments were designed to establish if the mode of GSK1034702 binding at the orthosteric pocket was equivalent to acetylcholine. In these studies the potentiation of orthosteric agonist binding by a positive allosteric modulator (PAM) was used to probe the nature of GSK1034702 and TBPB binding. BQCA, a PAM selective for the M₁ mAChR, has previously been shown to potentiate acetylcholine affinity by ~100 fold (Ma et al., 2009; Butcher et al., 2016). Consistent with these previous studies we show here that BQCA potentiates the ACh-mediated displacement of [³H]-NMS, thereby demonstrating positive cooperativity for acetylcholine binding of ~35-fold, consistent with previous reports of modulation according to a two-state model (Canals et al., 2012; Fig 6A). Such actions would predict a similar (if less substantial) effect on GSK1034702 or TBPB. However, the displacement of [³H]-NMS by GSK1034702 (Fig. 6B) or TBPB (Fig. 6C) was not modulated by BQCA. These data demonstrate the probe dependency of BQCA and indicate that either (a) there is neutral cooperativity between BQCA and GSK1034702/TBPB, or (b) that the binding site of GSK1034702 or TBPB simultaneously overlaps with those of both ACh and BQCA.
[$^3$H]-GSK1034702 binding studies further confirm novel orthosteric binding pose

To further characterise the binding site of GSK1034702 at the M₁ mAChR, we generated a radiolabelled version of the GSK1034702 compound ([$^3$H]-GSK1034702) and conducted binding interaction experiments in membranes expressing the human M₁ mAChR. [$^3$H]-GSK1034702 bound in a monophasic manner with moderate affinity ($K_D = 550$ nM; $B_{\text{max}} = 2.6$ pmol/mg protein) (Supplementary Fig. 3A-C). Membranes were incubated with 20 nM [$^3$H]-GSK1034702 in the absence and presence of increasing concentrations of unlabelled GSK1034702, TBPB, ACh and xanomeline (Fig. 7). GSK1034702 and TBPB fully displaced specific [$^3$H]-GSK1034702 binding to the M₁ mAChR in a monophasic manner, whereas ACh and xanomeline only partially displaced [$^3$H]-GSK1034702 binding. These data indicate either an allosteric interaction between GSK1034702 and ACh / xanomeline or a bitopic mechanism consistent with GSK1034702 spanning a binding pocket at the M₁ mAChR partially shared with that of ACh.

DREADD pharmacology confirms atypical mechanism of action of GSK1034702

By introducing point mutations (Y106C and A196G) into the orthosteric binding pocket of the M₁ mAChR, a M₁ DREADD mutant is created (Abdul-Ridha et al., 2013) that displays reduced responsiveness to acetylcholine but instead is activated by clozapine-N-oxide (CNO), a ligand that shows little activity at the wild type M₁ mAChR. We investigated the ability of GSK1034702 to interact with the M₁ DREADD by conducting [$^3$H]-NMS binding, functional IP₁ accumulation and ERK1/2 phosphorylation studies in CHO Flp-In cells expressing the humanised M₁ DREADD (Figs. 8, 9; Supplementary Tables 1-2). The affinity of GSK1034702 for the M₁ DREADD was not significantly different from the affinity for binding at the wild-type M₁ mAChR (wild-type $pK_i = 6.5 \pm 0.2$; DREADD $6.0 \pm 0.2$; Fig. 8A, B) and, as reported previously (Armbruster et al., 2007) (Abdul-Ridha et al., 2013), M₁ mAChR orthosteric agonists, ACh and xanomeline, showed a significant reduction in potency at the M₁ DREADD (Fig. 9). In contrast, GSK1034702 activated the M₁ DREADD with comparable potency and efficacy compared to its activity at the wild-type receptor. Similar results were obtained with TBPB, confirming previous
observations for this compound (Abdul-Ridha et al., 2014) (Fig. 9D-E). Furthermore, we assessed the ability of ACh and GSK1034702 to stimulate phosphorylation of the M₁ mAChR at serine 228 using a phosphorylation-specific antibody (Butcher et al., 2016) Both ACh and GSK1034702 stimulated a concentration-dependent increase in pSer228 immunoreactivity at the wild-type receptor (Fig. 10A-B). The potency of GSK1034702 to stimulate phosphorylation at serine 228 was unchanged at the M₁ DREADD, whereas ACh failed to stimulate a response. These data support the notion that GSK1034702 has a distinct binding mode at the orthosteric site from that of acetylcholine.

**GSK1034702 and TBPB are differentially biased agonists at M₁ mAChR**

Convergent evidence from radioligand binding and functional studies (vide supra and Supplementary Fig. 4; Supplementary Table 3) suggests that GSK1034702 and TBPB bind to the orthosteric site of the M₁ mAChR, but with an orientation or pose that distinguishes them from ACh and xanomeline. To further interrogate their pharmacology, we assessed their ability to engender biased signalling by application of an operational model of agonism to the concentration-response curves of either xanomeline, GSK1034702 or TBPB in both IP₁ and ERK1/2 phosphorylation assays (Evans et al., 2011; Kenakin et al., 2012; Keov et al., 2011) (Table 4). These analyses generated transduction coefficient values for each of these agonists at the two different pathways and allowed us to calculate the bias factor between IP₁ and ERK1/2 phosphorylation, revealing significant differences between ACh and xanomeline/TBPB (P<0.001; one-way ANOVA), with the latter displaying bias toward IP₁ responses (bias factor IP₁ – ERK1/2 phosphorylation = 1.1 and 1.4 for xanomeline and TBPB, respectively). However, no significant differences were revealed for GSK1034702 and ACh, suggesting that GSK1034702 and TBPB, despite apparently similar binding modes, engender differential signalling from the receptor.

**GSK1034702 shows lack of selectivity for M₁ mAChRs**

We evaluated the ability of GSK1034702 to bind to other mAChR subtypes by conducting equilibrium-binding studies on membranes expressing the M₁-, M₂-, M₃-, M₄ or M₅ mAChR, and found that GSK1034702 could inhibit [³H]-NMS binding at
all muscarinic receptor subtypes, albeit with much lower affinity for the M₃ mAChR (Fig. 11A; Table 5). We further assessed the functional activity of GSK1034702 at the M₂-, M₃-, M₄ and M₅ mAChRs in the ERK1/2 phosphorylation assay. GSK1034702 exhibited partial agonist activity at M₂, M₄ and M₅ mAChRs, but was devoid of activity at the M₃ mAChR in this assay (Fig. 11B). Finally, we investigated the ability of GSK1034702 to stimulate negative inotropic responses in isolated rat atria (Fig. 12A-B) or contraction of rat ileum (Fig. 12C-D), indicative of activity at M₂- and M₃ mAChR receptors, respectively. GSK1034702 elicited a robust response in the rat atria, reaching a maximal response equivalent to that of methacholine, with micromolar potency (Fig. 12A). Furthermore, GSK1034702 could inhibit methacholine-induced responses with an IC₅₀ of 8 µM (Fig. 12B). In the rat ileum, GSK1034702 stimulated approximately 50% of the maximal methacholine-induced contraction, with an EC₅₀ of 7 µM (Fig. 12C), and inhibited methacholine-induced contraction with an IC₅₀ of 46 µM (Fig. 12D).
Discussion

The development of the selective M₁ mAChR allosteric agonist GSK1034702 provided the opportunity to test the hypothesis that allosteric M₁ mAChR drugs might provide a clinical advantage over orthosteric M₁ mAChR agents due to increased selectivity whilst yielding fewer side effects. In the nicotine abstinence model of cognitive dysfunction, GSK1034702 significantly improved immediate memory recall but also induced adverse responses consistent with activation of other muscarinic receptor subtypes (Nathan et al., 2013). On face value these data might suggest that allosteric M₁ mAChR drugs offer little or no safety benefit compared to previous investigational agents targeting mAChRs. However, here we provide direct pharmacological evidence that GSK1034702 is not a pure allosteric agonist as previously reported, but rather interacts with the orthosteric binding site and broadly mimics the pharmacology of the known bitopic ligand, TBPB. Based on radioligand binding and functional studies, coupled with the structural similarity between GSK1034702 and TBPB, we conclude that GSK1034702 likely interacts concomitantly with both allosteric and orthosteric sites on the M₁ mAChR in a bitopic manner.

The conclusion that GSK1034702 interacts with the orthosteric site is primarily based on the full inhibition of [³H]-N-methyl scopolamine (NMS) binding and lack of co-operative effects on [³H]-NMS - features consistent with an orthosteric, rather than prototypical allosteric mechanism. In functional assays after receptor alkylation (to diminish its agonist response), GSK1034702 causes a non-saturable, concentration-dependent parallel rightward shift in the ACh-mediated IP response, further confirming an orthosteric mode of action.

That GSK1034702 might also interact with a site distinct from the orthosteric site was indicated most clearly in functional assays at the M₁ mAChR DREADD and by characterising the binding of [³H]-GSK1034702 to the wild-type receptor. The M₁ mAChR DREADD contains mutations at key residues within the orthosteric binding pocket that yields a receptor which is poorly responsive to the cognate ligand, ACh, but instead is activated by an otherwise inert chemical ligand, clozapine-n-oxide (Armbruster et al., 2007; Roth, 2016). As predicted from previous reports (Abdul-Ridha et al., 2014) the potencies of orthosteric ligands acetylcholine and xanomeline in inositol phosphate signaling, ERK1/2 phosphorylation and M₁ receptor
phosphorylation were significantly reduced at the M₁ DREADD. However, the potency and efficacy of GSK1034702 and TBPB were unaffected by the DREADD mutations, suggesting that GSK1034702, like TBPB, is able to activate the M₁ mAChR with a binding mode that is subtly distinct from that of prototypical orthosteric ligands. In support of this conclusion, GSK1034702 and TBPB fully inhibit the binding of [³H]-GSK1034702 to the M₁ mAChR, whereas the prototypical orthosteric agonists, ACh and xanomeline, only partially inhibit its binding. This indicates that GSK1034702 can still bind to the M₁ mAChR when ACh or xanomeline occupy the orthosteric site, suggesting that it can interact with the receptor via an allosteric binding site. The display of apparently both orthosteric (competitive) and allosteric behaviours depending on test system is typical of bitopic ligands that are able to “flip-flop” between binding poses (Valant et al., 2012).

Greater clarity around the receptor mechanism of action of GSK1034702, revealed here, has implications for drug design aimed at the treatment of AD. We have previously demonstrated that the learning and memory deficit observed in murine prion disease is due to a loss of cholinergic signalling in the hippocampus and as such this model replicates one of the key pathological hallmarks associated with human AD (Bradley et al., 2017). In the prion model we found that both M₁ mAChR orthosteric agonists and allosteric modulators completely rescue the learning and memory deficit observed in prion disease. However, we also found that the orthosteric ligand, xanomeline, gave adverse responses consistent with the activation of other muscarinic receptor subtypes whereas the positive allosteric modulator, BQCA, gave no detectable adverse responses at doses that rescued learning and memory (Bradley et al., 2017). Whereas these results together with other studies on the cognitive responses of M₁ mAChR allosteric modulators (Lange et al., 2015; Ma et al., 2009; Puri et al., 2015; Vardigan et al., 2015) support the potential clinical benefit of this class of ligand it is also clear that in rodent models allosteric modulators that either show direct agonism in addition to co-operativity result in adverse effects (Alt et al., 2016; Davoren et al., 2017; Davoren et al., 2016). Hence, we conclude here that to avoid adverse effects clinical candidates targeting the M₁ mAChR in AD would require the following properties (i) high levels of receptor subtype selectivity as would be seen with an allosteric modulator and (ii) low levels of intrinsic agonist activity.
Acknowledgements

We acknowledge the BSU facilities at the Cancer Research UK Beatson Institute (C596/A17196).
Author Contributions

Participated in research design: Bradley, Sexton, Felder, Christopoulos, Broad, Tobin and Langmead
Conducted experiments: Bradley, Molloy, Bundgaard, Mogg, Thompson, Dwomoh, Sanger, Crabtree and Brooke
Contributed new reagents or analytic tools: Felder and Broad
Performed data analysis: Bradley, Christopoulos, Tobin and Langmead
Wrote or contributed to the writing of the manuscript: Bradley, Christopoulos, Broad, Tobin and Langmead
MOL #111872

References

Abdul-Ridha A, Lane JR, Sexton PM, Canals M and Christopoulos A (2013) Allosteric modulation of a chemogenetically modified G protein-coupled receptor. *Mol Pharmacol* **83**(2): 521-530.

Abdul-Ridha A, Lopez L, Keov P, Thal DM, Mistry SN, Sexton PM, Lane JR, Canals M and Christopoulos A (2014) Molecular determinants of allosteric modulation at the M1 muscarinic acetylcholine receptor. *J Biol Chem* **289**(9): 6067-6079.

Alt A, Pendri A, Bertekap RL, Jr., Li G, Benitex Y, Nophsker M, Rockwell KL, Burford NT, Sum CS, Chen J, Herbst JJ, Ferrante M, Hendricson A, Cvijic ME, Westphal RS, O’Connell J, Banks M, Zhang L, Gentles RG, Jenkins S, Loy J and Macor JE (2016) Evidence for Classical Cholinergic Toxicity Associated with Selective Activation of M1 Muscarinic Receptors. *J Pharmacol Exp Ther* **356**(2): 293-304.

Anagnostaras SG, Murphy GG, Hamilton SE, Mitchell SL, Rahnama NP, Nathanson NM and Silva AJ (2003) Selective cognitive dysfunction in acetylcholine M1 muscarinic receptor mutant mice. *Nat Neurosci* **6**(1): 51-58.

Armbruster BN, Li X, Pausch MH, Herlitze S and Roth BL (2007) Evolving the lock to fit the key to create a family of G protein-coupled receptors potently activated by an inert ligand. *Proc Natl Acad Sci U S A* **104**(12): 5163-5168.

Bodick NC, Offen WW, Levey AI, Cutler NR, Gauthier SG, Satlin A, Shannon HE, Tollefson GD, Rasmussen K, Bymaster FP, Hurley DJ, Potter WZ and Paul SM (1997) Effects of xanomeline, a selective muscarinic receptor agonist, on cognitive function and behavioral symptoms in Alzheimer disease. *Archives of neurology* **54**(4): 465-473.

Bradley SJ, Bourgognon JM, Sanger HE, Verity N, Mogg AJ, White DJ, Butcher AJ, Moreno JA, Molloy C, Macedo-Hatch T, Edwards JM, Wess J, Pawlak R, Read DJ, Sexton PM, Broad LM, Steinert JR, Mallucci GR, Christopoulos A, Felder CC and Tobin AB (2017) M1 muscarinic allosteric modulators slow prion neurodegeneration and restore memory loss. *J Clin Invest* **127**(2): 487-499.

Buckley NJ, Bonner TI and Brann MR (1988) Localization of a family of muscarinic receptor mRNAs in rat brain. *J Neurosci* **8**(12): 4646-4652.

Budzik B, Garzya V, Shi D, Walker G, Woolley-Roberts M, Pardoe J, Lucas A, Tehan B, Rivero RA, Langmead CJ, Watson J, Wu Z, Forbes IT and Jin J (2010) Novel N-Substituted Benzimidazolones as Potent, Selective, CNS-Penetrant, and Orally Active M1 mAChR Agonists. *ACS Med Chem Lett* **1**(6): 244-248.

Butcher AJ, Bradley SJ, Prihandoko R, Brooke SM, Mogg A, Bourgognon JM, Macedo-Hatch T, Edwards JM, Bottrill AR, Challiss RA, Broad LM, Felder CC and Tobin AB (2016) An Antibody Biosensor Establishes the Activation of the M1 Muscarinic Acetylcholine Receptor during Learning and Memory. *J Biol Chem* **291**(17): 8862-8875.

Conn PJ, Christopoulos A and Lindsley CW (2009) Allosteric modulators of GPCRs: a novel approach for the treatment of CNS disorders. *Nat Rev Drug Discov* **8**(1): 41-54.

Davoren JE, Garnsey M, Pettersen B, Brodney MA, Edgerton JR, Fortin JP, Grimwood S, Harris AR, Jenkinson S, Kenakin T, Lazzaro JT, Lee CW,
Lotarski SM, Nottebaum L, O’Neil SV, Popiolek M, Ramsey S, Steyn SJ, Thorn CA, Zhang L and Webb D (2017) Design and Synthesis of gamma- and delta-Lactam M1 Positive Allosteric Modulators (PAMs): Convulsion and Cholinergic Toxicity of an M1-Selective PAM with Weak Agonist Activity. *Journal of medicinal chemistry* **60**(15): 6649-6663.

Davoren JE, Lee CW, Garnsey M, Brodney MA, Cordes J, Dlugolenski K, Edgerton JR, Harris AR, Helal CJ, Jenkinson S, Kauffman GW, Kenakin TP, Lazzaro JT, Lotarski SM, Mao Y, Nason DM, Northcott C, Nottebaum L, O’Neil SV, Pettersen B, Popiolek M, Reinhart V, Salomon-Ferrer R, Steyn SJ, Webb D, Zhang L and Grimwood S (2016) Discovery of the Potent and Selective M1 PAM-Agonist N-[(3R,4S)-3-Hydroxytetrahydro-2H-pyran-4-yl]-5-methyl-4-[(4-(1,3-thiazol-4-yl)benzyl]pyridine-2-carboxamide (PF-06767832): Evaluation of Efficacy and Cholinergic Side Effects. *Journal of medicinal chemistry* **59**(13): 6313-6328.

Evans BA, Broxton N, Merlin J, Sato M, Hutchinson DS, Christopoulos A and Summers RJ (2011) Quantification of functional selectivity at the human alpha(1A)-adrenoceptor. *Mol Pharmacol* **79**(2): 298-307.

Jones CK, Brady AE, Davis AA, Xiang Z, Bubser M, Tantawy MN, Kane AS, Bridges TM, Kennedy JP, Bradley SR, Peterson TE, Ansari MS, Baldwin RM, Kessler RM, Deutch AY, Lah JJ, Levey AI, Lindsley CW and Conn PJ (2008) Novel selective allosteric activator of the M1 muscarinic acetylcholine receptor regulates amyloid processing and produces antipsychotic-like activity in rats. *J Neurosci* **28**(41): 10422-10433.

Kenakin T, Watson C, Muniz-Medina V, Christopoulos A and Novick S (2012) A simple method for quantifying functional selectivity and agonist bias. *ACS chemical neuroscience* **3**(3): 193-203.

Keov P, Lopez L, Devine SM, Valant C, Lane JR, Scammells PJ, Sexton PM and Christopoulos A (2014) Molecular mechanisms of bitopic ligand engagement with the M1 muscarinic acetylcholine receptor. *J Biol Chem* **289**(34): 23817-23837.

Keov P, Sexton PM and Christopoulos A (2011) Allosteric modulation of G protein-coupled receptors: a pharmacological perspective. *Neuropsychopharmacology* **36**(1): 24-35.

Lange HS, Cannon CE, Drott JT, Kuduk SD and Uslaner JM (2015) The M1 Muscarinic Positive Allosteric Modulator PQCA Improves Performance on Translatable Tests of Memory and Attention in Rhesus Monkeys. *J Pharmacol Exp Ther* **355**(3): 442-450.

Langmead CJ and Christopoulos A (2006) Allosteric agonists of 7TM receptors: expanding the pharmacological toolbox. *Trends Pharmacol Sci* **27**(9): 475-481.

Langmead CJ, Watson J and Reavill C (2008) Muscarinic acetylcholine receptors as CNS drug targets. *Pharmacol Ther* **117**(2): 232-243.

Leach K, Loiacono RE, Felder CC, McKinzie DL, Mogg A, Shaw DB, Sexton PM and Christopoulos A (2010) Molecular mechanisms of action and in vivo validation of an M4 muscarinic acetylcholine receptor allosteric modulator with potential antipsychotic properties. *Neuropsychopharmacology* **35**(4): 855-869.
Lebon G, Langmead CJ, Tehan BG and Hulme EC (2009) Mutagenic mapping suggests a novel binding mode for selective agonists of M1 muscarinic acetylcholine receptors. *Mol Pharmacol* **75**(2): 331-341.

Levey AI, Edmunds SM, Koliatsos V, Wiley RG and Heilman CJ (1995) Expression of m1-m4 muscarinic acetylcholine receptor proteins in rat hippocampus and regulation by cholinergic innervation. *J Neurosci* **15**(5 Pt 2): 4077-4092.

Ma L, Seager MA, Wittmann M, Jacobson M, Bickel D, Burno M, Jones K, Graufelds VK, Xu G, Pearson M, McCampbell A, Gaspar R, Shughrue P, Danziger A, Regan C, Flick R, Pasquarella D, Garson S, Doran S, Kreatsoulas C, Veng L, Lindsley CW, Shipe W, Kuduk S, Sur C, Kinney G, Seabrook GR and Ray WJ (2009) Selective activation of the M1 muscarinic acetylcholine receptor achieved by allosteric potentiation. *Proc Natl Acad Sci U S A* **106**(37): 15950-15955.

May LT, Leach K, Sexton PM and Christopoulos A (2007) Allosteric modulation of G protein-coupled receptors. *Annu Rev Pharmacol Toxicol* **47**: 1-51.

Nathan PJ, Watson J, Lund J, Davies CH, Peters G, Dodds CM, Swirski B, Lawrence P, Bentley GD, O’Neill BV, Robertson J, Watson S, Jones GA, Maruff P, Croft RJ, Laruelle M and Bullmore ET (2013) The potent M1 receptor allosteric agonist GSK1034702 improves episodic memory in humans in the nicotine abstinence model of cognitive dysfunction. *Int J Neuropsychopharmacol* **16**(4): 721-731.

Puri V, Wang X, Vardigan JD, Kuduk SD and Uslaner JM (2015) The selective positive allosteric M1 muscarinic receptor modulator PQCA attenuates learning and memory deficits in the Tg2576 Alzheimer’s disease mouse model. *Behav Brain Res* **287**: 96-99.

Roth BL (2016) DREADDs for Neuroscientists. *Neuron* **89**(4): 683-694.

Spalding TA, Trotter C, Skjaerbaek N, Messier TL, Currier EA, Burstein ES, Li D, Hacksell U and Brann MR (2002) Discovery of an ectopic activation site on the M(1) muscarinic receptor. *Mol Pharmacol* **61**(6): 1297-1302.

Valant C, Robert Lane J, Sexton PM and Christopoulos A (2012) The best of both worlds? Bitopic orthosteric/allosteric ligands of g protein-coupled receptors. *Annu Rev Pharmacol Toxicol* **52**: 153-178.

van der Westhuizen ET, Breton B, Christopoulos A and Bouvier M (2014) Quantification of ligand bias for clinically relevant beta2-adrenergic receptor ligands: implications for drug taxonomy. *Mol Pharmacol* **85**(3): 492-509.

Vardigan JD, Cannon CE, Puri V, Dancho M, Koser A, Wittmann M, Kuduk SD, Renger JJ and Uslaner JM (2015) Improved cognition without adverse effects: novel M1 muscarinic potentiator compares favorably to donepezil and xanomeline in rhesus monkey. *Psychopharmacology (Berl)* **232**(11): 1859-1866.

Witkin JM, Ornstein PL, Mitch CH, Li R, Smith SC, Heinz BA, Wang XS, Xiang C, Carter JH, Anderson WH, Li X, Broad LM, Pasqui F, Fitzjohn SM, Sanger HE, Smith JL, Catlow J, Swanson S and Monn JA (2017) In vitro pharmacological and rat pharmacokinetic characterization of LY3020371, a potent and selective mGlu2/3 receptor antagonist. *Neuropharmacology* **115**: 100-114.
Young SL, Bohenek DL and Fanselow MS (1995) Scopolamine impairs acquisition and facilitates consolidation of fear conditioning: differential effects for tone vs context conditioning. *Neurobiol Learn Mem* 63(2): 174-180.
Footnotes

This work was supported in part by a Wellcome Trust Collaborative Award [201529/Z/16/Z], a Royal Society International Exchanges Scheme award [IE131060], a Medical Research Council MICA award [MR/P019366/1] and by the Eli Lilly Company.

Please send reprint requests to Prof. Andrew B. Tobin, Institute of Molecular, Cell and Systems Biology, College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow, Scotland, G12 8QQ. Email: andrew.tobin@glasgow.ac.uk.
Figure Legends

Figure 1
Effects of (A) xanomeline (1, 3, 10 and 30 mg/kg), (B) GSK1034702 (0.3, 1, 3, 10 and 30 mg/kg) and (C) TBPB (1, 3, 10 and 30 mg/kg) on scopolamine (1.5 mg/kg) induced impairments in contextual fear conditioning (inset chemical structures of compounds). Data shown are means ± S.E.M. of ≥ 8 mice per group. Data were analysed using a one-way ANOVA with Tukey’s multiple comparison test, where *P<0.05, **P<0.01, ***P<0.001 versus vehicle alone and #P<0.05 versus 1.5 mg/kg scopolamine. Total concentration (Log M) of (D) xanomeline, (E) GSK1034702 and (F) TBPB measured in plasma or brain samples 30 minutes after i.p. injection with increasing concentrations of respective compound. Data shown are means ± S.E.M. of 3 mice per concentration.

Figure 2
Displacement of [$^3$H]-NMS binding by ACh, xanomeline, GSK1034702 or TBPB at the human M1 mAChR expressed in CHO Flp-In cell monolayers. Experiments were performed against a K_D concentration of [$^3$H]-NMS. Non-specific binding was determined by addition of 10 µM atropine. Data are the means ± S.E.M. of 3-5 independent experiments performed in duplicate.

Figure 3
Dissociation of [$^3$H]-NMS with atropine in the absence or presence of (A) GSK1034702 or (B) TBPB in membranes expressing the human M1 receptor. Membranes were incubated with a K_D concentration of [$^3$H]-NMS for 60 minutes at 37°C, followed by dissociation of bound radioligand with atropine (10 µM) alone or in the presence of GSK1034702 (10 µM) or TBPB (10 µM). Data shown are mean of three independent experiments performed in duplicate.

Figure 4
(A) Inositol phosphate accumulation elicited by ACh, xanomeline, GSK1034702 or TBPB via the human M<sub>1</sub> receptor expressed in CHO Flp-In cells. Data are expressed as means ± S.E.M. of 3-10 independent experiments performed in duplicate. (B) [<sup>35</sup>S]-GTPγS binding to rat frontal cortex membranes. Data are the percentage of the maximal [<sup>35</sup>S]-GTPγS binding stimulated by oxotremorine-M (mean pEC<sub>50</sub> = 6.68 ± 0.13, n=3).

**Figure 5**

GSK1034702 (A) or TBPB (B) antagonism of ACh-stimulated inositol phosphate accumulation in CHO Flp-In cells expressing the human M<sub>1</sub> receptor. Cells were incubated with 3 µM phenoxybenzamine to irreversibly reduce receptor expression prior to addition of GSK1034702 or TBPB. Data are means ± S.E.M. of 3 independent experiments performed in duplicate.

**Figure 6**

Displacement of [<sup>3</sup>H]-NMS binding by (A) ACh, (B) GSK1034702 or (C) TBPB in the presence of increasing concentrations of BQCA at the human M<sub>1</sub> mAChR expressed in CHO Flp-In cell monolayers. Experiments were performed against a K<sub>D</sub> concentration of [<sup>3</sup>H]-NMS. Non-specific binding was determined by addition of 10 µM atropine. Data are the means ± S.E.M. of 3 independent experiments performed in duplicate.

**Figure 7**

Displacement of [<sup>3</sup>H]-GSK1034702 binding by ACh, xanomeline, GSK1034702 or TBPB at membranes expressing the human M<sub>1</sub> mAChR. Experiments were performed against 20 nM [<sup>3</sup>H]-GSK1034702 and non-specific binding was determined in the presence of 100 µM non-radiolabelled GSK1034702. Data are expressed as means ± S.E.M. of 3 independent experiments performed in duplicate.

**Figure 8**

This article has not been copyedited and formatted. The final version may differ from this version.
Displacement of $[^3]$H-NMS binding by ACh, CNO, xanomeline, GSK1034702 or TBPB at the humanised M$_1$ DREADD expressed in CHO Flp-In cell monolayers. Experiments were performed against a $K_D$ concentration of $[^3]$H-NMS. Non-specific binding was determined by addition of 10 $\mu$M atropine. Data are the means ± S.E.M. of 3-5 independent experiments performed in duplicate. (B) Comparison of p$K_i$ values for each of the compounds used at the wild-type M$_1$ mAChR or the mutant M$_1$ DREADD.

Figure 9

Inositol phosphate accumulation (left) or ERK1/2 phosphorylation (right) elicited by (A) ACh, (B) xanomeline, (C) CNO, (D) GSK1034702 or (E) TBPB via the humanised M$_1$ DREADD expressed in CHO Flp-In cells. The dashed curve represents the response of the ligand at the wild-type M$_1$ mAChR. Data are expressed as means ± S.E.M. of 3-4 independent experiments performed in duplicate.

Figure 10

(A) Phosphorylation at serine 228 elicited by ACh (left) or GSK1034702 (right) in CHO Flp-In cells expressing either M$_1$ WT (top) or M$_1$ DREADD (bottom). (B) Mean densitometric data showing phosphorylation at serine 228 as a % of the maximal response. Data were normalised to the total receptor expression, assessed using a HA antibody. Data are expressed as means ± S.E.M. of 3 independent experiments.

Figure 11

(A) Displacement of $[^3]$H-NMS binding by GSK1034702 in CHO membranes expressing M$_1$-, M$_2$-, M$_3$-, M$_4$- or M$_5$ mAChRs. Experiments were performed against a $K_D$ concentration of $[^3]$H-NMS. Non-specific binding was determined by addition of 10 $\mu$M atropine. Data are the means ± S.E.M. of 3 independent experiments. (B) ERK1/2 phosphorylation elicited by GSK1034702 at the M$_1$-, M$_2$-, M$_3$- M$_4$- or M$_5$ mAChR expressed in CHO cells. Data are expressed as a percentage of the maximum response stimulated by ACh, and are means ± S.E.M. of 3 experiments performed in duplicate.
Figure 12

Assessment of the effects of GSK1034703 activity at M₂- (A-B) and M₃ (C-D) mAChRs in *ex vivo* tissue preparations. The ability of GSK1034702 to stimulate negative inotropy in rat atria (A) or inhibit methacholine (1µM)-induced responses (B) was assessed. Activity of GSK1034702 at M₃ mAChR was assessed by measurement of rat ileum contraction relative to methacholine responses (C) or inhibition of methacholine-induced contraction (D). Data shown are a single experiment.
Table 1

|                | pKᵢ      | n  |
|----------------|----------|----|
| ACh            | 4.1 ± 0.3| 5  |
| Xanomeline     | 7.0 ± 0.1| 4  |
| GSK1034702     | 6.5 ± 0.2| 3  |
| TBPB           | 6.8 ± 0.1| 3  |

Affinity estimates for the competition between [³H]-NMS and ACh, xanomeline, GSK1034702 or TBPB at the M₁ mAChR. Values stated are the negative logarithms of the equilibrium dissociation constant (pKᵢ). Data are calculated from the means ± S.E.M. of 3-5 independent experiments performed in duplicate.
Table 2

|        | E<sub>max</sub> | pEC<sub>50</sub> | n  |
|--------|----------------|-----------------|----|
| ACh    | 100            | 7.1 ± 0.1       | 10 |
| Xanomeline | 98.0 ± 1.5   | 8.2 ± 0.1       | 3  |
| GSK1034702 | 90.1 ± 2.9   | 7.1 ± 0.1       | 3  |
| TBPB   | 55.2 ± 3.1     | 7.6 ± 0.2       | 4  |

Maximum agonist effect and potency of ACh, xanomeline, GSK1034702 and TBPB at stimulating IP<sub>1</sub> accumulation in CHO Flp-In human M<sub>1</sub> cells. Data are expressed as means ± S.E.M. of 3-10 independent experiments performed in duplicate.
Table 3

|              | GSK1034702 | TBPB  | n  |
|--------------|------------|-------|----|
| $pA_2$       | 6.2 ± 0.2  | 7.0 ± 0.1 | 3  |
| Schild slope | 1.1 ± 0.1  | 1.2 ± 0.1 | 3  |

Potency estimates of the antagonism of ACh-stimulated IP$_1$ accumulation by GSK1034702 or TBPB in CHO Flp-In cells expressing the human M$_1$ mAChR. Data are means ± S.E.M. of 3 independent experiments performed in duplicate.
Table 4

|               | IP<sub>1</sub> accumulation | ERK 1/2 phosphorylation | Log Bias Factor |
|---------------|----------------------------|-------------------------|-----------------|
|               | Log<sub>10</sub>(τ/K<sub>A</sub>) | ΔLog<sub>10</sub>(τ/K<sub>A</sub>) | Log<sub>10</sub>(τ/K<sub>A</sub>) | ΔLog<sub>10</sub>(τ/K<sub>A</sub>) | ΔΔLog<sub>10</sub>(τ/K<sub>A</sub>) |
| ACh          | 7.10± 0.1 | 0.0 ± 0.1 | 7.1 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.1 |
| Xanomeline   | 8.2 ± 0.1 | 1.1 ± 0.1 | 7.1 ± 0.3 | 0.0 ± 0.3 | 1.1 ± 0.1 |
| GSK1034702   | 7.4 ± 0.2 | 0.3 ± 0.2 | 7.2 ± 0.1 | 0.1 ± 0.1 | 0.2 ± 0.2 |
| TBPB         | 8.2 ± 0.2 | 1.1 ± 0.2 | 6.9 ± 0.4 | -0.3 ± 0.4 | 1.4 ± 0.2 |

Transduction coefficients (Log<sub>10</sub>(τ/K<sub>A</sub>)), normalised (reference ligand ACh) transduction coefficients (ΔLog<sub>10</sub>(τ/K<sub>A</sub>)) and bias factors (ΔΔLog<sub>10</sub>(τ/K<sub>A</sub>)) for IP<sub>1</sub> accumulation and phosphorylation of ERK1/2 at the wild-type M<sub>1</sub> mAChR.
Table 5

|      | pKᵢ       | n  |
|------|-----------|----|
| M₁   | 6.0 ± 0.1 | 3  |
| M₂   | 5.4 ± 0.1 | 3  |
| M₃   | n.d.      | 3  |
| M₄   | 5.7 ± 0.1 | 3  |
| M₅   | 5.2 ± 0.1 | 3  |

Values stated are the negative logarithms of the equilibrium dissociation constant (pKᵢ) of GSK1034702 binding to M₁-M₅ mAChRs. Data are calculated from the means ± S.E.M. of 3 independent experiments performed in duplicate. n.d.; not determined.
Figure 1

A. Xanomeline

B. GSK1034702

C. TBPB

D. Unbound [Xanomeline] (Log M) vs. [Xanomeline] (mg/kg; i.p.)

E. Unbound [GSK1034702] (Log M) vs. [GSK1034702] (mg/kg; i.p.)

F. Total [TBPB] (Log M) vs. [TBPB] (mg/kg; i.p.)
Figure 2

% $[^3H]$-NMS binding vs. Log [Agonist] (M).

- ○ ACh
- △ Xanomeline
- □ GSK1034702
- ◇ TBPB
Figure 4

A. 

\[ \text{IP}_1 \frac{A_{665nm}}{B_{620nm}} \times 100 \% \text{ of max. ACh} \]

- \( \circ \) ACh
- \( \triangle \) Xanomeline
- \( \square \) GSK1034702
- \( \Diamond \) TBPB

Log [Agonist] (M)

B. 

\[ [\text{S}]^3 \text{GTP} \gamma \text{S Bound} \times 100 \% \text{ of max. OXO-M} \]

Log [GSK1034702] (M)

This article has not been copyedited and formatted. The final version may differ from this version.
Figure 5

A.

B.

\[
\text{IP}_1 \text{A}_{665\text{nm}}/\text{B}_{620\text{nm}} (\% \text{ of max. ACh})
\]

\[
\text{Log [ACh] (M)}
\]

\[
\text{[GSK1034702] (μM)}
\]

0

0.01

0.1

1

3

10

30

\[
\text{Log [ACh] (M)}
\]

\[
\text{[TBPB] (μM)}
\]

0

0.01

0.1

0.3

1

3

10
Figure 6

A. %[^3]H-NMS binding vs Log [ACH] (M)

B. %[^3]H-NMS binding vs Log [GSK1034702] (M)

C. %[^3]H-NMS binding vs Log [TBPB] (M)

[BQCA] (μM)
- 0
- 1
- 10
- 100
Figure 7

The graph shows the log of agonist concentration (M) on the x-axis and the percent of [3H]-GSK1034702 binding on the y-axis. Different markers represent different agonists:

- Circles: ACh
- Triangles: Xanomeline
- Squares: GSK1034702
- Diamonds: TBPB

The graph demonstrates the binding affinity of various agonists across different concentrations.

This article has not been copyedited and formatted. The final version may differ from this version.

Molecular Pharmacology Fast Forward. Published on April 25, 2018 as DOI: 10.1124/mol.118.111872
Figure 10

A. 

| ACh (nM) | GSK1034702 (nM) |
|----------|-----------------|
| 0        | 0               |
| 0.1      | 0.1             |
| 1.0      | 1.0             |
| 10       | 10              |
| 100      | 100             |
| 1000     | 1000            |

Phosphorylated M₁-receptor (Anti-pS228)

Total M₁-receptor (Anti-HA)

B. 

Phosphorylation at pS228 (% of max. response)

Log [ACh] (M)

Log [GSK1034702] (M)
Figure 12

A. Rat atria (M<sub>2</sub>) stimulation

B. Rat atria (M<sub>2</sub>) inhibition

C. Rat ileum (M<sub>3</sub>) stimulation

D. Rat ileum (M<sub>3</sub>) inhibition

% Response vs. [GSK1034702] (µM)

% Inhibition vs. [GSK1034702] (µM)