

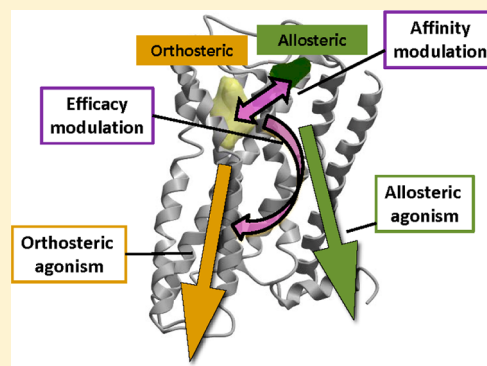
# Development of $M_1$ mAChR Allosteric and Bitopic Ligands: Prospective Therapeutics for the Treatment of Cognitive Deficits

Briana J. Davie,<sup>‡,†</sup> Arthur Christopoulos,<sup>\*,‡</sup> and Peter J. Scammells<sup>\*,†</sup>

<sup>†</sup>Medicinal Chemistry and <sup>‡</sup>Drug Discovery Biology, Monash Institute of Pharmaceutical Sciences, Monash University, 381 Royal Parade, Parkville VIC 3052, Australia

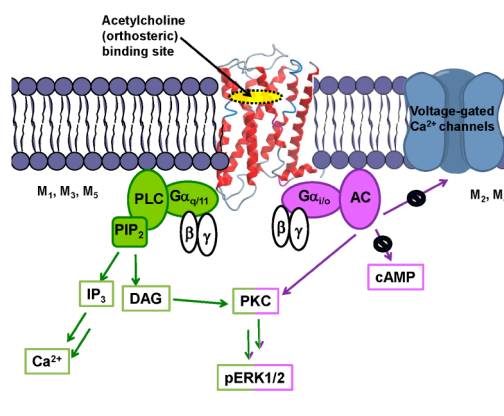
**ABSTRACT:** Since the cholinergic hypothesis of memory dysfunction was first reported, extensive research efforts have focused on elucidating the mechanisms by which this intricate system contributes to the regulation of processes such as learning, memory, and higher executive function. Several cholinergic therapeutic targets for the treatment of cognitive deficits, psychotic symptoms, and the underlying pathophysiology of neurodegenerative disorders, such as Alzheimer's disease and schizophrenia, have since emerged. Clinically approved drugs now exist for some of these targets; however, they all may be considered suboptimal therapeutics in that they produce undesirable off-target activity leading to side effects, fail to address the wide variety of symptoms and underlying pathophysiology that characterize these disorders, and/or afford little to no therapeutic effect in subsets of patient populations. A promising target for which there are presently no approved therapies is the  $M_1$  muscarinic acetylcholine receptor ( $M_1$  mAChR). Despite avid investigation, development of agents that selectively activate this receptor via the orthosteric site has been hampered by the high sequence homology of the binding site between the five muscarinic receptor subtypes and the wide distribution of this receptor family in both the central nervous system (CNS) and the periphery. Hence, a plethora of ligands targeting less structurally conserved allosteric sites of the  $M_1$  mAChR have been investigated. This Review aims to explain the rationale behind allosterically targeting the  $M_1$  mAChR, comprehensively summarize and critically evaluate the  $M_1$  mAChR allosteric ligand literature to date, highlight the challenges inherent in allosteric ligand investigation that are impeding their clinical advancement, and discuss potential methods for resolving these issues.

**KEYWORDS:**  $M_1$  mAChR, allosteric ligands, Alzheimer's disease, schizophrenia, cognitive deficits, memory



The muscarinic acetylcholine receptor (mAChR) family is a group of rhodopsin-like (Family A) G protein-coupled receptors (GPCRs) consisting of five distinct subtypes  $M_1$ – $M_5$ . Activation of the  $M_1$ ,  $M_3$ , and  $M_5$  receptor subtypes primarily results in coupling to the  $G_{q/11}$  family of G proteins, activation of phospholipase C (PLC), release of inositol-1,4,5-trisphosphate ( $IP_3$ ), and subsequent mobilization of intracellular calcium  $Ca^{2+}$ . Activation of the  $M_2$  and  $M_4$  receptor subtypes primarily results in coupling to the  $G_{i/o}$  family of G proteins, inhibition of adenylate cyclase (AC), reduction in cyclic AMP (cAMP), and a decrease in neurotransmitter release via the blockage of voltage-gated calcium channels (Figure 1). These pathways represent a generalized view of each receptor's coupling capacity, as all five subtypes couple to a broader range of G protein- and non-G protein-mediated signaling and regulatory pathways,<sup>1</sup> ultimately leading to the regulation of enzymes and neurotransmitters critical for intercellular chemical communication and biological function. In a broader molecular sense, this diversity is largely facilitated by the ability of GPCRs to adopt a range of conformational states that can lead to distinct functional outcomes.<sup>2</sup>

The relative distribution of the five mAChR subtypes in both the CNS and peripheral tissues was elucidated by mRNA hybridization and immunocytochemical methods.<sup>3,4</sup> The  $M_1$



**Figure 1.** Common signal transduction pathways of the five muscarinic acetylcholine receptors.

mAChR is predominantly expressed postsynaptically in the CNS, particularly in regions of the hippocampus, prefrontal cortex, and striatum. The  $M_2$  and  $M_3$  mAChRs are expressed

Received: April 11, 2013

Accepted: May 9, 2013

Published: May 9, 2013



both pre- and postsynaptically in different brain regions, most notably the basal forebrain, the thalamus, and the hippocampus. Furthermore, the peripheral tissues mostly express the  $M_2$  and  $M_3$  subtypes, particularly in cardiac and smooth muscle tissues. The  $M_4$  and  $M_5$  mAChRs are almost exclusively expressed in the CNS, the former presynaptically in the striatum and the hippocampus, and the latter in the substantia nigra.

This broad distribution is suggestive of muscarinic receptors mediating a diverse range of biological functions and hence playing potentially critical roles in a number of central and peripheral disease processes. This Review will henceforth focus on the role of the muscarinic system in the regulation of learning and memory in the CNS, specifically, the utility of targeting the  $M_1$  mAChR for the treatment of neurodegenerative disorders, such as Alzheimer's disease (AD) and schizophrenia.

AD is a condition of the CNS with no definitive cause and no known cure. Four key "hallmarks" of this disease are cognitive decline in memory and learning, the dysfunction and eventual death of cholinergic neurons, the accumulation of  $\beta$ -amyloid plaques, and the formation of neurofibrillary tangles as a result of tau-protein hyperphosphorylation.<sup>5</sup> Schizophrenia is a debilitating disorder most likely caused by a combination of genetic and environmental factors, though the biological origin is unknown. The disease manifests as four "symptom domains":<sup>6</sup> positive (hallucinations, delusions), negative (social withdrawal, lack of initiative), cognitive (attention deficit and impaired memory), and affective (anxiety, depression, aggression, and suicidal tendencies).

A poorly treated symptom of both disorders is cognitive impairment, which manifests as dysfunctions in memory consolidation, learning, and higher-order processing. While disruption of multiple neurotransmitter systems may be implicated in these deficits,<sup>7</sup> considerable evidence suggests that impaired cortical-hippocampal cholinergic signaling plays an integral part in the manifestation of these conditions.<sup>6</sup> The advent of research tools such as receptor knockout (KO) mice and subtype-selective muscarinic ligands has afforded significant advances in our understanding of the role of the cholinergic system in the physiology and pathophysiology of cognition.<sup>8,9</sup> In particular,  $M_1$  mAChR activation by subtype-selective allosteric enhancement has shown considerable promise as a means of ameliorating cognitive decline and treating disease state pathophysiology.<sup>10</sup> However, the lack of structural information about the  $M_1$  mAChR allosteric binding sites and the complex behavior of allosteric ligands is such that rational drug design and preclinical pharmacological evaluation are fraught with challenges impeding the development of viable therapeutics. This Review will discuss essential considerations in the classification and pharmacological evaluation of allosteric ligands, summarize the  $M_1$  mAChR allosteric ligands reported to date, and offer a perspective on the pharmacological methods employed in their development.

## ■ THE CHOLINERGIC HYPOTHESIS OF MEMORY DYSFUNCTION

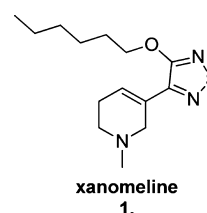
The culmination of considerable biochemical, electrophysiological, and pharmacological evidence gained from studies in elderly patients gave rise to the cholinergic hypothesis of memory dysfunction some decades ago.<sup>11</sup> Four critical observations that unequivocally validated this hypothesis were as follows:

- (i) the substantial loss of presynaptic cholinergic basal forebrain cortical projection neurons in human post-mortem Alzheimer's-diseased brains;<sup>12</sup>
- (ii) the dysfunction of cholinergic markers, such as acetylcholine (ACh), choline (a precursor of ACh), and choline acetyl transferase (an enzyme for ACh production), observed in the human post-mortem brains of patients suffering from cognitive deficits;<sup>13</sup>
- (iii) the behavioral cognitive impairments observed upon pharmacological disruption of cholinergic activity;<sup>11</sup> and
- (iv) the improvement in cognition observed in elderly patients upon artificial enhancement of cholinergic activity<sup>11</sup>

Accordingly, cholinergic enhancement via inhibition of the enzyme responsible for ACh degradation, cholinesterase, was pursued, giving rise to the four anticholinesterase drugs currently approved for the treatment of the various stages of AD: tacrine, donepezil, rivastigmine, and galantamine. While widely used, these drugs have a propensity for nonselective activity, leading to a swathe of side effects associated with excessive cholinergic stimulation, such as diarrhea, hypersalivation, and bradycardia. The failing of the anticholinesterases is unsurprising considering that they target an enzyme present at the synapses of all cholinergic neurons, likely resulting in broad spectrum, abrupt muscarinic activation in whatever tissue compartment that these drugs are distributed. Furthermore, these therapies have been shown to elicit little to no therapeutic efficacy in up to 75% of the patient population,<sup>14</sup> which is possibly due to the progressive degeneration of presynaptic cholinergic nerve terminals as the disease state progresses.

## ■ XANOMELINE: AN IMPORTANT PROOF-OF-CONCEPT COMPOUND

An alternative method by which cholinergic enhancement may be achieved is to mimic the actions of ACh, namely, by selective mAChR activation (and nAChR activation, which is beyond the scope of this Review). Important proof-of-concept for selective mAChR activation as a potential therapy for the cognitive deficits associated with neurodegenerative conditions came with the discovery of the compound xanomeline (1, Figure 2),



**Figure 2.** Structure of the  $M_1/M_4$  mAChR-preferring agonist xanomeline (1).

a  $M_1/M_4$ -preferring agonist (see Mirza et al.<sup>15</sup> for a comprehensive review). This compound produced increased brain ACh levels, demonstrated a reversal of antagonist-induced memory block following IP administration in mice, and attenuated cognitive decline in a 6 month clinical trial in human AD patients following oral administration.<sup>16</sup>

Studies in  $M_1$ - $M_5$  muscarinic receptor KO mice directly linking the absence of each receptor subtype to specific biochemical, physiological, and behavioral changes have enabled assignment of each receptor with likely biological

functions.<sup>8</sup>  $M_1$  mAChR KO mice exhibited deficits in tasks most likely involving neural communication between the cerebral cortex and the hippocampus. Moreover, activation of the  $M_1$  mAChR was found to be the predominant mediator of the effects on cognition, attention, and learning that were observed following xanomeline administration;<sup>17</sup> these effects were attenuated in  $M_1$  mAChR KO mice (but not  $M_4$  mAChR KOs).

In addition to validating the therapeutic utility of  $M_1$  mAChR targeting, xanomeline also validated  $M_4$  mAChR activation as a potential treatment for psychosis, demonstrating *in vivo* efficacy in both animal<sup>18</sup> and human<sup>19</sup> clinical trials, and exhibiting a similar therapeutic profile to the atypical antipsychotics clozapine and olanzapine.<sup>15</sup> The attenuation of amphetamine-induced psychotic behaviors observed with administration of xanomeline was absent in  $M_4$  mAChR KO mice (but remained present in  $M_1$  mAChR KOs).<sup>20</sup> This antipsychotic action is postulated to occur via indirect modulation of dopamine levels; the  $M_4$  mAChR is highly expressed within the mesolimbic dopaminergic system, commonly associated with positive symptoms of schizophrenia.<sup>21</sup>

Unfortunately, while xanomeline exhibits some degree of selectivity as a  $M_1/M_4$  mAChR agonist, binding studies in cloned human muscarinic receptors revealed no preferential binding affinity for these two subtypes in relation to the others,<sup>22</sup> a factor that likely contributed to the peripheral cholinergic side effects that prompted the withdrawal of 52% of AD subjects in a phase III trial of xanomeline.<sup>9</sup> This, combined with its vastly different efficacy and selectivity profiles depending on the *in vitro* experimental paradigm utilized,<sup>15</sup> its off-target activity at dopaminergic and 5-HT receptor subtypes,<sup>23</sup> and the compound's metabolic instability and high hepatic first pass effect leading to <1% oral bioavailability in both animals and humans,<sup>15</sup> has rendered xanomeline an insufficient clinical candidate. Despite this clinical failure, the  $M_1$  mAChR target validation afforded by xanomeline sparked considerable research interest into deconvoluting the mechanisms by which this receptor mediates cognitive processing.

### THE ROLE OF THE $M_1$ mAChR IN COGNITION

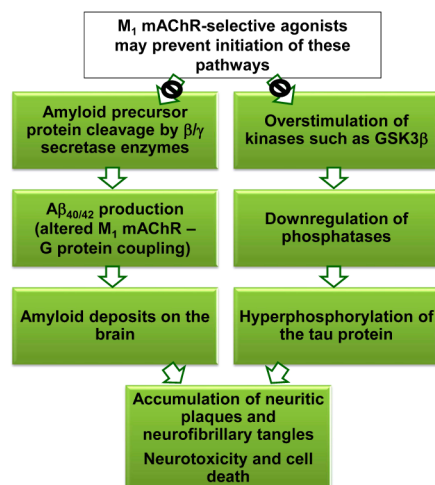
$M_1$  mAChRs are most abundantly expressed postsynaptically in regions of the forebrain such as the hippocampus, striatum, and cerebral cortex.<sup>3</sup> Stimulation of  $M_1$  mAChRs activates multiple signaling pathways leading to the production of numerous downstream effector molecules, each of which is likely to contribute to the overall physiological functioning of  $M_1$  mAChRs, as well as the utility of targeting these receptors for the treatment of cognitive deficits. One such example is the protein kinase C (PKC) family, important signaling enzymes that are produced following  $G\alpha_{q/11}$  pathway activation and are significantly reduced in the Alzheimer's diseased brain<sup>24</sup> (for a more detailed summary of the potential effects of PKC in cognition and Alzheimer's disease, see a recent publication by Fisher<sup>25</sup>). Additionally, it has been demonstrated that activation of  $M_1$  mAChRs leads to potentiation of currents through the *N*-methyl-D-aspartate (NMDA) receptor in hippocampal pyramidal cells,<sup>26</sup> a receptor shown to play a critical role in synaptic plasticity and memory consolidation.<sup>27</sup>

Herein lies one of the key theoretical advantages of postsynaptic  $M_1$  mAChR activation. Unlike the anticholinesterases, this mode of treatment does not rely on the presence of intact presynaptic cholinergic nerve terminals to be effective, neurons that are significantly compromised in neurodegener-

ative disorders.<sup>28</sup> Additionally, it has been shown that, in AD cortical tissue, postsynaptic  $M_1$  mAChR density is unaltered<sup>29</sup> (and in a more recent study significantly increased<sup>30</sup>), establishing the availability of the target in the most critically affected brain region of patients, regardless of the extent of cognitive decline.

### THE ROLE OF THE $M_1$ mAChR IN MODIFYING AD PATHOPHYSIOLOGY

A significant advantage of  $M_1$  mAChR activation for the treatment of Alzheimer's disease is the potential for positive modification of the underlying pathophysiology of the condition that leads to cognitive symptoms in the first place, affording a prospective dual action treatment for AD unable to be accomplished by anticholinesterase therapies. Two widely acknowledged pathophysiological hallmarks of AD are the accumulation of  $\beta$ -amyloid plaques and the formation of neurofibrillary tangles as a result of tau-protein hyperphosphorylation,<sup>5</sup> "vicious cycles" that both lead to substantive CNS cholinergic neural death in the cortical and hippocampal regions of the basal forebrain (flowchart depicted in Figure 3).

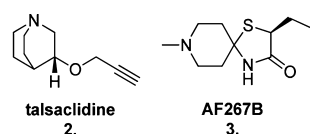


**Figure 3.** Two pathophysiological pathways underlying Alzheimer's disease.

Amyloid precursor protein (APP) has been linked to functions such as regulation of synaptic interactions and neuroplasticity.<sup>31</sup> APP can be differentially cleaved by secretase enzymes into amyloidogenic and nonamyloidogenic metabolites which forms the basis for the pathophysiology of Alzheimer's disease and the prospect of  $M_1$  mAChR activation as a potential treatment. The amyloidogenic pathway is characterized by  $\beta/\gamma$  secretase cleavage of APP, resulting in peptides such as  $A\beta_{40}$  and  $A\beta_{42}$ , the primary constituents of the aggregated amyloid neuritic plaques observed in the AD brain.<sup>32</sup> Accumulation of  $A\beta$  peptides is also postulated to impair mAChR coupling to G proteins,<sup>33</sup> which is likely to further perpetuate the disease state. In contrast, the non-amyloidogenic pathway is stimulated by the action of the  $\alpha$ -secretase enzyme, which cleaves APP within the  $A\beta$  peptide sequence, leading to harmless metabolites. The question of which pathway predominates at any given time depends on the relative expression of the secretase enzymes, which is largely dictated by the actions of cellular second messenger systems downstream of GPCRs. Specifically,  $M_1$  mAChR activation and

initiation of  $G_{q/11}$ -mediated activation of PKC has been linked to the increased expression of  $\alpha$ -secretase,<sup>34</sup> and consequently promotion of the nonamyloidogenic pathway and a reduction in  $A\beta$  peptide levels, which could potentially slow or halt the formation of amyloid plaques and hence, the progression of AD.

Further validation of the critical role of the  $M_1$  mAChR in modulating this process was obtained when the treatment of human AD patients with a selective  $M_1$  mAChR agonist, talsaclidine (**2**, Figure 4), led to an observed reduction in  $A\beta_{42}$



**Figure 4.** Structures of two  $M_1$  mAChR selective agonists, talsaclidine (**2**) and AF267B (**3**).

levels in their cerebrospinal fluid (CSF).<sup>32</sup> Additionally, in vivo studies in 3xTg mice (injected with transgenes for  $\beta$ -amyloid and tau, and therefore a model for AD) reported increased CSF levels of  $\alpha$ -secretase-producing enzyme, ADAM17, and a corresponding decrease in  $\beta$ -secretase formation following treatment with another  $M_1$  mAChR-selective agonist, AF267B (**3**, Figure 4).<sup>35</sup>

The second “vicious cycle” referred to earlier is the formation of neurofibrillary tangles that occurs when the protein essential for the growth of microtubules in the neuronal cytoskeleton, tau, becomes hyperphosphorylated. Both in vitro and in vivo studies<sup>33,35</sup> have demonstrated that activation of the  $M_1$  mAChR has the potential to decrease tau phosphorylation by PKC-mediated inhibition of kinases (particularly  $GSK3\beta$ ) and upregulation of phosphatases, subsequently decreasing the likelihood of fibrous tangle formation.

A comparative analysis of  $M_1$  mAChR expression and response in the post-mortem cortical tissue of humans observed to have no cognitive impairment, mild cognitive impairment, or Alzheimer’s disease was conducted to further understand the link between activation of this receptor and AD pathophysiology.<sup>30</sup> [ $^3H$ ]-Oxotremorine-M radioligand binding studies found  $M_1$  mAChR expression to be elevated in the AD cortical tissue, indicative of compensatory upregulation in response to a reduction in ACh levels. It was also found that while there was no correlation between function of  $M_1$  mAChRs and the degree of cognitive impairment, a negative correlation between  $M_1$  mAChR functional activity and severity of neuropathology (based on post-mortem neurofibrillary tangle analysis) was observed, adding further weight to the argument for selective targeting of this receptor in the treatment of AD.

## ■ THE ROLE OF THE $M_1$ mAChR IN TREATING SCHIZOPHRENIA

Currently available pharmaceutical treatments for schizophrenia largely target dopamine (D) receptors or a combination of dopamine and serotonin (5-HT) receptors. First generation antipsychotics, such as haloperidol and chlorpromazine, are predominantly  $D_2$ R antagonists, treating the positive symptoms of the disorder but also eliciting Parkinsonian-like motor dysfunction (extrapyramidal side effects (EPS)).<sup>36</sup> Second generation antipsychotics, such as clozapine and olanzapine, treat positive symptoms by acting as  $D_2$ R antagonists but also partially treat the negative symptoms and reduce EPS by acting

as cortical 5-HT<sub>2A</sub>R antagonists. However, these therapies still demonstrate poor efficacy and a lack of selectivity, resulting in a range of potentially dangerous side effects, including prolongation of QT interval, agranulocytosis, and severe weight gain.<sup>37</sup> Aripiprazole is a third generation antipsychotic exhibiting a unique mechanism of action:  $D_2$ R partial agonism, 5-HT<sub>1A</sub>R partial agonism, and 5-HT<sub>2A</sub>R antagonism, the combination of which results in increased and decreased neurotransmission in hypodopaminergic and hyperdopaminergic areas, respectively.<sup>38</sup> Thus, it is considered to be the first dopamine–serotonin system stabilizer. Despite reports of an excellent safety and tolerability profile, aripiprazole still demonstrates the potential to cause the same side effects as its first- and second-generation predecessors.

A major deficit of these three classes is that none were designed to treat, nor show substantial efficacy against, the cognitive deficits experienced as part of schizophrenia. This is despite the observation that the degree of cognitive impairment is the strongest predictor of clinical outcome for sufferers of this debilitating mental disorder,<sup>39</sup> and that cognitive symptoms are often evident in patients before the onset of psychosis.

The notion of treating schizophrenia by targeting the cholinergic system predates the widely regarded dopamine hypothesis,<sup>40</sup> the latter having given rise to the majority of schizophrenia medications to date. Decades-old clinical observations of muscarinic agonists exhibiting modest antipsychotic properties and anticholinergic exacerbation of schizophrenic symptoms formed the foundation of this theory, and more recent studies have demonstrated decreases in muscarinic receptor binding in both the prefrontal cortex and the hippocampus in schizophrenic patients,<sup>41,42</sup> regions rich in the  $M_1$  mAChR subtype. Taken together, this suggests that the  $M_1$  mAChR may represent a therapeutically beneficial target for both positive and cognitive symptom domains. Furthermore, the direct and indirect interactions between the dopaminergic and cholinergic neurotransmitter systems in the brain implicate a role for the  $M_1$  mAChR (and indeed other muscarinic receptor subtypes) in modulating the complex underlying pathophysiology of schizophrenia.<sup>43</sup> However, while a muscarinic hypothesis of schizophrenia certainly warrants further investigation, particularly the roles of the  $M_1$  and  $M_4$  mAChR subtypes, it must be acknowledged that schizophrenia is a collection of syndromes likely to be dictated by multiple neurotransmitter systems and ultimately requiring combination drug therapy if all symptoms are to be alleviated.

## ■ CAVEATS OF TARGETING THE $M_1$ mAChR

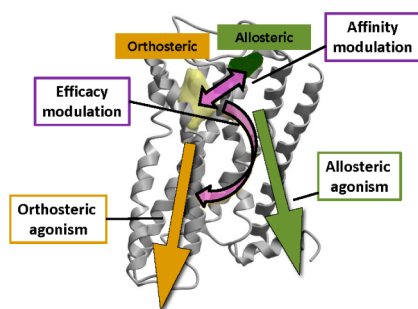
In principle, selective  $M_1$  mAChR agonists are less likely to exhibit side effects than anticholinesterases as they possess a more specific cholinergic objective, a single mAChR subtype. Despite this, there are presently no  $M_1$  mAChR agonists approved as neurodegenerative therapies. While several ligands have demonstrated sufficient  $M_1$  mAChR selectivity in in vitro studies supporting target validation, these compounds either show poor pharmacokinetic profiles and limited in vivo efficacy or fail to exhibit sufficient receptor subtype selectivity to avoid peripheral muscarinic side effects, such as gastrointestinal disruption, nausea, bradycardia, and excessive exocrine gland secretion.<sup>44</sup> This latter issue arises from their targeting the endogenous agonist-binding (orthosteric) site of the receptor, for which a high degree of sequence homology and structural conservation exists across the five mAChRs. This selectivity problem commonly manifests in therapeutics targeting GPCRs

with multiple receptor subtypes such as the adenosine, dopamine, and serotonin GPCRs. Another notable caveat of  $M_1$  mAChR activation is that it may not necessarily improve coupling between the receptor and the G protein, a critical event that is significantly compromised in the AD prefrontal cortex.<sup>45</sup>

### ■ THE UTILITY OF TARGETING $M_1$ mAChR ALLOSTERIC SITES

Allosteric ligands bind to a site topographically distinct from the orthosteric site of the receptor and are able to enhance or reduce the effect of the concomitantly bound orthosteric ligand.

The three mechanisms by which allosteric ligands exert their effects,<sup>46</sup> as depicted in Figure 5, are as follows:



**Figure 5.** Three modes by which allosteric ligands exert their effects. (1) Orthosteric ligand affinity modulation; (2) orthosteric ligand efficacy modulation; and (3) direct allosteric agonism.

- (i) by modulating the binding affinity of the orthosterically bound ligand;
- (ii) by modulating the downstream efficacy of the orthosterically bound ligand; and
- (iii) by activating the receptor in their own right, acting as allosteric agonists

Conformational changes in the receptor induced by allosteric ligand binding are the source of their modulatory activity. Localized alterations to the orthosteric site topography may either enhance or reduce the binding affinity of the orthosteric ligand, while a broader conformational shift in the GPCR may either enhance or reduce the overall signaling efficacy of the receptor. These modulatory effects, combined with any agonism conferred by the allosteric ligand alone, dictate the global change in receptor activity observed. For an elegant representation of the distinct allosteric ligand profiles that may arise from the combination of these three effects, see a recent review by Kenakin.<sup>47</sup> To gain an understanding of how the complex multidirectional interplay between an orthosteric ligand, allosteric ligand, and receptor (ternary) complex can be modeled and quantified, see the review by May et al.<sup>48</sup>

Recently marketed allosteric drugs afford practical validation for the development of allosteric ligands as therapeutic solutions. Cinacalcet<sup>49</sup> acts as an allosteric potentiator at the calcium-sensing receptor for the treatment of hyperparathyroidism, maraviroc<sup>50</sup> is an anti-HIV drug that acts as an allosteric inhibitor of the chemokine receptor CCR5, and the benzodiazepines,<sup>51</sup> used for the treatment of anxiety disorders, are allosteric modulators at GABA<sub>A</sub> receptors.

Allosteric ligands offer notable advantages over classic orthosteric drugs.<sup>52</sup> First, allosteric sites can have a less conserved amino acid sequence between receptor subclasses,<sup>53</sup>

potentially allowing for selective targeting and minimization of the side effects evident in the binding of pure orthosteric agonists, such as the undesirable peripheral muscarinic effects of xanomeline. Another advantage is the saturability of their effect. Drugs targeting the receptor orthosteric site frequently elicit an all-or-nothing response upon interaction, leading to complete activation or perturbation of downstream signaling and the associated biological function(s). The intensity of action of these ligands is directly proportional to their concentration in the receptor compartment;<sup>54</sup> so, depending on the dose that reaches the target receptor and the off-rate of the ligand from the receptor, the duration of effect of orthosteric drugs may carry the risks of side effects, overdose, or desensitization and tolerance.<sup>55</sup> In contrast, the effect of a positive or negative allosteric modulator is dictated by the degree of cooperativity they possess with the ligand occupying the orthosteric site,<sup>56</sup> such that the intensity of action reaches a limit once all allosteric binding sites are occupied; creating a “ceiling” for their biological effect and potentially avoiding the aforementioned risks.

A somewhat more contentious advantage is the precision of their effect. It is thought that “pure” allosteric modulators (that is, those devoid of agonist activity in their own right) afford the advantage of spatial and temporal specificity<sup>52,56</sup> in that they require the presence of the orthosteric ligand to exert their effect, confining their activity to the location and time in which physiological activity is occurring. However, it is now evident that an observed lack of allosteric agonism may also be a function of the ligand concentration, the degree of signal amplification of the pharmacological assay, and the coupling efficiency of the receptor for the signaling pathway being measured.<sup>57,58</sup> That is to say that if an allosteric ligand is assessed in an assay with minimal signal amplification that measures a pathway poorly coupled to the receptor of interest, any direct allosteric agonism of the ligand may go undetected. Indeed, it can be reasonably hypothesized that all positive allosteric modulators may display allosteric agonism and all negative allosteric modulators may display inverse agonism, under the right assay conditions.<sup>2</sup> Therefore, in order to more accurately predict the functional profile of an allosteric ligand in preclinical animal studies and subsequent clinical trials, *in vitro* cellular assays that follow initial hit detection ought to resemble the cellular background, native receptor expression levels, local neurotransmitter concentration, and biochemical outcomes of the human pathophysiological tissue of interest as closely as possible. This is likely to pose complex challenges, especially for CNS disorders in which multiple brain regions (with different receptor expression levels and neurotransmitter concentrations) are being targeted to achieve *in vivo* efficacy.

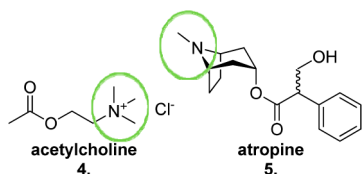
$M_1$  mAChR allosteric ligands may provide solutions to the problems hindering the development of an optimized cholinergic treatment for cognitive dysfunction. Their subtype selectivity will facilitate further investigation into the physiological and pathophysiological roles of this receptor in the brain, as well as offer the prospect of neurodegenerative disorder therapies devoid of side effects. Additionally, while their ability to modulate endogenous neurotransmitter activity affords the prospect of improved cholinergic tone, the potential for allosteric activation of the  $M_1$  mAChR in their own right becomes all the more critical as functional ACh levels decrease in tandem with neurodegeneration and disease state progression. Moreover, the ability of such ligands to stabilize distinct receptor conformations may lead to the discovery of

functional profiles that bias the receptor toward neurotrophic and pro-cognitive pathways while disfavoring pathophysiological and neurotoxic pathways.

### ■ STRUCTURAL STUDIES OF $M_1$ mAChR ORTHOSTERIC AND ALLOSTERIC SITES

Until recently, modeling of the muscarinic GPCR family has been largely based on the rhodopsin,<sup>59</sup>  $\beta$ -adrenergic,<sup>60</sup> and  $A_{2A}$  adenosine<sup>61</sup> GPCR crystal structures, other Family A GPCRs with which they share common structural motifs.<sup>62</sup> However, recent publication of the inactive human  $M_2$  and the rat  $M_3$  mAChR crystal structures<sup>63,64</sup> will undoubtedly provide unparalleled insight into the structures of the other muscarinic receptor subtypes going forward.

Studies of the cloned  $M_1$ – $M_5$  mAChRs have revealed a high degree of sequence homology between the subtypes, particularly in transmembrane (TM) units 2–7, a region containing a highly structurally conserved hydrophilic cavity.<sup>65</sup> Within this cavity projects a TM3 negatively charged aspartate residue common to all subtypes, which has been shown by binding and point-mutation studies to be critical for orthosteric ligand binding,<sup>66</sup> forming an ionic interaction with the polar, positively charged (or ionizable) headgroup of both agonists, such as acetylcholine, and antagonists, such as atropine (4 and 5, Figure 6). A combination of computational and molecular

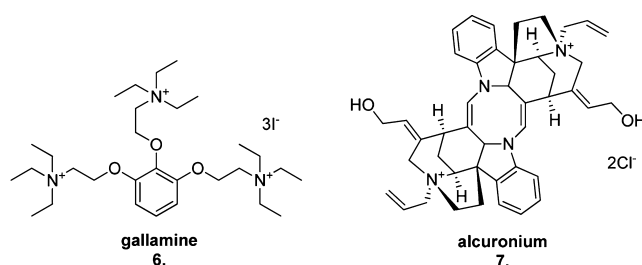


**Figure 6.** Structures of the muscarinic orthosteric agonist acetylcholine (4) and the muscarinic orthosteric antagonist atropine (5), highlighting the positively charged and ionizable head groups, respectively.

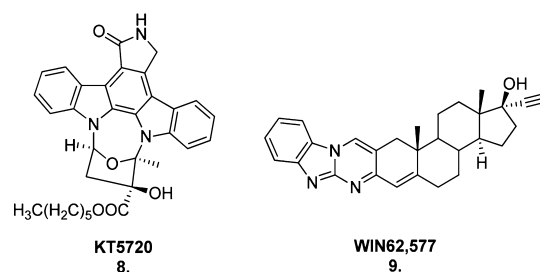
biological techniques have identified additional residues extending into this pocket as having key roles in orthosteric ligand binding, kinetics, and receptor activation.<sup>67</sup>

Similar approaches have been adopted to identify the key residues involved in the interaction of allosteric ligands with the  $M_1$  mAChR. To date, two  $M_1$  mAChR allosteric binding sites have been pharmacologically characterized. Early binding and site-directed mutagenesis studies using the allosteric muscarinic ligands gallamine (6) and alcuronium (7) provided evidence for the first site, an extracellular (EC) allosteric binding domain involving residues from EC loops 2 and 3 adjoining TMs 5, 6, and 7.<sup>68</sup> This site is often referred to as the “common” allosteric binding site<sup>69</sup> for mAChRs as its topographical location is preserved across the subtypes, while the nonconserved residues in this region confer allosteric ligand mAChR subtype selectivity. More specifically at the  $M_1$  mAChR, the positive charges present on both gallamine and alcuronium (Figure 7) are thought to be important for interaction with negatively charged EC3 residues (S388, D393, and E397) extending into the hydrophilic EC space, while the hydrophobic portions of these molecules are thought to form interactions with tryptophan residues (W101 and W400) within upper TM units.<sup>70</sup>

The existence of a second allosteric site was proposed when the ability of the indolocarbazole KT5720 (8, Figure 8) to allosterically enhance the binding of both the agonist ACh and



**Figure 7.** Structures of the two “common” muscarinic allosteric site binders, gallamine (6) and alcuronium (7).



**Figure 8.** Structures of the second muscarinic allosteric site binders, KT5720 (8) and WIN62,577 (9).

the antagonist *N*-methylscopolamine (NMS) at the  $M_1$ – $M_4$  mAChRs was unchanged in the presence of the allosteric ligand gallamine.<sup>71</sup> This is indicative of two separate allosteric binding sites and, as neither allosteric ligand altered the potency or relative effectiveness of the other when co-bound, they exhibit neutral cooperativity with one another.<sup>72</sup> Additional muscarinic allosteric ligands reported to bind to this second site are WIN62,577 (9) and its analogues (Figure 8).<sup>73</sup>

Using a rhodopsin-based homology model of the  $M_1$  mAChR, this second allosteric site was later postulated to be located on the intracellular (IC) face of the receptor close to the G protein-coupling domain, involving residues within TMs 2, 3, and 7, the C-terminal helix 8 and IC loop 3, a proposition that is yet to be experimentally validated.<sup>74</sup>

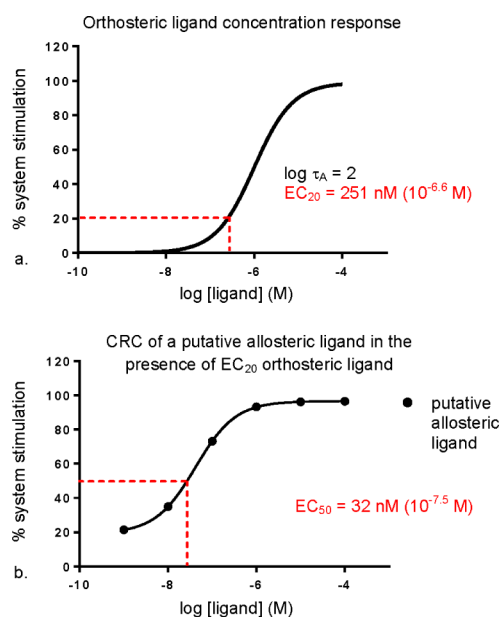
The future elucidation of the  $M_1$  mAChR structure by X-ray crystallography (and eventual cocrystallization with proposed allosteric ligands) will hopefully further validate the homology model-based structural studies undertaken so far and greatly enhance our understanding of ligand–receptor interactions. Regardless, the existence of multiple allosteric sites on the  $M_1$  mAChR adds another layer of complexity to the already complicated field of subtype-selective allosteric ligand design.

### ■ IMPORTANT CONSIDERATIONS IN ALLOSTERIC LIGAND CLASSIFICATION AND EVALUATION

**Positive Allosteric Modulators (PAMs) Possessing Intrinsic Agonism.** As alluded to earlier, it may be the case that all positive allosteric modulators may display allosteric agonism in their own right if evaluated in a sufficiently amplified pharmacological assay. Therefore, classifying a ligand as a “pure” allosteric modulator should be done tentatively and with due consideration to the context in which the ligand has been tested.

**Estimating Ligand Potency.** The  $M_1$  mAChR PAM literature regularly reports ligand potency by determining an  $EC_{50}$  value. This value is determined by first generating a concentration response curve (CRC) for an orthosteric agonist of the  $M_1$  mAChR (e.g., ACh) using the cellular background

and functional assay of choice (e.g., calcium mobilization in CHO cells). From this curve, a single concentration is selected depending on the type of allosteric modulation expected to be observed. If positive modulation is expected, as is the case with the  $M_1$  mAChR PAM studies described here, an  $EC_{20}$  of ACh may be selected (Figure 9a), the concentration at which 20% of the maximal system response to the ligand is elicited, affording a suitable “window” in which to detect the anticipated modulatory activity.



**Figure 9.** Simulated example of a common screening method for putative allosteric ligands. (a) A concentration–response curve of a known orthosteric ligand (e.g., ACh) used to derive an  $EC_{20}$  concentration value;  $\log \tau_A$  is the operational efficacy parameter of the orthosteric ligand (the higher the value, the greater the efficacy). (b) How a concentration response curve of a putative allosteric ligand might look in the presence of the derived  $EC_{20}$  concentration of orthosteric ligand.

This predetermined concentration of orthosteric ligand is then coadded with increasing concentrations of the test allosteric ligand in the same cellular background and functional assay, and, assuming the predicted modulation is observed, the potency of the allosteric modulator is equated to the  $EC_{50}$  value, the concentration at which 50% of the maximal system response to the allosteric ligand in the presence of a fixed concentration of orthosteric ligand is elicited (the point of inflection of the titration curve, see Figure 9b).

This is a typical and efficient method for screening for activity of putative ligands, and can provide preliminary information about the mode of action of the ligand. However, by virtue of screening against a single concentration of orthosteric ligand, this method examines a one-dimensional cross section of the putative ligand’s pharmacological profile, a cross section that may not necessarily capture the true behavior of the ligand. An interaction study in which the full CRC of the orthosteric ligand is assessed against increasing concentrations of the putative allosteric ligand is necessary to confidently classify the mode of action of the ligand. As demonstrated in Figure 10, a PAM, an allosteric agonist, and a competitive orthosteric agonist would all appear the same when coadded with a single orthosteric ligand concentration (note that Figure

9b and Figure 10d are indistinguishable). It is not until interaction studies over a range of orthosteric ligand concentrations are performed that the mode of action of the test ligands can begin to be deduced (Figure 10a–c).

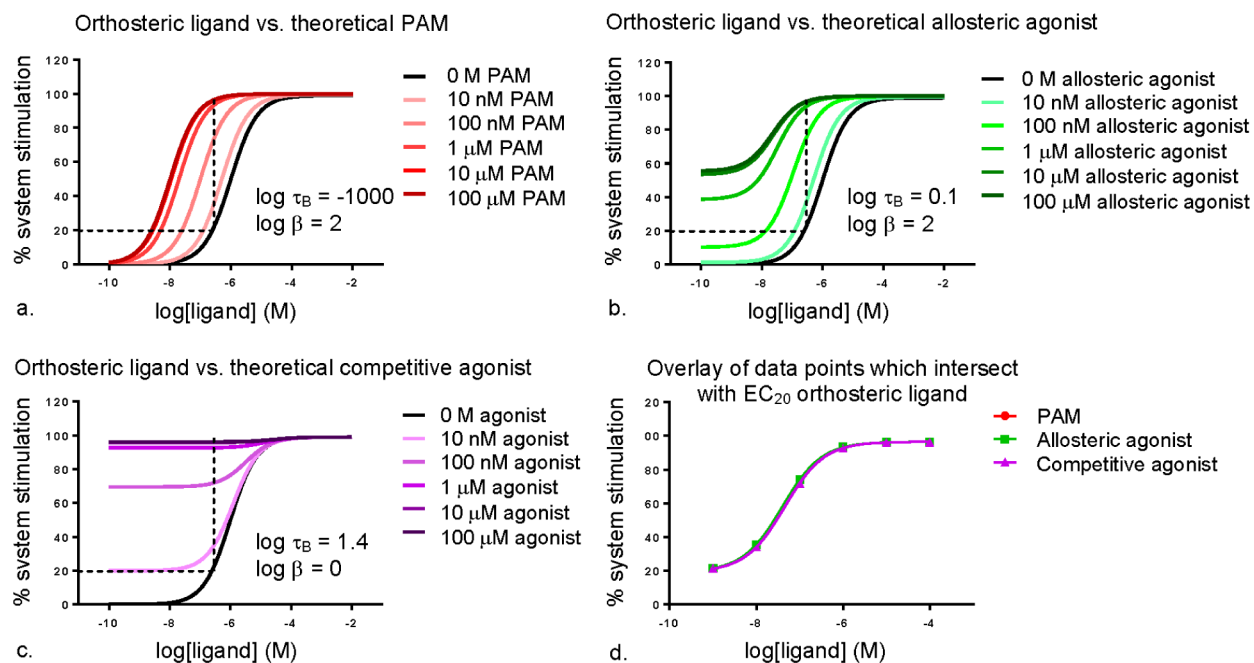
A combination of functional and radioligand binding assays is necessary to determine whether the allosteric ligand’s modulatory activity is elicited primarily through modulation of binding affinity, signaling efficacy, or a combination of the two. These experiments will also delineate its modulatory effects on the orthosteric ligand from any agonistic response in its own right. These parameters can be estimated via application of the allosteric ternary complex model for binding experiments and the operational model of allosterism for functional experiments.<sup>48</sup> It is important to note that, even if this more detailed pharmacological characterization of the allosteric ligand is performed, the estimates of affinity, efficacy, and cooperativity obtained are only applicable in the context of the orthosteric probe utilized, the functional assays performed, and the cellular background under investigation.

**Acknowledging Unique Allosteric Properties.** Allosteric ligands possess a number of inherent properties that necessitate broader pharmacological evaluation than their orthosteric counterparts. Two fundamental pharmacological properties that can be engendered by GPCR allosteric ligands are stimulus bias<sup>75</sup> and probe dependence.<sup>76</sup> These terms refer to the differing allosteric modulatory effects observed depending on the cellular signaling pathway being assessed by the assay, and the orthosteric ligand cobound to the receptor, respectively.

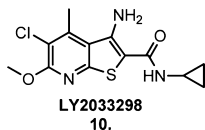
The foundation of stimulus bias is the stabilization of distinct receptor conformations. In the same way that different orthosteric agonists and antagonists stabilize multiple active and inactive receptor states, respectively, allosteric ligands may stabilize diverse conformations that result in differential activation of receptor signaling pathways, or collateral efficacy.<sup>54</sup> Such molecular tools may afford a greater understanding of the complex relationship between receptor conformations, discrete signaling profiles, and particular physiological and pathophysiological outcomes.

Probe dependence is an important property to consider during the processes of hit identification and lead optimization of allosteric ligands. If the orthosteric probe used to screen and elucidate the structure–activity relationships of novel allosteric chemical scaffolds is not the pathophysiologically relevant endogenous neurotransmitter, the risk is that the novel ligand may exhibit a different, potentially undesirable functional profile (or no function at all) in an *in vivo* setting.

An excellent mAChR exemplar of these two properties is the  $M_4$  mAChR allosteric ligand LY2033298 (**10**, Figure 11).<sup>77</sup> Leach et al.<sup>57</sup> performed radioligand binding, [<sup>35</sup>S]GTP $\gamma$ S, calcium mobilization, ERK1/2 phosphorylation, GSK-3 $\beta$  phosphorylation, and receptor internalization assays to fully assess the allosteric capabilities of LY2033298. They found that the degree of allosteric potentiation conferred by LY2033298 varied widely depending on the signaling pathway being examined, ranging from modest positive modulation of ACh activity in [<sup>35</sup>S]GTP $\gamma$ S assays to robust enhancement of ACh activity in receptor internalization studies. Estimates of the degree of allosteric agonist activity also varied, further illustrating the property of stimulus bias. This also disproved the original classification of the ligand as a positive allosteric modulator with no agonist activity in its own right.<sup>77</sup> Radioligand binding assays were used to highlight the probe



**Figure 10.** Simulated example of interaction studies between an orthosteric agonist and (a) a positive allosteric modulator (PAM), (b) an allosteric agonist, and (c) a competitive agonist, with broken lines indicating the intersection of the concentrations of test ligand with the  $EC_{20}$  of the orthosteric ligand. (d) An overlay of the data points from graphs (a)–(c) which intersect with the  $EC_{20}$  of the orthosteric ligand;  $\log \tau_B$  is the operational efficacy parameter of the allosteric ligand (the higher the value, the greater the efficacy; the high negative value represents a complete absence of allosteric ligand efficacy);  $\log \beta$  is the functional cooperativity factor between the orthosteric and allosteric ligands ( $\log \beta > 0$  = positive cooperativity,  $\log \beta = 0$  = neutral cooperativity or, in this instance, direct competition between two orthosteric ligands).



**Figure 11.** Structure of the  $M_4$  mAChR allosteric ligand LY2033298 (10).

dependence of LY2033298; its allosteric agonism and modulatory effects were only detected when cobound with an orthosteric agonist, not an antagonist.

LY2033298 also revealed the ability of allosteric ligands to exhibit species-dependent differences in cooperativity.<sup>78</sup> This later led to the hypothesis that the proposed subtype selectivity of the modulator may arise from differential cooperativity, rather than variability of the allosteric pockets between receptor subtypes.<sup>76</sup> Despite LY2033298 exhibiting selective ACh potentiation at the  $M_4$  mAChR, extensive evaluation of LY2033298 at wild-type and mutant  $M_2$  mAChRs revealed marked degrees of probe dependence with a range of agonists and antagonists in radioligand binding assays, as well as both positive and negative cooperativity in two functional assays, [ $^{35}S$ ]GTP $\gamma$ S binding and pERK1/2.

These findings highlight the highly contextual nature of allosteric effects and the importance of assessing binding, multiple functional pathways, different receptor subtypes, and a broad range of probes to fully characterize allosteric ligands.

**Identifying a Purely Allosteric Mode of Action.** For a ligand to be classified as an allosteric agonist at a particular receptor, it must possess intrinsic efficacy in its own right, regardless of whether an orthosteric ligand is co-bound to the receptor.<sup>46</sup> Its agonistic effect must be elicited solely via an

allosteric mechanism, and the absence of direct activation via orthosteric site binding must be established.

Comprehensive radioligand binding and functional assays unequivocally demonstrated that the agonistic and modulatory behavior of LY2033298 at the  $M_4$  mAChR was elicited via a purely allosteric mechanism,<sup>57</sup> with four key observations confirming this hypothesis.

In radioligand binding assays:

- LY2033298 exhibited a lack of significant affinity modulation of the muscarinic orthosteric antagonists NMS and QNB (3-quinuclidinyl benzilate), indicating no overlap with the orthosteric binding site

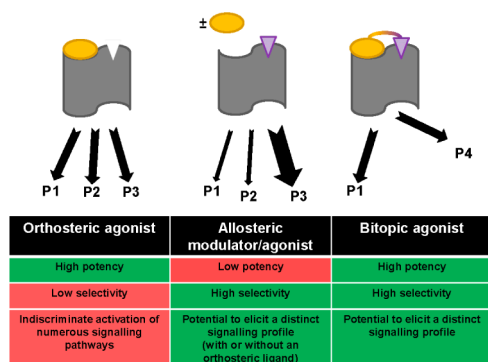
In [ $^{35}S$ ]GTP $\gamma$ S binding assays:

- coaddition of the mAChR antagonist atropine caused a substantial reduction in the maximal effect of LY2033298 with a minimal change in potency, characteristic of noncompetitive negative cooperativity
- LY2033298 demonstrated a competitive interaction with the mAChR negative allosteric modulator  $C_7/3$ -phth, known to bind the “common” muscarinic allosteric site<sup>79</sup>
- LY2033298 exhibited neutral cooperativity upon coaddition with WIN51708, a known “second allosteric site” mAChR ligand,<sup>73</sup> confirming the allosteric agonist’s site of action to be the “common” allosteric site on the  $M_4$  mAChR

**The Prospect of Bitopic Interactions.** While the  $M_1$  mAChR-selective ligand literature reviewed herein provides substantial evidence to suggest purely allosteric modes of action, there exist several examples where this evidence is inconclusive. Such ligands have hence been termed “putative bitopic  $M_1$  mAChR agonists” in this Review. It has been noted that these ligands exhibit unique binding modes and divergent

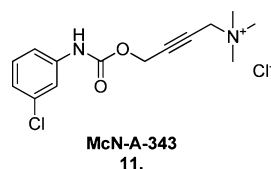


functionality when compared to  $M_1$  mAChR PAMs.<sup>80,81</sup> Depending on the experimental paradigm, many of these molecules have exhibited degrees of antagonistic interaction with different orthosteric mAChR ligands. While they may be acting as pure allosteric agonists possessing very high negative cooperativity with orthosterically bound ligands, it is equally if not more likely that they are interacting with the orthosteric site and an allosteric site simultaneously, behaving as “bitopic” ligands (also termed “dualsteric” or “multivalent”) (Figure 12).



**Figure 12.** Advantages and disadvantages of orthosteric and allosteric ligands, and the theoretical fusion of their respective advantages by bitopic ligands: high potency, high subtype selectivity, and the potential to elicit distinct signaling profiles (Ps) that may be of therapeutic value.

This unique mode of ligand–receptor interaction has been validated<sup>82</sup> by a number of proof-of-concept studies (see Valant et al.<sup>83</sup> for a review). Notably, the conclusive classification of McN-A-343 (11, Figure 13) as a bitopic ligand,<sup>84</sup> initially



**Figure 13.** Structure of the  $M_2$  mAChR bitopic ligand McN-A-343 (11).

thought to be an  $M_2$  mAChR allosteric partial agonist,<sup>85</sup> highlights the detailed level of synthetic manipulation and pharmacological characterization necessary to appropriately classify the mode of action of a bitopic ligand. Just as McN-A-343 was truncated to form small molecular derivatives that were used to identify the orthosteric and allosteric portions of the molecule, similar methods will need to be employed to confirm the true nature of the putative bitopic  $M_1$  mAChR agonists described within.

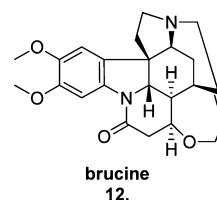
### ■ $M_1$ mAChR-SELECTIVE LIGANDS

Over the past two decades, a structurally diverse array of  $M_1$  mAChR-selective ligands have been identified and characterized. Two distinct groups of these ligands will be reviewed herein:  $M_1$  mAChR PAMs and putative bitopic  $M_1$  mAChR agonists. Orthosteric  $M_1$  mAChR ligands are beyond the scope of this Review. The methods used and the implications for the detection, optimization, and development of novel treatments for cognitive deficits of neurodegenerative conditions, and

indeed any allosterically derived treatment, will also be discussed.

**$M_1$  mAChR PAMs. Brucine.** In 1995, Lazareno and Birdsall<sup>86</sup> identified the naturally occurring alkaloid strychnine as an  $M_1$ ,  $M_2$ ,  $M_4$ -preferring allosteric ligand shown to possess low negative cooperativity with ACh, prompting the researchers to investigate a modified series of analogues with the intent of identifying ligands that possessed positive cooperativity with ACh as well as greater subtype selectivity.

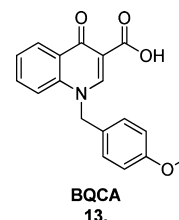
The result was the discovery that the structurally related natural product brucine, 10,11-dimethoxy strychnine (12, Figure 14), exhibits modest positive cooperativity with ACh



**Figure 14.** Structure of the  $M_1$ -preferring allosteric ligand brucine (12).

in both equilibrium binding experiments and functional assays (such as the [<sup>35</sup>S]-GTPγS assay and the calcium mobilization assay) at the  $M_1$  mAChR.<sup>87</sup> Unfortunately, brucine exhibited low affinity (0.1 mmol for the ACh-occupied receptor), minimal cooperativity (1.5–2-fold enhancement of ACh activity) and required high micromolar concentrations to produce any detectable activity. This, combined with its poor pharmacological profile, rendered the compound insufficient for use in native tissue and animal studies, and off-target activity suggested that the scaffold was unfavorable for further  $M_1$  mAChR PAM analogue development. However, brucine provided important validation for selective allosteric modulation of  $M_1$  mAChR activity as a viable field of therapeutic research.

**Benzyl Quinolone Carboxylic Acid, BQCA.** An exciting development in the pursuit of selective  $M_1$  mAChR allosteric potentiators was Merck's discovery of BQCA (13, Figure 15),<sup>88</sup>



**Figure 15.** Structure of the breakthrough  $M_1$ -selective mAChR PAM BQCA (13).

by far the most actively investigated scaffold in this field to date. BQCA is the first reported orally bioavailable  $M_1$  mAChR allosteric ligand exhibiting absolute subtype selectivity; no activity was detected at the other four muscarinic receptor subtypes in calcium mobilization assays (concentrations up to 100 μM). A notable feature of BQCA is its high degree of cooperativity; 100 μM of BQCA induced a robust 129-fold leftward shift in the ACh CRC (estimated from fluorometric imaging plate reader (FLIPR) calcium mobilization assays). Furthermore, BQCA produced an inflection point value of 845 nM in the presence of 3 nM ACh. A combination of molecular

modeling, site-directed mutagenesis, and radioligand binding studies revealed the residues Y179 and W400 to be important to the BQCA–receptor interaction, which is suggestive, but not conclusive, of the modulator exerting its effects via the “common” allosteric site.

Interestingly, it was recently reported that BQCA is the first allosteric GPCR ligand proven to abide by a strict two-state Monod-Wyman-Changeux (MWC) model of receptor activation,<sup>2</sup> in that its degree of modulation tracks with both the intrinsic efficacy of the cobound orthosteric ligand and the coupling efficiency of the receptor for a given intracellular signaling pathway.<sup>58</sup> Therefore, BQCA displays no stimulus bias, but still shows probe dependence by stabilizing the active receptor state favored by orthosteric agonists. As a result, it exhibits positive modulation when co-bound with orthosteric agonists and negative modulation when co-bound with orthosteric antagonists. It has been postulated that the inability of BQCA to stabilize a discrete receptor conformation arises from the ligand possessing few chemical functionalities capable of forming strong receptor interactions. Furthermore, the same study demonstrated that BQCA behaves as both a positive allosteric modulator and an allosteric agonist.<sup>58</sup>

BQCA possesses a sufficient pharmacological profile for analysis in both native tissue assays and in vivo animal studies. BQCA potentiated phosphorylated ERK production and RNA expression of the neuronal activation markers *c-fos* and *arc* in critical forebrain regions of the mouse brain where cholinergic degeneration in human AD patients is most pronounced.

Importantly, BQCA displayed efficacy in a contextual fear conditioning (CFC) mouse model of cognitive dysfunction. Mice dosed with 15–20 mg/kg BQCA exhibited behaviors suggesting a reversal of the scopolamine-induced block of memory formation.<sup>88</sup> As BQCA was shown to have no effect on [<sup>3</sup>H]NMS affinity in radioligand binding assays (at concentrations up to 30  $\mu$ M), this reversal of induced memory deficits was proposed to be solely due to BQCA's allosteric enhancement of endogenous ACh activity, decreasing the concentration of agonist required to displace the antagonist.

The short-term memory of acquired fear (as observed in the CFC model) is linked to the hippocampus, in which the M<sub>1</sub> mAChR is expressed in abundance. However, M<sub>1</sub> mAChR KO mice have shown intact cognitive processes associated with hippocampal-dependent learning<sup>89</sup> and no impairment in mAChR-mediated hippocampal pyramidal cell excitation.<sup>90</sup>

Shirey et al.<sup>91</sup> reported evidence indicating the M<sub>1</sub> mAChR is more likely to have a role in prefrontal cortex (PFC)-dependent learning, so the ability of BQCA to potentiate the carbachol (muscarinic agonist)-induced inward current (excitability) of medial (m)PFC pyramidal cells in rat brain slices was examined. BQCA increased the intensity and frequency of carbachol-induced spontaneous excitatory postsynaptic currents (sEPSCs), effects that were not observed in brain slices of M<sub>1</sub> mAChR KO mice. In support of these observations, another electrophysiology study demonstrated that coapplication of BQCA enhanced synaptic stimulation by carbachol in mPFC pyramidal cells, subsequently producing long-term depression (LTD), a critical process that, in conjunction with long-term potentiation (LTP), mediates synaptic plasticity.<sup>92</sup> These findings translated well to in vivo electrophysiology studies in which BQCA induced an elevation in the spontaneous firing rate of mPFC neurons in rats.<sup>91</sup>

In complement to these findings, further in vivo analysis using a mouse model of Alzheimer's disease (Tg2576 mice;

genetically modified to overexpress an amyloidogenic familial AD mutant form of APP) indicated that BQCA improved the performance of these mice in a discrimination reversal learning test, a PFC-dependent learning task.<sup>91</sup> The propensity for error was almost 7 times lower in BQCA-treated Tg2576 mice compared to controls. BQCA was found to be taken up into the brain between 30 min and 1 h after dosing, and maintained a constant level for 4 h; however, concentrations in the brain were found to be substantially lower than that in systemic circulation. Furthermore, BQCA (in the presence of carbachol) promoted the nonamyloidogenic pathway of APP cleavage in PC12 cells overexpressing human APP and M<sub>1</sub> mAChRs, as measured by increased levels of the protein fragments released by  $\alpha$ -secretase cleavage of APP.

Overall, these studies validate the hypothesis that selective enhancement of M<sub>1</sub> mAChR activity in the PFC may lead to improved cognitive function in human patients, whether by directly enhancing PFC function and/or by modulating hippocampal function via cortical projection neurons. BQCA has shown efficacy in additional animal models of cognition that lend support to this hypothesis. A 10 mg/kg dose of BQCA attenuated scopolamine-induced memory deficit in a spontaneous alternation task<sup>93</sup> and prevented natural forgetting<sup>94</sup> in rats, suggestive of enhancement of both hippocampal- and PFC-dependent memory processes. Taken together, these studies endorse the investigation of M<sub>1</sub> mAChR PAMs as therapeutics for the symptoms and pathophysiology of AD.

Despite its many advantageous properties, critical deficits of BQCA include its low affinity (105  $\mu$ M as estimated by Canals et al.<sup>58</sup>) and low free fraction (FF) of 1–4% (percentage of compound unbound by blood plasma proteins). However, the BQCA structure is highly amenable to optimization, affording the same benefits to medicinal chemists as conventional fragment hits do: a promising core scaffold with affinity for the target of interest, multiple points for diversification facilitating thorough structure–activity relationship (SAR) studies, and the ability to add functionalities for physicochemical optimization without exceeding a MW of 500 Da. This latter consideration is especially important for compounds like BQCA which require blood-brain barrier (BBB) permeability to reach their therapeutic target; a modified version of the Rule of Five suggests a MW < 450 Da for BBB passage.<sup>95</sup>

The multiple points for diversification on the BQCA scaffold have since resulted in a plethora of SAR studies that have generated a wide variety of analogues, some of which display substantial improvement on the characteristics of the parent compound. It is important to note however that all subsequent BQCA analogues described here were evaluated in only one functional assay against a single orthosteric ligand concentration, equating compound potencies to the EC<sub>50</sub> (point of inflection) of the compound's CRC in the presence of an EC<sub>20</sub> of ACh at human M<sub>1</sub> mAChR-expressing CHO cells using FLIPR calcium mobilization assays. Hence, the allosteric properties of stimulus bias and probe dependence remain untested.

After examining substituents with differing properties such as size, lipophilicity, and polarity, Yang et al.<sup>96</sup> derived some fundamental features they found to be critical to the scaffold's M<sub>1</sub> mAChR potency, summarized in Figure 17. Note that A-, B-, C-, and D-ring labeling will be used for ease of communication from this point onward.

Of the ~100 analogues synthesized and tested, the compound possessing the best balance of potency and FF

was compound **14** (Figure 16);  $EC_{50} = 61$  nM, hFF = 5.5%, rFF = 5.1%. However, subsequent evaluation of compound

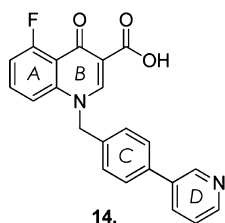
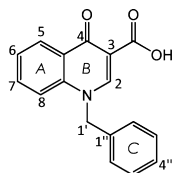


Figure 16. Structure of compound **14**.



A-ring - 5-fluoro, 8-fluoro and 5,8-difluoro substitutions preferred for potency  
 B/C-ring - longer linker or branching at 1' position not tolerated  
 C-ring - halogens and lipophilic moieties better tolerated than polar groups  
 - *para*-substitution (4'') preferred

Figure 17. Summary of the pharmacophoric features reported by Yang et al.<sup>96</sup>

concentration in CSF after oral dosing in rats (a surrogate measure for CNS exposure) yielded poor results, and was most likely the result of compound **14** being later confirmed as a P-glycoprotein (P-gp) substrate, one of the efflux transporters present in the BBB.

The most extensive BQCA analogue studies to date have been reported by Kuduk and co-workers.<sup>97–105</sup> Table 1 provides a summary of the most notable compounds from each series of structural modification; including their potency, favorable pharmacological properties and notable caveats.

Figure 18 provides a summary of the key SAR findings of these same publications. Challenging issues faced during these studies included the often confounding SAR, and difficulties in obtaining an optimal combination of pharmacological and pharmacokinetic properties. Additional patented structures discovered by this group not included in this Review are summarized in a recent publication.<sup>106</sup>

**Vanderbilt University Compounds.** Using high-throughput screening, researchers at Vanderbilt University investigated a broad range of chemical space to identify novel, structurally distinct chemical scaffolds that produce substantial allosteric modulatory activity at the  $M_1$  mAChR.<sup>107</sup>

At a concentration of  $10 \mu\text{M}$ , 1634 compounds significantly increased the muscarinic agonist carbachol's  $EC_{20}$  response above control levels in CHO cells expressing the rat  $M_1$  mAChR. Subsequent screens of the more potent ligands confirmed 105 compounds (at  $30 \mu\text{M}$  concentration) as positively modulating an  $EC_{20}$  concentration of ACh by measurement of intracellular calcium mobilization in the same cellular background. Four structurally diverse hits were selected for further analysis (**22–25**, Figure 19).

An allosteric mode of action was confirmed using [ $^3\text{H}$ ]NMS binding experiments; none of the test compounds demonstrated a competitive interaction with the orthosteric site-bound radio-labeled ligand. Using  $M_1$  mAChR calcium mobilization assays as described previously, all four compounds demonstrated positive allosteric modulation of the ACh-induced receptor response and no observed allosteric agonism in their

own right. The most substantial enhancements of ACh potency were reported for VU0029767 (**25**) (15-fold) and VU0119498 (**22**) (14-fold). Unfortunately, none of the test compounds showed absolute subtype selectivity for the  $M_1$  mAChR. As VU0029767 exhibited minimal modulation at the other four receptor subtypes and VU0090157 (**24**) showed only weak modulation at the  $M_4$  mAChR, these two  $M_1$  mAChR-preferring PAMs were further analyzed and found to exert their effects at the  $M_1$  mAChR primarily through enhancement of ACh affinity for the orthosteric site (as indicated by shifts in the ACh/[ $^3\text{H}$ ]NMS competition binding assay curve). The two compounds were tested at both wild-type  $M_1$  mAChRs and  $M_1$  mAChRs containing the single point mutation Y381A, which replaces a tyrosine residue critical for orthosteric ACh binding with an alanine residue, thereby reducing ACh affinity and potency.

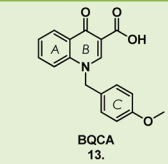
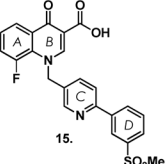
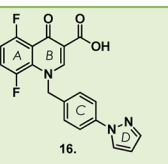
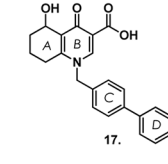
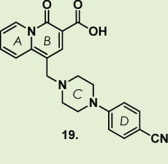
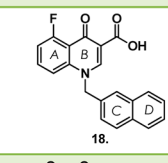
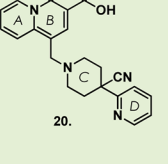
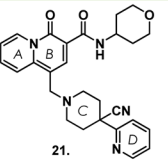
Interestingly, they found that the two ligands exhibited markedly different profiles. The potentiating ability of VU0029767 was preserved between wild-type and mutated receptors, while VU0090157 exhibited no potentiation of the ACh response at the mutated receptor. This suggests that these structurally diverse ligands may possess different degrees of cooperativity with ACh or two distinct binding modes at the  $M_1$  mAChR. A shared characteristic, however, is that both ligands appear to stabilize a receptor conformation that renders ACh uncharacteristically tolerant of the Y381A mutation, as evidenced by the preserved 20% basal ACh activity between wild type and mutated receptors. The actual binding modes and sites of both allosteric compounds are thus far unconfirmed.

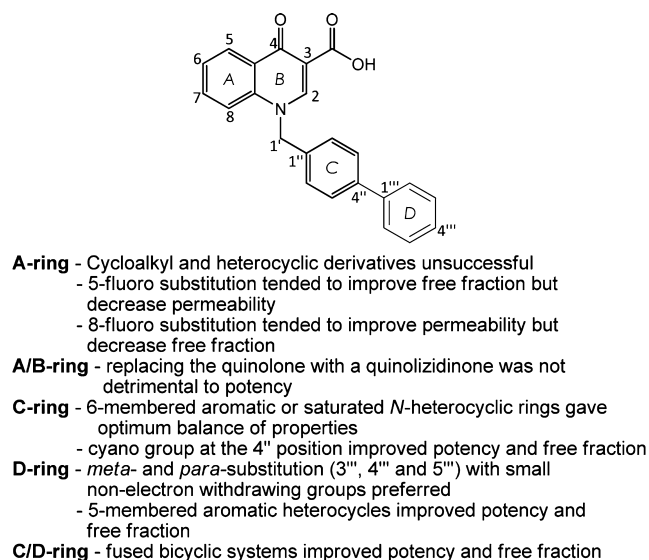
Additionally, the two ligands were found to induce different functional profiles in the ACh-mediated response of the wild-type receptor; the modulatory function of VU0090157 was preserved across three downstream signaling pathways linked to intracellular calcium release, phosphoinositide hydrolysis and phospholipase D activation respectively, while the degrees of potentiation of VU0029767 across these same pathways varied substantially. This finding highlights the importance of screening putative allosteric ligands across a broad range of functional assays to obtain thorough pharmacological characterization.

VU0119498 (**22**) was not pursued in earlier studies<sup>107</sup> as it potentiated ACh activity at all three  $G_{q/11}$ -coupled mAChRs,  $M_1$ ,  $M_3$ , and  $M_5$ . However, following publication of BQCA,<sup>88</sup> to which it bears a resemblance, this compound was pursued as a candidate for further optimization to improve subtype selectivity.<sup>108</sup> Previous optimization of this structure to develop an  $M_5$ -selective PAM demonstrated that modifications to the aromatic left side of the isatin scaffold tended to yield ligands of highly variable activation, potentiation, and selectivity profiles.<sup>109</sup> Hence, modifications to the right side, such as chain length alterations, additional benzyl substituents, and introduction of a diverse range of substituted biaryl moieties, were explored.

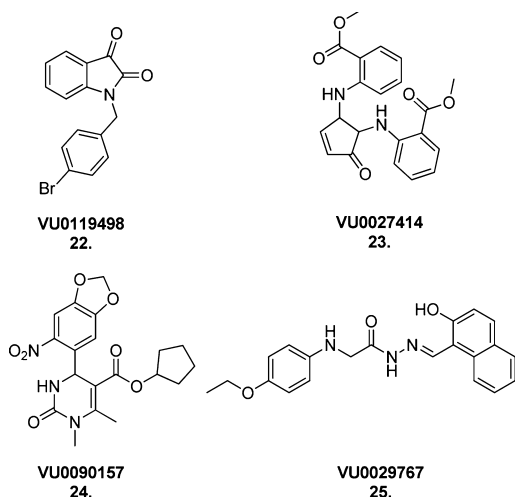
The result was VU0366369 (**26**, Figure 20), the second highly selective and potent  $M_1$  mAChR PAM reported.<sup>108</sup> BQCA and VU0366369 are reported to possess similar  $M_1$  mAChR potencies in the same cellular background ( $EC_{50} = 845$  and  $830$  nM respectively); however, the latter compound exhibited a markedly low degree of cooperativity (3-fold left shift of ACh CRC at  $30 \mu\text{M}$ ) compared to the former (129-fold left shift of ACh CRC at  $100 \mu\text{M}$ ). Taking into consideration the known promiscuity of isatin motifs in high-throughput screens, replacement with alternative structures was under-

Table 1. Summary of the BQCA Analogues Possessing the Best Balance of Properties, and Any Notable Caveats (as reported by Kuduk et al.)

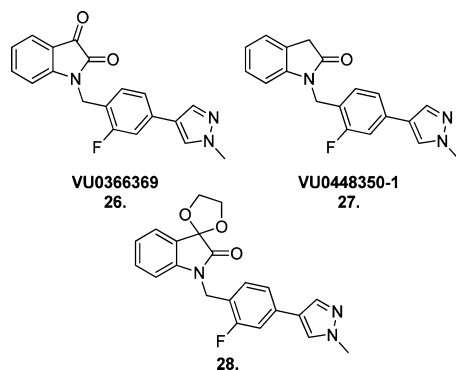
Reference	Structural modifications	Most promising compound	No. in paper	Potency (nM)	Favourable pharmacological properties	Notable caveats
Ma, L. <i>et al.</i> (2009), PNAS, 106, 15950 Shirey, J. <i>et al.</i> (2009), J. Neurosci., 29, 14271	N/A	 BQCA 13.	N/A	845	- 129-fold potentiation of ACh activity in Ca <sup>2+</sup> mobilisation assays (at 100 μM) - efficacy in a mouse contextual fear conditioning (CFC) study (15-20 mg/kg dose) and a discrimination reversal learning test in Tg2576 mice (30 mg/kg dose)	- low free fraction (1-4% - Yang <i>et al.</i> (2010), Bioorg. Med. Chem. Lett. 20, 531) - poor affinity (105 μM - Canals <i>et al.</i> (2012), JBC, 287, 650)
Kuduk, S. <i>et al.</i> (2010), Bioorg. Med. Chem. Lett., 20, 657	- Benzylic C-ring replacement with pyridine or diazines - D-ring benzyl substituents and heterocycles - A-ring fluorine substitution	 15.	7e	28	- free fraction 5-7% - not a P-gp substrate	poor CNS exposure in rats
Kuduk, S. <i>et al.</i> (2010), Bioorg. Med. Chem. Lett., 20, 1334	Introduction of N-linked D-rings	 16.	7	171	- free fraction 4-5% - 46-fold potentiation of ACh activity in Ca <sup>2+</sup> mobilisation assays (at 1 μM) - efficacy in a mouse CFC study (10 mg/kg dose)	some degree of rat P-gp susceptibility
Kuduk, S. <i>et al.</i> (2010), Bioorg. Med. Chem. Lett., 20, 2533 Kuduk, S. <i>et al.</i> (2010), Bioorg. Med. Chem. Lett., 20, 2538	Heterocyclic or cycloalkyl A-rings	 17.	10e	440	- free fraction 5-7% - excellent permeability (Papp = 20) - not a P-gp substrate - efficacy in a mouse CFC study (3 mg/kg dose)	
Kuduk, S. <i>et al.</i> (2010), ACS Med. Chem. Lett. 1, 263 Kuduk, S. <i>et al.</i> (2011), Bioorg. Med. Chem. Lett. 21, 1710	Replacement of quinoline with a quinolizidinone core	 19.	10i	520	- excellent permeability (Papp = 22) - not a P-gp substrate - minimal hERG channel affinity - CNS exposure in rats - acceptable pharmacokinetics in rats and dogs - efficacy in a mouse CFC study (30 mg/kg dose)	
Kuduk, S. <i>et al.</i> (2011) Bioorg. Med. Chem. Lett., 21, 2769	Fused bicyclic C/D rings	 18.	9a	69	- excellent permeability (Papp = 23) - not a P-gp substrate - 100-fold potentiation of ACh activity in Ca <sup>2+</sup> mobilisation assays (at 1 μM)	
Kuduk, S. D. <i>et al.</i> (2011), J. Med. Chem. 54, 4773 Uslaner, J.M. <i>et al.</i> (2013), Psychopharmacol. 225, 21	Introduction of cyano group to the C-ring para position	 20.	4i	135	- free fraction 29-32% - not a P-gp substrate - 146-fold potentiation of ACh activity in Ca <sup>2+</sup> mobilisation assays (at 1 μM) - increased cerebral blood flow in mice - significant CNS exposure in rats - acceptable pharmacokinetics in rats and dogs - improved cognitive performance in three different animal models of cognition	potential for substantial hepatic first-pass effect
Kuduk, S.D. <i>et al.</i> (2012), ACS Med. Chem. Lett. 3, 1070	-Investigation of carboxylic acid surrogates	 21.	4o	338	- free fraction 27-49% - excellent permeability (Papp = 33) - 52-fold potentiation of ACh activity in Ca <sup>2+</sup> mobilisation assays (at 34 μM) - significant CNS exposure in rats - efficacy in a mouse CFC study (10 mg/kg dose)	P-gp substrate



**Figure 18.** Summary of the pharmacophoric features reported by Kuduk et al.



**Figure 19.** Structures of the structurally diverse muscarinic allosteric ligands VU0119498 (22), VU0027414 (23), VU0090157 (24), and VU0029767 (25).

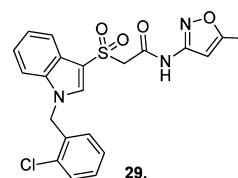


**Figure 20.** Structure of the BQCA-like PAMs VU0366369 (26), VU0448350-1 (27), and compound 28.

taken.<sup>110,111</sup> Replacement with an indolinone (27, Figure 20) resulted in a marked reduction in  $M_1$  mAChR potency compared to compound 26 ( $EC_{50} = 2.4 \mu M$ ) but a 10-fold

improvement in ACh cooperativity (27-fold left shift of ACh CRC at  $30 \mu M$ ).<sup>110</sup> Similarly, spirocyclic replacements for the isatin core largely resulted in potency reductions but improvements in ACh cooperativity, as well as increased subtype selectivity. An exemplar compound from this series was compound 28 (Figure 20).

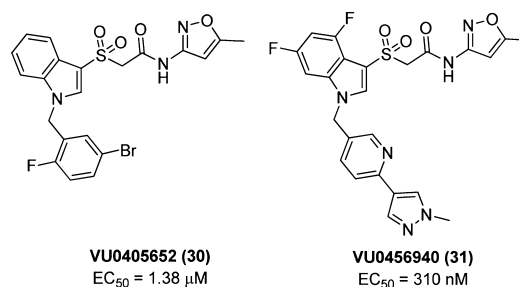
A novel chemical scaffold<sup>112</sup> derived from the same high-throughput screen as VU0366369 (26)<sup>108</sup> showing weak  $M_1$  mAChR PAM activity but high  $M_1$  mAChR selectivity was developed into a prospective lead, compound 29 (Figure 21).



**Figure 21.** Structure of the novel scaffold compound 29.

After modifications to the large northern portion of compound 29 proved detrimental to activity (evaluated in  $Ca^{2+}$  mobilization assays), synthetic efforts focused on introducing novel substituents to the southern benzyl moiety. 3-Chloro-, 3-bromo-, and 3-pyrazole-substitution gave the most potent analogues ( $EC_{50}s = 5.8, 3.8,$  and  $2.2 \mu M$ , respectively) with highly basic and sterically bulky groups not tolerated at this or any other position.

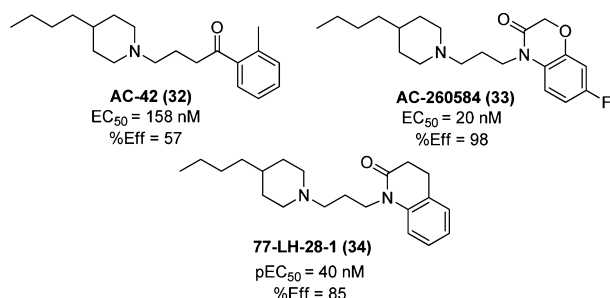
Extensive SAR efforts around this scaffold afforded further pharmacological improvements, resulting in  $M_1$  mAChR-selective compounds 30<sup>112</sup> and 31<sup>113</sup> (Figure 22). Compound



**Figure 22.** Structures of the novel scaffold analogues VU0405652 (30) and VU0456940 (31).

30 displayed relatively modest potency (when compared with BQCA and its optimized analogues) but promising positive modulation of ACh activity (at  $30 \mu M$ ). Compound 30 also displayed improved brain penetration compared to that of BQCA, minimal off-target activity and no orthosteric binding at any mAChR subtypes, and was observed to promote nonamyloidogenic processing of APP in conjunction with carbachol. Compound 31 displayed a substantial improvement in potency compared to BQCA and its predecessor compound 30, and potentiated  $M_1$  mAChR-mediated, carbachol-induced excitability of striatal medium spiny neurons as well as nonamyloidogenic APP processing.

**Putative Bitopic  $M_1$  mAChR Agonists.** *ACADIA Pharmaceuticals Compounds.* AC-42 (32, Figure 23) selectively activates the  $M_1$  mAChR subtype and was shown to bind to a novel combination of  $M_1$  mAChR residues within the N-terminus/TM1 and EC3/TM7 regions.<sup>114</sup> This novel



**Figure 23.** Structures of AC-42 (32), AC-260584 (33), and 77-LH-28-1 (34); R-SAT functional assays measuring  $\beta$ -galactosidase activity gave estimates of potency,  $EC_{50}$  (converted from  $pEC_{50}$ ), and efficacy, %Eff (normalized to the maximum response to the muscarinic agonist carbachol).

binding epitope cannot, however, be used as conclusive evidence of a purely allosteric binding mode.

Extensive pharmacological studies have revealed that AC-42 exhibits characteristics suggestive of both orthosteric and allosteric modes of action.<sup>114–118</sup> IP accumulation assays in CHO cells expressing the hM<sub>1</sub> mAChR revealed the orthosteric antagonist atropine to decrease the potency of AC-42 as evidenced by a concentration-dependent dextral shift in the AC-42 CRC, characteristic of a competitive interaction.<sup>116</sup> In equilibrium binding studies,<sup>115</sup> AC-42 almost completely inhibited binding of the radiolabeled [<sup>3</sup>H]NMS (0.2 nM), indicative of either competitive antagonism via the orthosteric site or a high degree of negative cooperativity via the allosteric site, or perhaps a combination of the two. Further studies confirmed the ability of AC-42 to interact allosterically with the M<sub>1</sub> mAChR; the ligand significantly slowed the rate of [<sup>3</sup>H]NMS dissociation from the M<sub>1</sub> mAChR.<sup>115</sup>

Notably, single point mutations of key orthosteric site residues Y381A and W101A,<sup>114,115,117</sup> while reducing the affinity of prototypical orthosteric muscarinic agonists ACh and pilocarpine, actually led to increases in the affinity of AC-42 from that recorded at the wild-type M<sub>1</sub> mAChR.<sup>115</sup> Furthermore, AC-42 exhibited a divergent signaling and regulatory profile to that of other orthosteric muscarinic agonists such as oxotremorine-M. AC-42 was unable to stimulate M<sub>1</sub> mAChR-G $\alpha_{i1/2}$  coupling<sup>119</sup> and did not result in M<sub>1</sub> mAChR internalization after prolonged (24 h) exposure. Rather, it actually upregulated total receptor expression.<sup>120</sup> Taken together, these observations support the hypothesis that AC-42 adopts a binding mode distinct from that of traditional nonselective agonists,<sup>121</sup> but one which places AC-42 in close proximity to the orthosteric site, implying that its agonistic properties may be derived from interactions with orthosteric residues (as well as allosteric residues); the definition of a bitopic ligand.

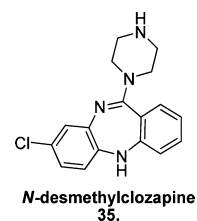
AC-42 was superseded by the more potent and efficacious structural analogue, AC-260584 (33, Figure 23), when it was found to exhibit the same high M<sub>1</sub> mAChR subtype selectivity as its predecessor but with the added advantages of oral bioavailability in rodents and the exhibition of antipsychotic and pro-cognitive effects in animal models of schizophrenia and AD.<sup>122,123</sup> While these results show great therapeutic promise, the ligand's classification as a purely allosteric agonist is likely premature. AC-260584 exhibited the same potential for binding orthosteric site residues as did AC-42.<sup>117</sup>

77-LH-28-1 (34, Figure 23), an M<sub>1</sub> mAChR-selective compound closely structurally related to the AC compounds,

demonstrated agonistic behavior at rat hippocampal M<sub>1</sub> mAChRs in vitro and CNS penetration leading to the stimulation of rat hippocampal cell firing in vivo.<sup>124</sup> Furthermore, activation of M<sub>1</sub> mAChRs by 77-LH-28-1 enhanced NMDA receptor activation in vitro, facilitating long-term potentiation.<sup>125</sup> Consistent with the interactions of AC-42, the mAChR orthosteric antagonists pirenzepine and scopolamine both produced a dextral displacement of the 77-LH-28-1 CRC;<sup>124</sup> however, 77-LH-28-1 has also been shown to compete with the prototypical muscarinic negative allosteric modulator C<sub>7</sub>/3-phth (at the NMS-occupied receptor),<sup>115</sup> adding weight to the theory that this scaffold interacts with portions of both orthosteric and allosteric sites on the M<sub>1</sub> mAChR.

The affinity of 77-LH-28-1, like AC-42, was improved at M<sub>1</sub> mAChRs containing the orthosteric mutations Y381A and W101A ( $pK_a = 7.16$  and  $8.64$ , respectively) from that recorded at the wild-type ( $pK_a = 6.74$ ). Additionally, a novel M<sub>1</sub> mAChR receptor mutation F77I resulted in substantially reduced efficacy of both AC-42 and 77-LH-28-1, while having little effect on the efficacy of traditional orthosteric ligands,<sup>115,118</sup> highlighting the importance of this residue in facilitating receptor activation by this scaffold, and the potential utility of this mutated receptor as a detection tool for this novel binding mode. Ligand docking studies performed on an M<sub>1</sub> mAChR homology model further support a bitopic binding mode for 77-LH-28-1.<sup>115</sup>

*N*-Desmethylozapine. Clozapine is a second-generation antipsychotic known for its unrivaled efficacy as a dual-action treatment for the positive and negative symptoms of schizophrenia,<sup>126</sup> and notorious for promiscuous off-target activity that has rendered it a risky therapeutic option. *N*-Desmethylozapine (35, Figure 24) is a biologically active metabolite of clozapine thought to play a role in the parent drug's unique therapeutic profile.



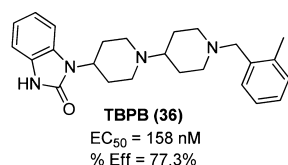
**Figure 24.** Structure of *N*-desmethylozapine (35).

Following an evaluation of the metabolite's effects at human cloned mAChRs,<sup>127</sup> *N*-desmethylozapine was described as an M<sub>1</sub> mAChR-preferring allosteric agonist, indirectly facilitating the potentiation of NMDA receptor activity, shown to be depleted in patients with psychotic disorders. In binding assays, *N*-desmethylozapine demonstrated a competitive interaction with the known M<sub>1</sub> mAChR allosteric ligand brucine (12), suggesting an allosteric mode of action; however, the activity of *N*-desmethylozapine in Ca<sup>2+</sup> mobilization assays was completely blocked when coinubated with atropine, suggesting an interaction with the orthosteric site as well. These observations certainly indicate that the ligand may be behaving in a bitopic manner.

*N*-Desmethylozapine has since been reported as a human native M<sub>1</sub> mAChR antagonist, despite its agonistic activity at human recombinant and rat native M<sub>1</sub> mAChRs,<sup>128</sup> rendering its therapeutic prospect as a treatment for cognitive deficits

obsolete. Disappointing outcomes such as this serve as a lesson for future development, highlighting the importance of evaluating lead compounds not just on rat and human recombinant receptors to assess their viability for in vitro and early in vivo trials, but to examine their activity at human native receptors to ensure their viability as future therapeutics.

**Vanderbilt University Compounds.** Compared to the scaffolds of previously reported compounds, TBPB ([1-(1'-(2-tolyl)-1,4'-bipiperidin-4-yl)-1H benzo[d]imidazol-2(3H)-one)] (36, Figure 25) represents a structurally unique



**Figure 25.** Structure of TBPB (36). Calcium mobilization assays in rat  $M_1$ -transfected CHO-K1 cells gave an estimate of potency,  $EC_{50}$ , and efficacy, %Eff (normalized to the maximum response to the muscarinic agonist carbachol).

mAChR ligand that exhibits selectivity for the  $M_1$  mAChR at the level of function. TBPB displayed agonistic behavior and potentiates NMDA receptor currents at rat hippocampal  $M_1$  mAChRs in vitro and was also shown to promote non-amyloidogenic APP processing in PC12 cells as measured by a decrease in  $A\beta_{42}$  levels and an increase in  $\alpha$ -secretase metabolites.<sup>129</sup> Furthermore, TBPB elicited antipsychotic-like behavior in rodent models of schizophrenia.

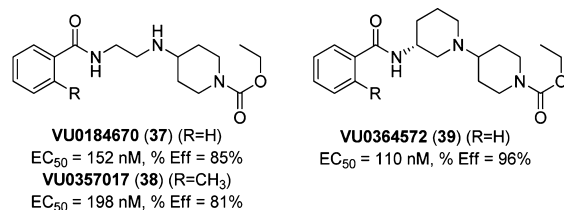
In calcium mobilization assays at the  $rM_1$  mAChR, TBPB displayed an apparent noncompetitive interaction with the orthosteric antagonist atropine. However, as this assay does not assess the ligands in an equilibrated state, this is not conclusive of an allosteric mode of action. Indeed the TBPB-mediated modulation of APP processing in PC12 cells expressing the  $hM_1$  mAChR was completely blocked by atropine which, as previously explained, could either be the result of a very high degree of negative cooperativity between the two ligands when concomitantly bound at the orthosteric site (atropine) and the allosteric site (TBPB), or evidence of a competitive interaction. Hence, the binding mode of TBPB is unclear and remains unresolved following the observed preservation of TBPB activity at  $M_1$  mAChRs bearing a mutation at a residue critical to orthosteric ligand binding (Y381A). Despite confirming that this residue is not essential to the TBPB- $M_1$  mAChR interaction, binding to other orthosteric site residues remains a valid possibility (as in the case of AC-42 and related compounds).

Further evidence for the potential orthosteric site-interaction of TBPB is that the  $M_1$  mAChR allosteric modulator VU0029767 (25) elicited a concentration-dependent potentiation of the cellular response to an  $EC_{20}$  concentration of TBPB "with a potency consistent with the potency of this compound for potentiating the response to ACh."<sup>107</sup> While this observation may be the result of positive cooperativity between two ligands binding to two distinct allosteric sites on the  $M_1$  mAChR, the possibility that a portion of TBPB is in fact occupying the orthosteric site in this interaction cannot be ignored.

SAR studies on this scaffold to date have proven unfruitful,<sup>130,131</sup> with a range of substitutions at both the distal basic piperidine nitrogen and the fused benzyl ring mostly

yielding compounds displaying either decreases in  $M_1$  mAChR potency, a loss of  $M_1$  mAChR selectivity, or undesirable off-target activity. Interestingly, a recent study identified "molecular switches" within the structure of TBPB that convert the ligand from a selective allosteric agonist to a *pan*-mAChR orthosteric antagonist.<sup>132</sup> Surprisingly, addition of a single fluorine atom to the central piperidine ring was sufficient to disrupt an interaction critical for allosteric binding. This result, combined with the finding that TBPB behaves as an orthosteric antagonist at the other mAChR subtypes (in  $Ca^{2+}$  mobilization assays), led to the first definitive classification of TBPB as a bitopic ligand; though additional supportive pharmacological data will add further weight to this classification.

Following high-throughput screening and synthetic optimization, VU0184670 and VU0357017/ML071<sup>133</sup> (37 and 38, Figure 26) were identified as potent ( $EC_{50} = 152$  and  $198 \text{ nM}$ ,



**Figure 26.** Structures of VU0184670 (37), VU0357017 (38), and VU0364572 (39). Calcium mobilization assays in rat  $M_1$ -transfected CHO-K1 cells gave an estimate of potency,  $EC_{50}$ , and efficacy, %Eff (normalized to the maximum response to the muscarinic agonist ACh).

respectively) and selective  $M_1$  mAChR agonists, producing maximum agonist effects of greater than 80% of the maximal response to ACh (as assessed by calcium mobilization assays). The activity of both VU0184670 and VU0357017 was preserved at receptors containing the mutated orthosteric site residue Y381A, and the ligands also demonstrated non-competitive interactions with the orthosteric antagonist atropine in calcium mobilization assays. In line with studies of TBPB, these findings suggest, but do not conclusively confirm, an allosteric mode of action for these compounds. While these compounds show minimal perturbation of [ $^3\text{H}$ ]NMS binding at concentrations required for activity in calcium mobilization assays, concentrations greater than  $1 \mu\text{M}$  displace radioligand binding, as evidenced by a clear reduction in the percentage of [ $^3\text{H}$ ]NMS receptor occupancy, suggestive of an orthosteric site interaction. Hence, a bitopic mode of action for these compounds remains a valid possibility.

These compounds demonstrated selective  $M_1$  mAChR-mediated potentiation of NMDA receptor currents in vitro and reversal of scopolamine-induced memory impairment in CFC in rats; however the need for improved potency and efficacy, as well as the elimination of functional  $D_2$  antagonism, prompted additional synthetic modifications in the form of cyclic constraints to be pursued.<sup>134</sup> This study resulted in VU0364572 (39, Figure 26), a compound which produced enantioselective  $M_1$  mAChR activation. This compound maintained the favorable in vitro characteristics observed previously but also displayed the desired improvements in activity and clean ancillary pharmacology. Moreover, VU0364572 possessed low plasma protein binding, increased oral bioavailability, and excellent CNS exposure. An alternative approach involving the introduction of a more diverse variety of

bicyclic constraints yielded some compounds with low nanomolar potency; however, a corresponding loss of muscarinic subtype selectivity was observed.<sup>135</sup> This led the authors to speculate that these compounds may possess a previously unappreciated bitopic mechanism of action, whereby lower potency analogues appear purely allosteric but higher potency analogues result in predominantly orthosteric interactions.

Interestingly, a recent publication demonstrated that VU0357017 and VU0364572 exhibit stimulus bias; both compounds behaved as agonists of  $M_1$  mAChR-mediated  $Ca^{2+}$  mobilization and ERK1/2 phosphorylation while having no effect on  $\beta$ -arrestin recruitment.<sup>136</sup> Studies in native tissues of  $M_1$  mAChR-mediated responses demonstrate that while both compounds potentiate NMDA receptor currents in hippocampal pyramidal cells and induce hippocampal LTP, they are devoid of electrophysiological activity in PFC pyramidal cells. These observations correlate well with the aforementioned study in which VU0357017 demonstrated efficacy in CFC, a hippocampal-dependent animal model of cognition.<sup>133</sup> As with TBPB, subtle “molecular switches” were also found within this scaffold,<sup>132,137</sup> where modifications such as inversion of chirality, reversed attachment, and benzyl ring replacement converted allosteric agonism to orthosteric antagonism. These observations prompted additional studies that ultimately led to the classification of VU0357017 and VU0364572 as bitopic ligands. Both ligands competitively displaced [ $^3H$ ]NMS binding at all five muscarinic receptor subtypes and behaved as orthosteric partial agonists at the  $M_1$  mAChR, but they also exhibited a characteristic allosteric effect of slowing the rate of [ $^3H$ ]NMS dissociation at  $M_1$  mAChR-expressing cell membranes.<sup>137</sup>

**Lu AE51090.** A more recent addition to the  $M_1$  mAChR-selective ligand literature is Lundbeck's Lu AE51090<sup>138</sup> (**40**, Figure 27). The original hit compound was found to be highly

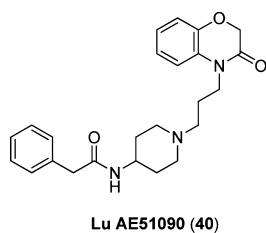


Figure 27. Structure of Lu AE51090 (**40**).

potent though not exclusively selective for the  $M_1$  mAChR subtype, so a number of analogues were synthesized to attempt to remedy this. Structural modifications included fluoro- and methoxy-substitution of the tetrahydroquinolinone ring system, replacement of this system with benzoxazolone and benzothiazinone ring systems, and the introduction of a range of alkyl chains and saturated and unsaturated rings at the amide position. The panel of analogues was screened against all five muscarinic receptor subtypes in functional  $Ca^{2+}$  mobilization assays. From this, Lu AE51090 was selected as an ideal lead compound based on both  $M_1$  mAChR activity preservation (the compound displayed a potency of 61 nM and a percentage efficacy of 83% compared to ACh) and  $M_1$  mAChR selectivity (the compound showed no agonism and negligible binding at the  $M_2$ – $M_5$  subtypes). The compound demonstrated acceptable absorption, distribution, metabolism, and excretion

(ADME) properties for in vivo analysis, and subsequently showed a dose-dependent reversal in delay-induced memory decay evident in mice performing a PFC and hippocampal-dependent task.

In a functional interaction assay against the orthosteric antagonist atropine, Lu AE51090 exhibited a saturable, noncompetitive profile suggesting an allosteric mode of action. As with the aforementioned “allosteric agonists”, Lu AE51090 activity was not compromised at Y381A mutated receptors. Once again, this evidence does not unequivocally rule out the possibility of a bitopic mechanism.

Interestingly, when screened against a broad range of 69 receptors, ion channels, transporters, and enzymes, Lu AE51090 exhibited only minor off-target activity. This is in contrast to AC-42 and TBPB, which displayed considerable affinity for other GPCRs including dopamine, serotonin, and adrenergic receptor subtypes, implying that Lu AE51090 may be a more useful molecular tool for exploring  $M_1$  mAChR activation in disease-specific tissues and in vivo cognition models.

With a view to designing an antipsychotic drug with pro-cognitive potential, the same group recently reported the in silico screening, synthesis, and evaluation of compounds displaying combined  $D_2$  R affinity and  $M_1$  mAChR agonism,<sup>139</sup> giving rise to compound **41** (Figure 28). While the binding

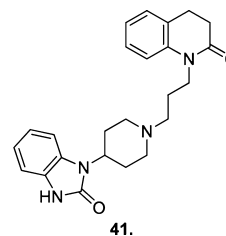


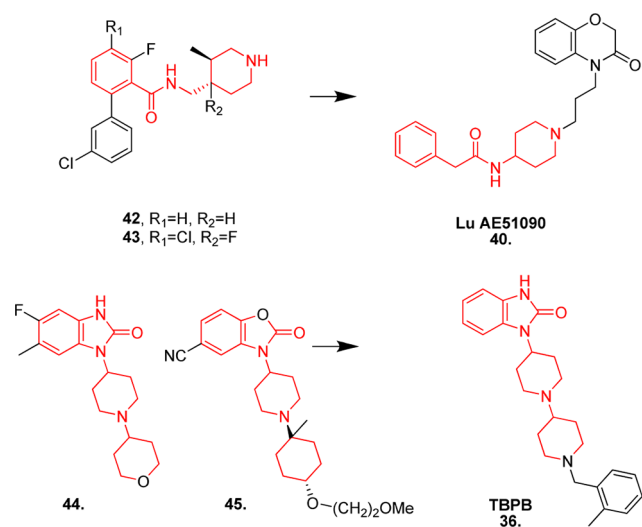
Figure 28. Structure of compound **41**.

mode and activity profile of this compound at the  $D_2$  R was unexamined, the  $M_1$  mAChR activity of compound **41** was unaffected by the Y381A orthosteric mutation, consistent with an allosteric or possibly bitopic mode of action at this receptor. A similar mutagenesis approach, or functional interaction studies, would have elucidated a potential binding mode for the compound at the  $D_2$  R, be it orthosteric, allosteric, or bitopic. The complete mode of action of this compound hence remains purely speculative. It may be an orthosteric  $D_2$ /allosteric  $M_1$  ligand, a pure allosteric ligand at both receptors, or a bitopic ligand at one or both targets. This ambiguity remains unexplored as, unfortunately, the compound also displayed hERG channel inhibition and synthetic strategies to overcome this adverse property were unsuccessful, resulting in the hit-to-lead campaign being abandoned.

**GlaxoSmithKline Compounds.** GlaxoSmithKline have contributed a number of other subtype-selective  $M_1$  mAChR agonists to this body of literature.<sup>140–144</sup> While these compounds are not purported specifically as either PAMs or allosteric agonists, their excellent subtype selectivity and striking similarities to some of the ligands previously described make them noteworthy.

Considerable SAR studies were performed around a 2' biaryl amide scaffold,<sup>140,141</sup> ultimately yielding compounds **42** and **43** (Figure 29), which showed favorable pharmacokinetic characteristics in addition to their high potency, intrinsic activity and





**Figure 29.** Structures of GSK compounds 42–45, with the motifs common to other M<sub>1</sub> mAChR-selective ligands highlighted in red.

exceptional selectivity against a panel of targets (as assessed by FLIPR calcium mobilization assays). In other studies,<sup>142,143</sup> optimization of a novel *N*-substituted benzimidazolone hit structure gave the CNS-penetrant, orally active, and subtype-selective compounds 44 and 45 (Figure 29). Recently, the M<sub>1</sub> mAChR allosteric agonist GSK1034702, an isomer of compound 44, has shown efficacy in improving episodic memory in humans in a nicotine abstinence model of cognitive dysfunction.<sup>144</sup> An 8 mg dose of GSK1034702 significantly improved immediate recall (but not delayed recall) following abstinence-induced impairment. Furthermore, in preclinical studies, the potency of GSK1034702 was sensitive to the M<sub>1</sub> mAChR F77I mutation, a mutation that also had a pronounced effect on the efficacy of AC-42 and 77-LH-28-1; suggesting a similar, potentially bitopic, binding mode.

More detailed pharmacological investigation would verify the binding mode and classification of these GSK ligands. However, simple visual comparison of these structures with both a putative bitopic ligand (Lu AE51090) and an acknowledged bitopic ligand (TBPB) reveals conspicuous similarities that support the notion of a shared mode of binding and activation at this receptor (Figure 29).

## PERSPECTIVE

Our knowledge of the intricate behaviors and interactions of M<sub>1</sub> mAChR allosteric ligands is rapidly expanding; however, despite the increasing depth of our understanding, researchers are still hampered by a number of difficulties that largely arise from the complexities of *screening*, *optimizing*, and *classifying* allosteric ligands.

With regards to initial library *screening*, each analogue is typically evaluated in one pharmacological assay and tested in the presence of a single concentration of one orthosteric ligand to gain a measure of ligand potency and degree of allosteric effect. While this is a valid method for the detection of putative ligand activity, the properties of stimulus bias and probe dependence that may be engendered by allosteric ligands remain unassessed. This may potentially reveal only one aspect of the ligand's pharmacological capabilities, recognizing only one "string" in the putative allosteric ligand's "bow". If the test ligand selectively activates different downstream receptor

signaling pathways, but not the pathway being assessed by the assay, pharmacologically desirable molecules may go undetected.<sup>54</sup> Or, as outlined earlier in Figures 9 and 10, this may lead to misclassification of the ligand itself. Another consideration with regard to screening is the nature of the receptor, be it rat, human recombinant, or human native. As previously highlighted with LY2033298 (10)<sup>76,78</sup> and *N*-desmethyloclozapine (35),<sup>128</sup> identification of species-dependent variability in both cooperativity and mode of action can lead to the discovery of altered selectivity and functional profiles that may have had deleterious results in a clinical setting if not identified earlier. Furthermore, the potential for allosteric ligands to elicit different physiological and toxicological outcomes in animal and human studies poses considerable challenges to the traditional drug discovery pipeline that will be difficult to resolve. In an ideal world, screening for novel allosteric drug candidates would involve a wide panel of orthosteric probes, assay outputs, and receptor targets. However, factoring in time, economic, and technological constraints, the best approach at present is to bear these considerations in mind and, following hit identification, apply this broader analysis in the hit-to-lead optimization process.

The potential issues surrounding the allosteric ligand screening process carry through into their *optimization*. While the evaluation of M<sub>1</sub> mAChR-selective allosteric ligands frequently encounters "flat" or "shallow" SAR as a challenge impeding the development of allosteric ligands,<sup>98,102,103,108,112</sup> it is possible that these observations are a consequence of too narrow a pharmacological analysis, given screening constraints. Broader evaluation of a limited cluster of novel scaffolds in radioligand binding and a variety of functional assays in the presence of multiple classes of orthosteric ligands at this stage of development may deconvolute the "confounding" SAR frequently observed in these studies and potentially reveal the capacity to differentially modulate discrete receptor signaling pathways and affect unique changes in the traditional behavior of orthosteric ligands. Additionally, quantification of properties such as binding cooperativity, functional cooperativity, and allosteric agonism would reduce the dependence on titration curve EC<sub>50</sub> values as the key determinant for future structural modifications. This would likely generate more detailed, "textured" SARs, advance our understanding of the relationship between allosteric ligand, orthosteric ligand, and receptor, and more accurately direct drug candidate optimization. Furthermore, as subtle changes in allosteric ligand structure have been shown to dramatically modify receptor subtype selectivity,<sup>87</sup> all promising analogues to emerge from the optimization process ought to be rescreened to ensure preservation of the desired selectivity profile.

Finally, the highly contextual (and sometimes premature) *classification* of allosteric ligands as PAMs, NAMs, and agonists further complicates this field of research. Misclassification typically results from incomplete investigation of the binding mode and functional profile of these ligands. Notable examples already described include the potential over-reliance on specific receptor mutants (e.g., orthosteric site Y381A mutation) as a means of discriminating orthosteric and allosteric binding modes, and the difficulty in distinguishing competitive and noncompetitive interactions, particularly when recording functional response data at a time point prior to the ligands reaching a state of equilibrium (e.g., the calcium mobilization assay). The prospect of bitopic modes of action further confounds this issue.

There is no simple means to overcoming this classification challenge. Confirming a purely allosteric mode of action and further investigating the exact nature of the putative bitopic  $M_1$  mAChR agonists already described cannot be achieved using one experimental technique alone, such as a mutagenesis study or a single functional assay. More comprehensive synthetic and pharmacological studies need to be undertaken.<sup>57,84</sup> Knowledge of each ligand's true binding mode with the  $M_1$  mAChR will facilitate rational drug design and will likely result in the more productive synthesis of potent, efficacious therapeutic leads. Additionally, adopting a global, more detailed lexicon to describe allosteric ligand activity<sup>56</sup> may aid communication between researchers.

## CONCLUSION

The unique properties of allosteric ligands require more thorough pharmacological evaluation if we are to advance our understanding of their interactions and comprehend the complete therapeutic potential of these molecules. Their complex behavior also demands a more detailed classification system that is globally recognized. The sooner these necessities are acknowledged and embraced, the more fruitful our labors will be.

In the field of selective  $M_1$  mAChRs specifically, this would translate to an improved ability to map the key binding residues of the multiple allosteric sites present on the receptor, more informed design of putative allosteric ligands, and a greater understanding of the intricate role of this muscarinic receptor subtype in the normal functioning of the CNS and the pathophysiology of CNS diseases. As a consequence, it will be possible to fully appreciate the scope of  $M_1$  mAChR activation as a potential neurodegenerative disorder therapy.

## AUTHOR INFORMATION

### Corresponding Author

\*Telephone: +61 3 9903 9542 (P.J.S.); +61 3 9903 9067 (A.C.). E-mail: peter.scammells@monash.edu (P.J.S.); arthur.christopoulos@monash.edu (A.C.).

### Author Contributions

All authors have given approval to the final version of the manuscript.

### Funding

The authors acknowledge financial support from the Australian Research Council (Discovery Grant DP110100687) and the National Health and Medical Research Council (NHMRC) of Australia (Program Grant 519461). A.C. is a Principal Research Fellow of the NHMRC, and B.J.D. is supported by an Australian Postgraduate Award.

### Notes

The authors declare no competing financial interest.

## ABBREVIATIONS

mAChR, muscarinic acetylcholine receptor; CNS, central nervous system; GPCR, G protein-coupled receptor; PLC, phospholipase C; IP<sub>3</sub>, inositol trisphosphate; AC, adenylate cyclase; cAMP, cyclic adenosine monophosphate; AD, Alzheimer's disease; ACh, acetylcholine; KO, knockout; PKC, protein kinase C; NMDA, *N*-methyl-D-aspartate; APP, amyloid precursor protein; CSF, cerebrospinal fluid; EPS, extrapyramidal side effects; TM, transmembrane; EC, extracellular; NMS, *N*-methylscopolamine; IC, intracellular; PAM, positive allosteric modulator; CRC, concentration response curve;

QNB, 3-quinuclidinyl benzilate; BQCA, benzyl quinolone carboxylic acid; FLIPR, fluorometric imaging plate reader; MWC, Monod-Wyman-Changeux; CFC, contextual fear conditioning; PFC, prefrontal cortex; sEPSCs, spontaneous excitatory postsynaptic currents; FF, free fraction; SAR, structure–activity relationship; BBB, blood-brain barrier; P-gp, P-glycoprotein; TBPB, [1-(1'-(2-tolyl)-1,4'-bipiperidin-4-yl)-1H benzo[*d*]imidazol-2(3H)-one]; ADME, absorption, distribution, metabolism, and excretion

## REFERENCES

- (1) van Koppen, C. J., and Kaiser, B. (2003) Regulation of muscarinic acetylcholine receptor signaling. *Pharmacol. Ther.* 98, 197–220.
- (2) Canals, M., Sexton, P. M., and Christopoulos, A. (2011) Allosterism in GPCRs: “MWC” revisited. *Trends Biochem. Sci.* 36, 663–672.
- (3) Caulfield, M. P. (1993) Muscarinic Receptors-Characterization, Coupling and Function. *Pharmacol. Ther.* 58, 319–379.
- (4) Nathanson, N. M. (2008) Synthesis, trafficking and localization of muscarinic acetylcholine receptors. *Pharmacol. Ther.* 119, 33–43.
- (5) Fisher, A. (2008)  $M_1$  Muscarinic Agonists Target Major Hallmarks of Alzheimer's Disease - The Pivotal Role of Brain  $M_1$  Receptors. *Neurodegener. Dis.* 5, 237–240.
- (6) Langmead, C. J., Watson, J., and Reavill, C. (2008) Muscarinic acetylcholine receptors as CNS drug targets. *Pharmacol. Ther.* 117, 232–243.
- (7) Xu, Y., Yan, J., Zhou, P., Li, J., Gao, H., Xia, Y., and Wang, Q. (2012) Neurotransmitter receptors and cognitive dysfunction in Alzheimer's disease and Parkinson's disease. *Prog. Neurobiol.* 97, 1–13.
- (8) Wess, J. (2004) Muscarinic Acetylcholine Receptor Knockout Mice: Novel Phenotypes and Clinical Implications. *Annu. Rev. Pharmacol. Toxicol.* 44, 423–450.
- (9) Bodick, N. C., Offen, W. W., Levey, A. I., Cutler, N. R., Gauthier, S. G., Satlin, A., Shannon, H. E., Tollefson, G. D., Rasmussen, K., Bymaster, F. P., Hurley, D. J., Potter, W. Z., and Paul, S. M. (1997) Effects of Xanomeline, a Selective Muscarinic Receptor Agonist, on Cognitive Function and Behavioral Symptoms in Alzheimer Disease. *Arch. Neurol.* 54, 465–473.
- (10) Digby, G. J., Shirey, J. K., and Conn, P. J. (2010) Allosteric activators of muscarinic receptors as novel approaches for treatment of CNS disorders. *Mol. Biosyst.* 6, 1345–1354.
- (11) Bartus, R. T., Dean, R. L., Beer, B., and Lippa, A. S. (1982) The Cholinergic Hypothesis of Geriatric Memory Dysfunction. *Science* 217, 408–417.
- (12) Whitehouse, P. J., Price, D. L., Struble, R. G., Clark, A. W., Coyle, J. T., and Delon, M. R. (1982) Alzheimer's disease and senile dementia: loss of neurons in the basal forebrain. *Science* 215, 1237–1239.
- (13) Francis, P. T., Palmer, A. M., Snape, M., and Wilcock, G. K. (1999) The cholinergic hypothesis of Alzheimer's disease: a review of progress. *J. Neurol. Neurosurg. Psychiatry* 66, 137–147.
- (14) Giacobini, E. (2000) Cholinesterase Inhibitors Stabilize Alzheimer's Disease. *Ann. N.Y. Acad. Sci.* 920, 321–327.
- (15) Mirza, N. R., Peters, D., and Sparks, R. G. (2003) Xanomeline and the Antipsychotic Potential of Muscarinic Receptor Subtype Selective Agonists. *CNS Drug Rev.* 9, 159–186.
- (16) Bymaster, F. P., Whitesitt, C. A., Shannon, H. E., DeLapp, N., Ward, J. S., Calligaro, D. O., Shipley, L. A., Buelke-Sam, J. L., Bodick, N. C., Farde, L., Sheardown, M. J., Olesen, P. H., Hansen, K. T., Suzdak, P. D., Swedberg, M. D. B., Sauerberg, P., and Mitch, C. H. (1997) Xanomeline: A Selective Muscarinic Agonist for the Treatment of Alzheimer's Disease. *Drug Dev. Res.* 40, 158–170.
- (17) Bymaster, F. P., McKinzie, D. L., Felder, C. C., and Wess, J. (2003) Use of  $M_1$ - $M_5$  Muscarinic Receptor Knockout Mice as Novel Tools to Delineate the Physiological Roles of the Muscarinic Cholinergic System. *Neurochem. Res.* 28, 437–442.
- (18) Shannon, H. E., Rasmussen, K., Bymaster, F. P., Hart, J. C., Peters, S. C., Swedberg, M. D. B., Jeppeson, L., Sheardown, M. J., Sauerberg, P., and Fink-Jensen, A. (2000) Xanomeline, an  $M_1/M_4$

preferring muscarinic cholinergic receptor agonist, produces anti-psychotic-like activity in rats and mice. *Schizophr. Res.* 42, 249–259.

(19) Shekhar, A., Potter, W. Z., Lightfoot, J., Lienemann, J., Dube, S., Mallinckrodt, C., Bymaster, F. P., McKinzie, D. L., and Felder, C. C. (2008) Selective Muscarinic Receptor Agonist Xanomeline as a Novel Treatment Approach for Schizophrenia. *Am. J. Psychiatry* 165, 1033–1039.

(20) Woolley, M. L., Carter, H. J., Gartlon, J. E., Watson, J. M., and Dawson, L. A. (2009) Attenuation of amphetamine-induced activity by the non-selective muscarinic receptor agonist, xanomeline, is absent in muscarinic M<sub>4</sub> receptor knockout mice and attenuated in muscarinic M<sub>1</sub> receptor knockout mice. *Eur. J. Pharmacol.* 603, 147–149.

(21) Tzavara, E. T., Bymaster, F. P., Davis, R. J., Wade, M. R., Perry, K. W., Wess, J., McKinzie, D. L., Felder, C., and Nomikos, G. G. (2004) M<sub>4</sub> muscarinic receptors regulate the dynamics of cholinergic and dopaminergic neurotransmission: relevance to the pathophysiology and treatment of related central nervous system pathologies. *FASEB J.* 18, 1410–1427.

(22) Wood, M. D., Murkitt, K. L., Ho, M., Watson, J. M., Brown, F., Hunter, A. J., and Middlemiss, D. N. (1999) Functional comparison of muscarinic partial agonists at muscarinic receptor subtypes hM<sub>1</sub>, hM<sub>2</sub>, hM<sub>3</sub>, hM<sub>4</sub> and hM<sub>5</sub> using microphysiometry. *Br. J. Pharmacol.* 126, 1620–1624.

(23) Farrell, M., and Roth, B. L. (2010) Allosteric Antipsychotics: M4Muscarinic Potentiators as Novel Treatments for Schizophrenia. *Neuropsychopharmacology* 35, 851–852.

(24) Tsang, S. W. Y., Pomakian, J., Marshall, G. A., Vinters, H. V., Cummings, J. L., Chen, C. P. L.-H., Wong, P. T.-H., and Lai, M. K. P. (2007) Disrupted muscarinic M<sub>1</sub> receptor signaling correlates with loss of protein kinase C activity and glutamatergic deficit in Alzheimer's disease. *Neurobiol. Aging* 28, 1381–1387.

(25) Fisher, A. (2012) Cholinergic modulation of amyloid precursor protein processing with emphasis on M1 muscarinic receptor: perspectives and challenges in treatment of Alzheimer's disease. *J. Neurochem.* 120, 22–33.

(26) Marino, M. J., Rouse, S. T., Levey, A. I., Potter, L. T., and Conn, P. J. (1998) Activation of the genetically defined m1 muscarinic receptor potentiates N-methyl-D-aspartate (NMDA) receptor currents in hippocampal pyramidal cells. *Proc. Natl. Acad. Sci. U.S.A.* 95, 11465–11470.

(27) Collingridge, G. L., Volianskis, A., Bannister, N., France, G., Hanna, L., Mercier, M., Tidball, P., Fang, G., Irvine, M. W., Costa, B. M., Monaghan, D. T., Bortolotto, Z. A., Molnar, E., Lodge, D., and Jane, D. E. (2013) The NMDA receptor as a target for cognitive enhancement. *Neuropharmacology* 64, 13–26.

(28) Davies, P., and Maloney, A. J. F. (1976) Selective Loss of Central Cholinergic Neurons in Alzheimer's Disease. *Lancet* 2, 1403.

(29) Flynn, D. D., Ferrari-DiLeo, G., Mash, D. C., and Levey, A. I. (1995) Differential Regulation of Molecular Subtypes of Muscarinic Receptors in Alzheimer's Disease. *J. Neurochem.* 64, 1888–1891.

(30) Overk, C. R., Felder, C. C., Tu, Y., Schober, D. A., Bales, K. R., Wu, J., and Mufson, E. J. (2010) Cortical M<sub>1</sub> receptor concentration increases without a concomitant change in function in Alzheimer's disease. *J. Chem. Neuroanat.* 40, 63–70.

(31) Turner, P. R., O'Connor, K., Tate, W. P., and Abraham, W. C. (2003) Roles of amyloid precursor protein and its fragments in regulating neural activity, plasticity and memory. *Prog. Neurobiol.* 70, 1–32.

(32) Hock, C., Maddalena, A., Raschig, A., Muller-Spahn, F., Eschweiler, G., Hager, K., Heuser, I., Hampel, H., Muller-Thomsen, T., Oertel, W., Wienrich, M., Signorell, A., Gonzalez-Agosti, C., and Nitsch, R. M. (2003) Treatment with the selective muscarinic m1 agonist talsaclidine decreases cerebrospinal fluid levels of Ab<sub>42</sub> in patients with Alzheimer's disease. *Amyloid* 10, 1–6.

(33) Fisher, A., Michaelson, D. M., Brandeis, R., Haring, R., Chapman, S., and Pittel, Z. (2000) M1Muscarinic Agonists as Potential Disease-Modifying Agents in Alzheimer's Disease: Rationale and Perspectives. *Ann. N.Y. Acad. Sci.* 920, 315–320.

(34) Buxbaum, J. D., Gandy, S. E., Cicchetti, P., Ehrlich, M. E., Czernik, A. J., Fracasso, R. P., Ramabhadran, T. V., Unterbeck, A. J., and Greengard, P. (1990) Processing of Alzheimer beta/A4 amyloid precursor protein: Modulation by agents that regulate protein phosphorylation. *Proc. Natl. Acad. Sci. U.S.A.* 87, 6003–6006.

(35) Caccamo, A., Oddo, S., Billings, L. M., Green, K. N., Martinez-Coria, H., Fisher, A., and LaFerla, F. M. (2006) M<sub>1</sub> Receptors Play a Central Role in Modulating AD-like Pathology in Transgenic Mice. *Neuron* 49, 671–682.

(36) Farde, L., Nordstrom, A.-L., Wiesel, F.-A., Pauli, S., Halldin, C., and Sedvall, G. (1992) Positron Emission Tomographic Analysis of Central D<sub>1</sub> and D<sub>2</sub> Dopamine Receptor Occupancy in Patients Treated With Classical Neuroleptics and Clozapine. *Arch. Gen. Psychiatry* 49, 538–544.

(37) Serretti, A., De Ronchi, D., Lorenzi, C., and Berardi, D. (2004) New Antipsychotics and Schizophrenia: A Review on Efficacy and Side Effects. *Curr. Med. Chem.* 11, 343–358.

(38) DeLeon, A., Patel, N. C., and Crismon, M. L. (2004) Aripiprazole: A Comprehensive Review of Its Pharmacology, Clinical Efficacy, and Tolerability. *Clin. Ther.* 26, 649–666.

(39) Minzenberg, M. J., and Carter, C. S. (2012) Developing treatments for impaired cognition in schizophrenia. *Trends Cognit. Sci.* 16, 35–42.

(40) Felder, C. C., Bymaster, F. P., and DeLapp, N. (2000) Therapeutic Opportunities for Muscarinic Receptors in the Central Nervous System. *J. Med. Chem.* 43, 4333–4353.

(41) Crook, J. M., Tomaskovic-Crook, E., Copolov, D. L., and Dean, B. (2000) Decreased Muscarinic Receptor Binding in Subjects with Schizophrenia: A Study of the Human Hippocampal Formation. *Biol. Psychiatry* 48, 381–388.

(42) Crook, J. M., Tomaskovic-Crook, E., Copolov, D. L., and Dean, B. (2001) Low Muscarinic Receptor Binding in Prefrontal Cortex From Subjects With Schizophrenia: A Study of Brodmann's Areas 8, 9, 10 and 46 and the Effects of Neuroleptic Drug Treatment. *Am. J. Psychiatry* 158, 918–925.

(43) Raedler, T. J., Bymaster, F. P., Tandon, R., Copolov, D., and Dean, B. (2007) Towards a muscarinic hypothesis of schizophrenia. *Mol. Psychiatry* 12, 232–246.

(44) Korczyn, A. D. (2000) Muscarinic M<sub>1</sub> agonists in the treatment of Alzheimer's disease. *Expert Opin. Invest. Drug* 9, 2259–2267.

(45) Flynn, D. D., Weinstein, D. A., and Mash, D. C. (1991) Loss of High-Affinity Agonist Binding to M1Muscarinic Receptors in Alzheimer's Disease: Implications for the Failure of Cholinergic Replacement Therapies. *Ann. Neurol.* 29, 256–262.

(46) Langmead, C. J., and Christopoulos, A. (2006) Allosteric agonists of 7TM receptors: expanding the pharmacological toolbox. *Trends Pharmacol. Sci.* 27, 475–481.

(47) Kenakin, T. P. (2012) Biased signalling and allosteric machines: new vistas and challenges for drug discovery. *Br. J. Pharmacol.* 165, 1659–1669.

(48) May, L. T., Leach, K., Sexton, P. M., and Christopoulos, A. (2007) Allosteric Modulation of G Protein-Coupled Receptors. *Annu. Rev. Pharmacol. Toxicol.* 47, 1–51.

(49) Dong, B. J. (2005) Cinacalcet: An Oral Calcimimetic Agent for the Management of Hyperparathyroidism. *Clin. Ther.* 27, 1725–1751.

(50) Garcia-Perez, J., Rueda, P., Staropoli, I., Kellenberger, E., Alcami, J., Arenzana-Seisdedos, F., and Lagane, B. (2011) New Insights into the Mechanisms whereby Low Molecular Weight CCR5 Ligands Inhibit HIV-1 Infection. *J. Biol. Chem.* 286, 4978–4990.

(51) Ehlert, F. J., Roeske, W. R., Gee, K. W., and Yamamura, H. I. (1983) An Allosteric Model For Benzodiazepine Receptor Function. *Biochem. Pharmacol.* 32, 2375–2383.

(52) Christopoulos, A. (2002) Allosteric Binding Sites On Cell-Surface Receptors: Novel Targets For Drug Discovery. *Nat. Rev. Drug Discovery* 1, 198–210.

(53) Conn, P. J., Jones, C. K., and Lindsley, C. W. (2009) Subtype-selective allosteric modulators of muscarinic receptors for the treatment of CNS disorders. *Trends Pharmacol. Sci.* 30, 148–155.

- (54) Kenakin, T. (2005) New Concepts In Drug Discovery: Collateral Efficacy And Permissive Antagonism. *Nat. Rev. Drug Discovery* 4, 919–927.
- (55) Kenakin, T. (2003) Ligand-selective receptor conformations revisited: the promise and the problem. *Trends Pharmacol. Sci.* 24, 346–354.
- (56) Keov, P., Sexton, P. M., and Christopoulos, A. (2011) Allosteric modulation of G protein-coupled receptors: A pharmacological perspective. *Neuropharmacology* 60, 24–35.
- (57) Leach, K., Loiacono, R. E., Felder, C. C., McKinzie, D. L., Mogg, A., Shaw, D. B., Sexton, P. M., and Christopoulos, A. (2010) Molecular Mechanisms of Action and *In Vivo* Validation of an M<sub>4</sub> Muscarinic Acetylcholine Receptor Allosteric Modulator with Potential Antipsychotic Properties. *Neuropsychopharmacology* 35, 855–869.
- (58) Canals, M., Lane, J. R., Wen, A., Scammells, P. J., Sexton, P. M., and Christopoulos, A. (2012) A Monod-Wyman-Changeux Mechanism Can Explain G Protein-coupled Receptor (GPCR) Allosteric Modulation. *J. Biol. Chem.* 287, 650–659.
- (59) Palczewski, K., Kumasaka, T., Hori, T., Behnke, C. A., Motoshima, H., Fox, B. A., Le Trong, I., Teller, D. C., Okada, T., Stenkamp, R. E., Yamamoto, M., and Miyano, M. (2000) Crystal Structure of Rhodopsin: A G Protein-Coupled Receptor. *Science* 289, 739–745.
- (60) Rasmussen, S. G. F., Choi, H.-J., Rosenbaum, D. M., Kobilka, T. S., Thian, F. S., Edwards, P. C., Burghammer, M., Ratnala, V. R. P., Sanishvili, R., Fischetti, R. F., Schertler, G. F. X., Weis, W. I., and Kobilka, B. K. (2007) Crystal structure of the human b<sub>2</sub> adrenergic G-protein-coupled receptor. *Nature* 450, 383–388.
- (61) Jaakola, V.-P., Griffith, M. T., Hanson, M. A., Cherezov, V., Chien, E. Y. T., Lane, J. R., Ijzerman, A. P., and Stevens, R. C. (2008) The 2.6 Angstrom Crystal Structure of a Human A<sub>2A</sub> Adenosine Receptor Bound to an Antagonist. *Science* 322, 1211–1217.
- (62) Strotmann, R., Schrock, K., Boselt, I., Staubert, C., Russ, A., and Schoneberg, T. (2011) Evolution of GPCR: Change and continuity. *Mol. Cell. Endocrinol.* 331, 170–178.
- (63) Haga, K., Kruse, A. C., Asada, H., Yurugi-Kobayashi, T., Shiroishi, M., Zhang, C., Weis, W. I., Okada, T., Kobilka, B. K., Haga, T., and Kobayashi, T. (2012) Structure of the human M2 muscarinic acetylcholine receptor bound to an antagonist. *Nature* 482, 547–552.
- (64) Kruse, A. C., Hu, J., Pan, A. C., Arlow, D. H., Rosenbaum, D. M., Rosemond, E., Green, H. F., Liu, T., Chae, P. S., Dror, R. O., Shaw, D. E., Weis, W. I., Wess, J., and Kobilka, B. K. (2012) Structure and dynamics of the M3 muscarinic acetylcholine receptor. *Nature* 482, 552–559.
- (65) Hulme, E. C., Birdsall, N. J. M., and Buckley, N. J. (1990) Muscarinic Receptor Subtypes. *Annu. Rev. Pharmacol. Toxicol.* 30, 633–673.
- (66) Spalding, T. A., Birdsall, N. J. M., Curtis, C. A. M., and Hulme, E. C. (1994) Acetylcholine Mustard Labels the Binding Site Aspartate in Muscarinic Acetylcholine Receptors. *J. Biol. Chem.* 269, 4092–4097.
- (67) Goodwin, J. A., Hulme, E. C., Langmead, C. J., and Tehan, B. G. (2007) Roof and Floor of the Muscarinic Binding Pocket: Variations in the Binding Modes of Orthosteric Ligands. *Mol. Pharmacol.* 72, 1484–1496.
- (68) Ellis, J. (1997) Allosteric Binding Sites on Muscarinic Receptors. *Drug Dev. Res.* 40, 193–204.
- (69) Wess, J. (2005) Allosteric Binding Sites on Muscarinic Acetylcholine Receptors. *Mol. Pharmacol.* 68, 1506–1509.
- (70) Gnagey, A. L., Seidenberg, M., and Ellis, J. (1999) Site-Directed Mutagenesis Reveals Two Epitopes Involved in the Subtype Selectivity of the Allosteric Interactions of Gallamine at Muscarinic Acetylcholine Receptors. *Mol. Pharmacol.* 56, 1245–1253.
- (71) Lazareno, S., Popham, A., and Birdsall, N. J. M. (2000) Allosteric Interactions of Staurosporine and Other Indolocarbazoles with N-[methyl-<sup>3</sup>H]Scopolamine and Acetylcholine at Muscarinic Receptor Subtypes: Identification of a Second Allosteric Site. *Mol. Pharmacol.* 58, 194–207.
- (72) Birdsall, N. J. M., Lazareno, S., Popham, A., and Saldanha, J. (2001) Multiple allosteric sites on muscarinic receptors. *Life Sci.* 68, 2517–2524.
- (73) Lazareno, S., Popham, A., and Birdsall, N. J. M. (2002) Analogs of WIN 62,577 Define a Second Allosteric Site on Muscarinic Receptors. *Mol. Pharmacol.* 62, 1492–1505.
- (74) Espinoza-Fonseca, L. M., and Trujillo-Ferrara, J. G. (2006) The existence of a second allosteric site on the M<sub>1</sub> muscarinic acetylcholine receptor and its implications for drug design. *Bioorg. Med. Chem. Lett.* 16, 1217–1220.
- (75) Kenakin, T., and Christopoulos, A. (2013) Signalling bias in new drug discovery: detection, quantification and therapeutic impact. *Nat. Rev. Drug Discovery* 12, 205–216.
- (76) Valant, C., Felder, C. C., Sexton, P. M., and Christopoulos, A. (2012) Probe Dependence in the Allosteric Modulation of a G Protein-Coupled Receptor: Implications for Detection and Validation of Allosteric Ligand Effects. *Mol. Pharmacol.* 81, 41–52.
- (77) Chan, W. Y., McKinzie, D. L., Bose, S., Mitchell, S. N., Witkin, J. M., Thompson, R. C., Christopoulos, A., Lazareno, S., Birdsall, N. J. M., Bymaster, F. P., and Felder, C. C. (2008) Allosteric modulation of the muscarinic M<sub>4</sub> receptor as an approach to treating schizophrenia. *Proc. Natl. Acad. Sci. U.S.A.* 105, 10978–10983.
- (78) Suratman, S., Leach, K., Sexton, P. M., Felder, C. C., Loiacono, R. E., and Christopoulos, A. (2011) Impact of species variability and 'probe dependence' on the detection and *in vivo* validation of allosteric modulation at the M<sub>4</sub> muscarinic acetylcholine receptor. *Br. J. Pharmacol.* 162, 1659–1670.
- (79) Lanzafame, A., Christopoulos, A., and Mitchelson, F. (1997) Three Allosteric Modulators Act at a Common Site, Distinct from that of Competitive Antagonists, at Muscarinic Acetylcholine M<sub>2</sub> Receptors. *J. Pharmacol. Exp. Ther.* 282, 278–285.
- (80) May, L. T., Avlani, V. A., Langmead, C. J., Herdon, H. J., Wood, M. D., Sexton, P. M., and Christopoulos, A. (2007) Structure-Function Studies of Allosteric Agonism at M<sub>2</sub> Muscarinic Acetylcholine Receptors. *Mol. Pharmacol.* 72, 463–476.
- (81) Antony, J., Kellershohn, K., Mohr-Andra, M., Kebig, A., Prilla, S., Muth, M., Heller, E., Disingrini, T., Dallanocce, C., Bertoni, S., Schrobang, J., Trankle, C., Kostenis, E., Christopoulos, A., Holtje, H.-D., Barocelli, E., De Amici, M., Holzgrabe, U., and Mohr, K. (2009) Dualsteric GPCR targeting: a novel route to binding and signaling pathway selectivity. *FASEB J.* 23, 442–450.
- (82) Lane, J. R., Sexton, P. M., and Christopoulos, A. (2013) Bridging the gap: bitopic ligands of G-protein-coupled receptors. *Trends Pharmacol. Sci.* 34, 59–66.
- (83) Valant, C., Sexton, P. M., and Christopoulos, A. (2009) Orthosteric/Allosteric Bitopic Ligands - Going Hybrid at GPCRs. *Mol. Interventions* 9, 125–135.
- (84) Valant, C., Gregory, K. J., Hall, N. E., Scammells, P. J., Lew, M. J., Sexton, P. M., and Christopoulos, A. (2008) A Novel Mechanism of G Protein-coupled Receptor Functional Selectivity - Muscarinic Partial Agonist McN-A-343 As A Bitopic Orthosteric/Allosteric Ligand. *J. Biol. Chem.* 283, 29312–29321.
- (85) Birdsall, N. J. M., Burgen, A. S. V., Hulme, E. C., Stockton, J. M., and Zigmund, M. J. (1983) The effect of McN-A-343 on muscarinic receptors in the cerebral cortex and heart. *Br. J. Pharmacol.* 78, 257–259.
- (86) Lazareno, S., and Birdsall, N. J. M. (1995) Detection, Quantitation, and Verification of Allosteric Interactions of Agents with Labeled and Unlabeled Ligands at G Protein-Coupled Receptors: Interactions of Strychnine and Acetylcholine at Muscarinic Receptors. *Mol. Pharmacol.* 48, 362–378.
- (87) Birdsall, N. J. M., Farries, T., Gharagozloo, P., Kobayashi, S., Kuonen, D., Lazareno, S., Popham, A., and Sugimoto, M. (1997) Selective Allosteric Enhancement of the Binding and Actions of Acetylcholine at Muscarinic Receptor Subtypes. *Life Sci.* 60, 1047–1052.
- (88) Ma, L., Seager, M. A., Wittman, M., Jacobson, M., Bickel, D., Burno, M., Jones, K., Graufelds, V. K., Xu, G., Pearson, M., McCampbell, A., Gaspar, R., Shughrue, P., Danziger, A., Regan, C.,

Flick, R., Pascarella, D., Garson, S., Doran, S., Kreatsoulas, C., Veng, L., Lindsley, C. W., Shipe, W., Kuduk, S., Sur, C., Kinney, G., Seabrook, G. R., and Ray, W. J. (2009) Selective activation of the M<sub>1</sub> muscarinic acetylcholine receptor achieved by allosteric potentiation. *Proc. Natl. Acad. Sci. U.S.A.* 106, 15950–15955.

(89) Miyakawa, T., Yamada, M., Duttaroy, A., and Wess, J. (2001) Hyperactivity and Intact Hippocampus-Dependent Learning in Mice Lacking the M<sub>1</sub> Muscarinic Acetylcholine Receptor. *J. Neurosci.* 21, 5239–5250.

(90) Rouse, S. T., Hamilton, S. E., Potter, L. T., Nathanson, N. M., and Conn, P. J. (2000) Muscarinic-induced modulation of potassium conductances is unchanged in mouse hippocampal pyramidal cells that lack functional M<sub>1</sub> receptors. *Neurosci. Lett.* 278, 61–64.

(91) Shirey, J. K., Brady, A. E., Jones, P. J., Davis, A. A., Bridges, T. M., Kennedy, J. P., Jadhav, S. B., Menon, U. N., Xiang, Z., Watson, M. L., Christian, E. P., Doherty, J. J., Quirk, M. C., Snyder, D. H., Lah, J. J., Levey, A. I., Nicolle, M. M., Lindsley, C. W., and Conn, P. J. (2009) A Selective Allosteric Potentiator of the M<sub>1</sub> Muscarinic Acetylcholine Receptor Increases Activity of Medial Prefrontal Cortical Neurons and Restores Impairments in Reversal Learning. *J. Neurosci.* 29, 14271–14286.

(92) Caruana, D. A., Warburton, E. C., and Bashir, Z. I. (2011) Induction of Activity-Dependent LTD Requires Muscarinic Receptor Activation in Medial Prefrontal Cortex. *J. Neurosci.* 31, 18464–18478.

(93) Chambon, C., Jatzke, C., Wegener, N., Gravius, A., and Danysz, W. (2012) Using cholinergic M<sub>1</sub> receptor positive allosteric modulators to improve memory via enhancement of brain cholinergic communication. *Eur. J. Pharmacol.* 697, 73–80.

(94) Chambon, C., Wegener, N., Gravius, A., and Danysz, W. (2011) A new automated method to assess the rat recognition memory: Validation of the method. *Behav. Brain Res.* 222, 151–157.

(95) Reichel, A. (2006) The Role of Blood-Brain Barrier Studies in the Pharmaceutical Industry. *Curr. Drug Metab.* 7, 183–203.

(96) Yang, F. V., Shipe, W. D., Bunda, J. L., Nolt, M. B., Wisnoski, D. D., Zhao, Z., Barrow, J. C., Ray, W. J., Ma, L., Wittman, M., Seager, M. A., Koeplinger, K. A., Hartman, G. D., and Lindsley, C. W. (2010) Parallel synthesis of N-biaryl quinolone carboxylic acids as selective M<sub>1</sub> positive allosteric modulators. *Bioorg. Med. Chem. Lett.* 20, 531–536.

(97) Kuduk, S. D., Chang, R. K., Di Marco, C. N., Ray, W. J., Ma, L., Wittman, M., Seager, M. A., Koeplinger, K. A., Thompson, C. D., Hartman, G. D., and Bilodeau, M. T. (2010) Quinolizidinone Carboxylic Acids as CNS Penetrant, Selective M<sub>1</sub> Allosteric Muscarinic Receptor Modulators. *ACS Med. Chem. Lett.* 1, 263–267.

(98) Kuduk, S. D., Chang, R. K., Di Marco, C. N., Ray, W. J., Ma, L., Wittman, M., Seager, M. A., Koeplinger, K. A., Thompson, C. D., Hartman, G. D., and Bilodeau, M. T. (2011) Quinolizidinone carboxylic acid selective M<sub>1</sub> allosteric modulators: SAR in the piperidine series. *Bioorg. Med. Chem. Lett.* 21, 1710–1715.

(99) Kuduk, S. D., Di Marco, C. N., Chang, R. K., Ray, W. J., Ma, L., Wittman, M., Seager, M. A., Koeplinger, K. A., Thompson, C. D., Hartman, G. D., and Bilodeau, M. T. (2010) Heterocyclic fused pyridone carboxylic acid M<sub>1</sub> positive allosteric modulators. *Bioorg. Med. Chem. Lett.* 20, 2533–2537.

(100) Kuduk, S. D., Di Marco, C. N., Cofre, V., Pitts, D. R., Ray, W. J., Ma, L., Wittman, M., Seager, M., Koeplinger, K., Thompson, C. D., Hartman, G. D., and Bilodeau, M. T. (2010) Pyridine containing M<sub>1</sub> positive allosteric modulators with reduced plasma protein binding. *Bioorg. Med. Chem. Lett.* 20, 657–661.

(101) Kuduk, S. D., Di Marco, C. N., Cofre, V., Pitts, D. R., Ray, W. J., Ma, L., Wittman, M., Veng, L., Seager, M. A., Koeplinger, K., Thompson, C. D., Hartman, G. D., and Bilodeau, M. T. (2010) N-Heterocyclic derived M<sub>1</sub> positive allosteric modulators. *Bioorg. Med. Chem. Lett.* 20, 1334–1337.

(102) Kuduk, S. D., Di Marco, C. N., Cofre, V., Ray, W. J., Ma, L., Wittman, M., Seager, M. A., Koeplinger, K. A., Thompson, C. D., Hartman, G. D., and Bilodeau, M. T. (2011) Fused heterocyclic M<sub>1</sub> positive allosteric modulators. *Bioorg. Med. Chem. Lett.* 21, 2769–2772.

(103) Kuduk, S. D., DiPardo, R. M., Beshore, D. C., Ray, W. J., Ma, L., Wittman, M., Seager, M. A., Koeplinger, K. A., Thompson, C. D.,

Hartman, G. D., and Bilodeau, M. T. (2010) Hydroxy cycloalkyl fused pyridone carboxylic acid M<sub>1</sub> positive allosteric modulators. *Bioorg. Med. Chem. Lett.* 20, 2538–2541.

(104) Kuduk, S. D., Chang, R. K., Greshock, T. J., Ray, W. J., Ma, L., Wittmann, M., Koeplinger, K. A., Seager, M. A., Thompson, C. D., Hartman, G., and Bilodeau, M. T. (2012) Identification of Amides as Carboxylic Acid Surrogates for Quinolizidinone-Based M<sub>1</sub> Positive Allosteric Modulators. *ACS Med. Chem. Lett.* 3, 1070–1074.

(105) Uslaner, J. M., Eddins, D., Puri, V., Cannon, C. E., Sutcliffe, J., Chew, C. S., Pearson, M., Vivian, J. A., Chang, R. K., Ray, W. J., Kuduk, S. D., and Wittmann, M. (2013) The muscarinic M<sub>1</sub> receptor positive allosteric modulator PQCA improves cognitive measures in rat, cynomolgus macaque, and rhesus macaque. *Psychopharmacology* 225, 21–30.

(106) Kuduk, S. D., and Beshore, D. C. (2012) Novel M<sub>1</sub> allosteric ligands: a patent review. *Expert Opin. Ther. Pat.* 22, 1385–1398.

(107) Marlo, J. E., Niswender, C. M., Days, E. L., Bridges, T. M., Xiang, Y., Rodriguez, A. L., Shirey, J. K., Brady, A. E., Nalywajko, T., Luo, Q., Austin, C. A., Baxter Williams, M., Kim, K., Williams, R., Orton, D., Brown, H. A., Lindsley, C. W., Weaver, C. D., and Conn, P. J. (2009) Discovery and Characterization of Novel Allosteric Potentiators of M<sub>1</sub> Muscarinic Receptors Reveals Multiple Modes of Activity. *Mol. Pharmacol.* 75, 577–588.

(108) Bridges, T. M., Kennedy, J. P., Noetzel, M. J., Breining, M. L., Gentry, P. R., Conn, P. J., and Lindsley, C. W. (2010) Chemical lead optimization of a pan G<sub>q</sub> mAChR M<sub>1</sub>, M<sub>3</sub>, M<sub>5</sub> positive allosteric modulator (PAM) lead. Part II: Development of a potent and highly selective M<sub>1</sub> PAM. *Bioorg. Med. Chem. Lett.* 20, 1972–1975.

(109) Bridges, T. M., Kennedy, J. P., Cho, H. P., Breining, M. L., Gentry, P. R., Hopkins, C. R., Conn, P. J., and Lindsley, C. W. (2010) Chemical lead optimization of a pan G<sub>q</sub> mAChR M<sub>1</sub>, M<sub>3</sub>, M<sub>5</sub> positive allosteric modulator (PAM) lead. Part I: Development of the first highly selective M<sub>5</sub> PAM. *Bioorg. Med. Chem. Lett.* 20, 558–562.

(110) Melancon, B. J., Poslusney, M. S., Gentry, P. R., Tarr, J. C., Sheffler, D. J., Mattmann, M. E., Bridges, T. M., Utley, T. J., Daniels, J. S., Niswender, C. M., Conn, P. J., Lindsley, C. W., and Wood, M. R. (2013) Isatin replacements applied to the highly selective, muscarinic M<sub>1</sub> PAM ML137: Continued optimization of an MLPCN probe molecule. *Bioorg. Med. Chem. Lett.* 23, 412–416.

(111) Poslusney, M. S., Melancon, B. J., Gentry, P. R., Sheffler, D. J., Bridges, T. M., Utley, T. J., Daniels, J. S., Niswender, C. M., Conn, P. J., Lindsley, C. W., and Wood, M. R. (2013) Spirocyclic replacements for the isatin in the highly selective, muscarinic M<sub>1</sub> PAM ML137: the continued optimization of an MLPCN probe molecule. *Bioorg. Med. Chem. Lett.* 23, 1860–1864.

(112) Reid, P. R., Bridges, T. M., Sheffler, D. J., Cho, H. P., Lewis, L. M., Days, E., Daniels, J. S., Jones, C. K., Niswender, C. M., Weaver, C. D., Conn, P. J., Lindsley, C. W., and Wood, M. R. (2011) Discovery and optimization of a novel, selective and brain penetrant M<sub>1</sub> positive allosteric modulator (PAM): The development of ML169, an MLPCN probe. *Bioorg. Med. Chem. Lett.* 21, 2697–2701.

(113) Tarr, J. C., Turlington, M. L., Reid, P. R., Utley, T. J., Sheffler, D. J., Cho, H. P., Klar, R., Pancani, T., Klein, M. T., Bridges, T. M., Morrison, R. D., Blobaum, A. L., Xiang, Z., Daniels, J. S., Niswender, C. M., Conn, P. J., Wood, M. R., and Lindsley, C. W. (2012) Targeting Selective Activation of M<sub>1</sub> for the Treatment of Alzheimer's Disease: Further Chemical Optimization and Pharmacological Characterization of the M<sub>1</sub> Positive Allosteric Modulator ML169. *ACS Chem. Neurosci.* 3, 884–895.

(114) Spalding, T. A., Trotter, C., Skjaerbaek, N., Messier, T. L., Currier, E. A., Burstein, E. S., Li, D., Hacksell, U., and Brann, M. R. (2002) Discovery of an Ectopic Activation Site on the M<sub>1</sub> Muscarinic Receptor. *Mol. Pharmacol.* 61, 1297–1302.

(115) Avlani, V. A., Langmead, C. J., Guida, E., Wood, M. D., Tehan, B. G., Herdon, H. J., Watson, J. M., Sexton, P. M., and Christopoulos, A. (2010) Orthosteric and Allosteric Modes of Interaction of Novel Selective Agonists of the M<sub>1</sub> Muscarinic Acetylcholine Receptor. *Mol. Pharmacol.* 78, 94–104.

- (116) Langmead, C. J., Fry, V. A. H., Forbes, I. T., Branch, C. L., Christopoulos, A., Wood, M. D., and Herdon, H. J. (2006) Probing the Molecular Mechanism of Interaction between 4-*n*-Butyl-1-[4-(2-methylphenyl)-4-oxo-1-butyl]-piperidine (AC-42) and the Muscarinic M<sub>1</sub> Receptor: Direct Pharmacological Evidence That AC-42 Is an Allosteric Agonist. *Mol. Pharmacol.* 69, 236–246.
- (117) Spalding, T. A., Ma, J.-N., Ott, T. R., Friberg, M., Bajpai, A., Bradley, S. R., Davis, R. E., Brann, M. R., and Burstein, E. S. (2006) Structural Requirements of Transmembrane Domain 3 for Activation by the M<sub>1</sub> Muscarinic Receptor Agonists AC-42, AC-260584, Clozapine, and *N*-Desmethylclozapine: Evidence for Three Distinct Modes of Receptor Activation. *Mol. Pharmacol.* 70, 1974–1983.
- (118) Jacobson, M. A., O'Brien, J. A., Pascarella, D., Mallorga, P. J., Scolnick, E. M., and Sur, C. (2004) Mapping the interaction site of M<sub>1</sub> muscarinic receptor allosteric agonists. *Soc. Neurosci. Abstr.* 30, 846.16.
- (119) Thomas, R. L., Mistry, R., Langmead, C. J., Wood, M. D., and Challiss, R. A. J. (2008) G Protein Coupling and Signaling Pathway Activation by M<sub>1</sub> Muscarinic Acetylcholine Receptor Orthosteric and Allosteric Agonists. *J. Pharmacol. Exp. Ther.* 327, 365–374.
- (120) Thomas, R. L., Langmead, C. J., Wood, M. D., and Challiss, R. A. J. (2009) Contrasting Effects of Allosteric and Orthosteric Agonists on M<sub>1</sub> Muscarinic Acetylcholine Receptor Internalization and Down-regulation. *J. Pharmacol. Exp. Ther.* 331, 1086–1095.
- (121) Lebon, G., Langmead, C. J., Tehan, B. G., and Hulme, E. C. (2009) Mutagenic Mapping Suggests a Novel Binding Mode for Selective Agonists of M<sub>1</sub> Muscarinic Acetylcholine Receptors. *Mol. Pharmacol.* 75, 331–341.
- (122) Bradley, S. R., Lameh, J., Ohrmund, L., Son, T., Bajpai, A., Nguyen, D., Friberg, M., Burstein, E. S., Spalding, T. A., Ott, T. R., Schiffer, H. H., Tabatabaei, A., McFarland, K., Davis, R. E., and Bonhaus, D. W. (2010) AC-260584, an orally bioavailable M<sub>1</sub> muscarinic receptor allosteric agonist, improves cognitive performance in an animal model. *Neuropharmacology* 58, 365–373.
- (123) Vanover, K. E., Veinbergs, I., and Davis, R. E. (2008) Antipsychotic-Like Behavioural Effects and Cognitive Enhancement by a Potent and Selective Muscarinic M<sub>1</sub> Receptor Agonist AC-260584. *Behav. Neurosci.* 122, 570–575.
- (124) Langmead, C. J., Austin, N. E., Branch, C. L., Brown, J. T., Buchanan, K. A., Davies, C. H., Forbes, I. T., Fry, V. A. H., Hagan, J. J., Herdon, H. J., Jones, G. A., Jeggo, R., Kew, J. N. C., Mazzali, A., Melarange, R., Patel, N., Pardoe, J., Randall, A. D., Roberts, C., Roopun, A., Starr, K. R., Teriakidis, A., Wood, M. D., Whittington, M., Wu, Z., and Watson, J. (2008) Characterization of a CNS penetrant, selective M<sub>1</sub> muscarinic receptor agonist, 77-LH-28-1. *Br. J. Pharmacol.* 154, 1104–1115.
- (125) Buchanan, K. A., Petrovic, M. M., Chamberlain, S. E. L., Marrion, N. V., and Mellor, J. R. (2010) Facilitation of Long-Term Potentiation by Muscarinic M<sub>1</sub> Receptors Is Mediated by Inhibition of SK Channels. *Neuron* 68, 948–963.
- (126) Kuroki, T., Nagao, N., and Nakahara, T. (2008) Neuropharmacology of second-generation antipsychotic drugs: a validity of the serotonin-dopamine hypothesis. *Prog. Brain Res.* 172, 199–212.
- (127) Sur, C., Mallorga, P. J., Wittmann, M., Jacobson, M. A., Pascarella, D., Williams, J. B., Brandish, P. E., Pettibone, D. J., Scolnick, E. M., and Conn, P. J. (2003) *N*-Desmethylclozapine, an allosteric agonist at muscarinic 1 receptor, potentiates *N*-methyl-D-aspartate receptor activity. *Proc. Natl. Acad. Sci. U.S.A.* 100, 13674–13679.
- (128) Thomas, D. R., Dada, A., Jones, G. A., Deisz, R. A., Gigout, S., Langmead, C. J., Werry, T. D., Hendry, N., Hagan, J. J., Davies, C. H., and Watson, J. M. (2010) *N*-Desmethylclozapine (NDMC) is an antagonist at the human native muscarinic M<sub>1</sub> receptor. *Neuropharmacology* 58, 1206–1214.
- (129) Jones, C. K., Brady, A. E., Davis, A. A., Xiang, Z., Bubser, M., Noor Tantawy, M., Kane, A. S., Bridges, T. M., Kennedy, J. P., Bradley, S. R., Peterson, T. E., Ansari, M. S., Baldwin, R. M., Kessler, R. M., Deutch, A. Y., Lah, J. J., Levey, A. I., Lindsley, C. W., and Conn, P. J. (2008) Novel Selective Allosteric Activator of the M<sub>1</sub> Muscarinic Acetylcholine Receptor Regulates Amyloid Processing and Produces Antipsychotic-Like Activity in Rats. *J. Neurosci.* 28, 10422–10433.
- (130) Bridges, T. M., Brady, A. E., Kennedy, J. P., Daniels, R. N., Miller, N. R., Kim, K., Breining, M. L., Gentry, P. R., Brogan, J. T., Jones, C. K., Conn, P. J., and Lindsley, C. W. (2008) Synthesis and SAR of analogues of the M<sub>1</sub> allosteric agonist TBPB. Part I: Exploration of alternative benzyl and privileged structure moieties. *Bioorg. Med. Chem. Lett.* 18, 5439–5442.
- (131) Miller, N. R., Daniels, R. N., Bridges, T. M., Brady, A. E., Conn, P. J., and Lindsley, C. W. (2008) Synthesis and SAR of analogs of the M<sub>1</sub> allosteric agonist TBPB. Part II: Amides, sulfonamides and ureas—The effect of capping the distal basic piperidine nitrogen. *Bioorg. Med. Chem. Lett.* 18, 5443–5447.
- (132) Sheffler, D. J., Sevel, C., Le, U., Lovett, K. M., Tarr, J. C., Carrington, S. J. S., Cho, H. P., Digby, G. J., Niswender, C. M., Conn, P. J., Hopkins, C. R., Wood, M. R., and Lindsley, C. W. (2013) Further exploration of M<sub>1</sub> allosteric agonists: Subtle structural changes abolish M<sub>1</sub> allosteric agonism and result in *pan*-mAChR orthosteric antagonism. *Bioorg. Med. Chem. Lett.* 23, 223–227.
- (133) Lebois, E. P., Bridges, T. M., Lewis, L. M., Dawson, E. S., Kane, A. S., Xiang, Z., Jadhav, S. B., Yin, H., Kennedy, J. P., Meiler, J., Niswender, C. M., Jones, C. K., Conn, P. J., Weaver, C. D., and Lindsley, C. W. (2010) Discovery and Characterization of Novel Subtype-Selective Allosteric Agonists for the Investigation of M<sub>1</sub> Receptor Function in the Central Nervous System. *ACS Chem. Neurosci.* 1, 104–121.
- (134) Lebois, E. P., Digby, G. J., Sheffler, D. J., Melancon, B. J., Tarr, J. C., Cho, H. P., Miller, N. R., Morrison, R., Bridges, T. M., Xiang, Z., Daniels, J. S., Wood, M. R., Conn, P. J., and Lindsley, C. W. (2011) Development of a highly selective, orally bioavailable and CNS penetrant M<sub>1</sub> agonist derived from the MLPNCN probe ML071. *Bioorg. Med. Chem. Lett.* 21, 6451–6455.
- (135) Melancon, B. J., Gogliotti, R. D., Tarr, J. C., Saleh, S. A., Chauder, B. A., Lebois, E. P., Cho, H. P., Utley, T. J., Sheffler, D. J., Bridges, T. M., Morrison, R. D., Daniels, J. S., Niswender, C. M., Conn, P. J., Lindsley, C. W., and Wood, M. R. (2012) Continued optimization of the MLPNCN probe ML071 into highly potent agonists of the hM<sub>1</sub> muscarinic acetylcholine receptor. *Bioorg. Med. Chem. Lett.* 22, 3467–3472.
- (136) Digby, G. J., Noetzel, M. J., Bubser, M., Utley, T. J., Walker, A. G., Byun, N. E., Lebois, E. P., Xiang, Z., Sheffler, D. J., Cho, H. P., Davis, A. A., Nemirovsky, N. E., Mennenga, S. E., Camp, B. W., Bimonte-Nelson, H. A., Bode, J., Italiano, K., Morrison, R., Daniels, J. S., Niswender, C. M., Olive, M. F., Lindsley, C. W., Jones, C. K., and Conn, P. J. (2012) Novel Allosteric Agonists of M<sub>1</sub> Muscarinic Acetylcholine Receptors Induce Brain Region-Specific Responses That Correspond with Behavioural Effects in Animal Models. *J. Neurosci.* 32, 8532–8544.
- (137) Digby, G. J., Utley, T. J., Lamsal, A., Sevel, C., Sheffler, D. J., Lebois, E. P., Bridges, T. M., Wood, M. R., Niswender, C. M., Lindsley, C. W., and Conn, P. J. (2012) Chemical Modification of the M<sub>1</sub> Agonist VU0364572 Reveals Molecular Switches in Pharmacology and a Bitopic Binding Mode. *ACS Chem. Neurosci.* 3, 1025–1036.
- (138) Sams, A. G., Hentzer, M., Mikkelsen, G. K., Larsen, K., Bundgaard, C., Plath, N., Christoffersen, C. T., and Bang-Andersen, B. (2010) Discovery of *N*-{1-[3-(3-Oxo-2,3-dihydrobenzo[1,4]oxazin-4-yl)propyl]piperidin-4-yl}-2-phenylacetamide (Lu AE51090): An Allosteric Muscarinic M<sub>1</sub> Receptor Agonist with Unprecedented Selectivity and Procognitive Potential. *J. Med. Chem.* 53, 6386–6397.
- (139) Sams, A. G., Larsen, K., Mikkelsen, G. K., Hentzer, M., Christoffersen, C. T., Jensen, K. G., Frederiksen, K., and Bang-Andersen, B. (2012) Hit-to-lead investigation of a series of novel combined dopamine D<sub>2</sub> and muscarinic M<sub>1</sub> receptor ligands with putative antipsychotic and pro-cognitive potential. *Bioorg. Med. Chem. Lett.* 22, 5134–5140.
- (140) Budzik, B., Garzya, V., Shi, D., Foley, J. J., Rivero, R. A., Langmead, C. J., Watson, J., Wu, Z., Forbes, I. T., and Jin, J. (2010) 2' Biaryl amides as novel and subtype selective M<sub>1</sub> agonists. Part I: Identification, synthesis, and initial SAR. *Bioorg. Med. Chem. Lett.* 20, 3540–3544.

(141) Budzik, B., Garzya, V., Shi, D., Walker, G., Lauchart, Y., Lucas, A. J., Rivero, R. A., Langmead, C. J., Watson, J., Wu, Z., Forbes, I. T., and Jin, J. (2010) 2' Biaryl amides as novel and subtype selective M<sub>1</sub> agonists. Part II: Further optimization and profiling. *Bioorg. Med. Chem. Lett.* *20*, 3545–3549.

(142) Budzik, B., Garzya, V., Shi, D., Walker, G., Woolley-Roberts, M., Pardoe, J., Lucas, A., Tehan, B., Rivero, R. A., Langmead, C. J., Watson, J., Wu, Z., Forbes, I. T., and Jin, J. (2010) Novel N-Substituted Benzimidazolones as Potent, Selective, CNS-Penetrant, and Orally Active M<sub>1</sub> mAChR Agonists. *ACS Med. Chem. Lett.* *1*, 244–248.

(143) Johnson, D. J., Forbes, I. T., Watson, S. P., Garzya, V., Stevenson, G. I., Walker, G. R., Mudhar, H. S., Flynn, S. T., Wyman, P. A., Smith, P. W., Murkitt, G. S., Lucas, A. J., Mookherjee, C. R., Watson, J. M., Gartlon, J. E., Bradford, A. M., and Brown, F. (2010) The discovery of a series of N-substituted 3-(4-piperidinyl)-1,3-benzoxazolinones and oxindoles as highly brain penetrant, selective muscarinic M<sub>1</sub> agonists. *Bioorg. Med. Chem. Lett.* *20*, 5434–5438.

(144) Nathan, P. J., Watson, J., Lund, J., Davies, C. H., Peters, G., Dodds, C. M., Swirski, B., Lawrence, P., Bentley, G. D., O'Neill, B. V., Robertson, J., Watson, S., Jones, G. A., Maruff, P., Croft, R. J., Laruelle, M., and Bullmore, E. T. (2013) The potent M<sub>1</sub> receptor allosteric agonist GSK1034702 improves episodic memory in humans in the nicotine abstinence model of cognitive dysfunction. *Int. J. Neuro-psychopharmacol.* *16*, 721–731.