*Allosteric modulation of a variety of GPCRs, targeting glutamatergic dysfunction, represents a significant area of research for the treatment of schizophrenia. Here, we summarize a group of selective M_1 agonists including activity at other muscarinic receptors, ancillary pharmacology and relevant DMPK profiles, results from **in vitro** assays and native tissue experiments, as well as important **in vivo** findings.*

Allosteric modulation of the M_1 muscarinic acetylcholine receptor: improving cognition and a potential treatment for schizophrenia and Alzheimer's disease

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Allosteric modulation of AMPA, NR2B, $mGlu_2$, $mGlu_5$ and M_1, targeting glutamatergic dysfunction, represents a significant area of research for the treatment of schizophrenia. Of these targets, clinical promise has been demonstrated using muscarinic activators for the treatment of Alzheimer's disease (AD) and schizophrenia. These diseases have inspired researchers to determine the effects of modulating cholinergic transmission in the forebrain, which is primarily regulated by one of five subtypes of muscarinic acetylcholine receptor (mAChR), a subfamily of G-protein-coupled receptors (GPCRs). Of these five subtypes, M_1 is highly expressed in brain regions responsible for learning, cognition and memory. Xanomeline, an orthosteric muscarinic agonist with modest selectivity, was one of the first compounds that displayed improvements in behavioral disturbances in AD patients and efficacy in schizophrenics. Since these initial clinical results, many scientists, including those in our laboratories, have strived to elucidate the role of M_1 with compounds that display improved selectivity for this receptor by targeting allosteric modes of receptor activation. A survey of selected compounds in this area will be presented.

Introduction

Schizophrenia is a devastating psychiatric illness that afflicts approximately 1% of the population and presents with three classical symptom clusters: positive symptoms, negative symptoms and cognitive impairments [1,2]. Cognitive (including deficits in attention, memory and executive function) and negative (social withdrawal, anhedonia and apathy) deficits that precede the first psychotic episode (delusions, hallucinations and thought disorders) are not effectively treated by current antipsychotic drugs, and account for the lifelong disability and poor outcomes associated with schizophrenia [2–5]. A host of data suggests that dysfunction in glutamatergic synaptic transmission in frontal cortical networks underlies the complex

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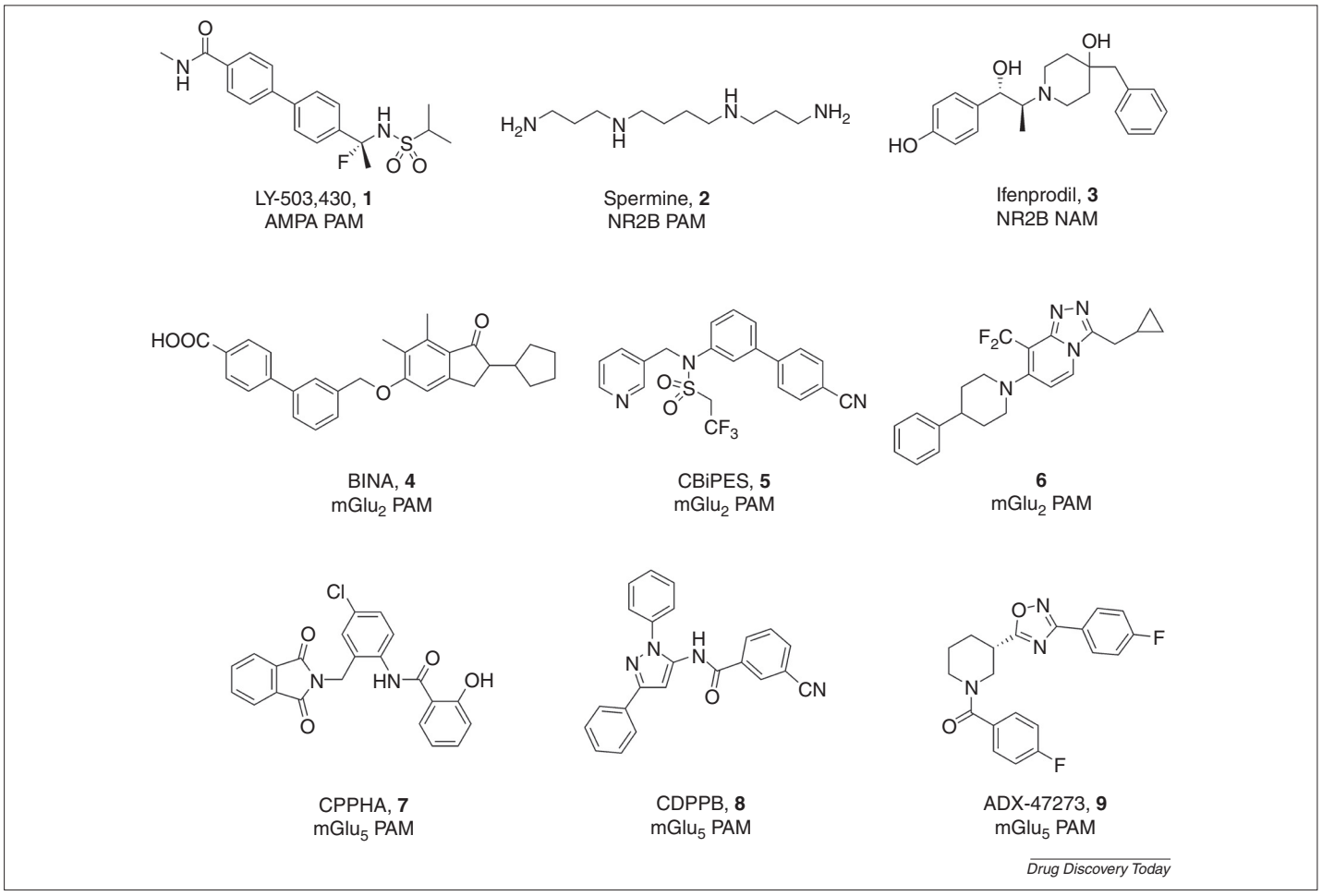

FIGURE 1

Representative allosteric modulators of AMPA, NR2B, $mGlu_2$ and $mGlu_5$ that address dysfunction in glutamatergic neurotransmission in cortical brain regions.

symptom clusters of schizophrenia, as opposed to altered neurotransmitter levels [4–7]. These findings have led researchers to evaluate multiple pre- and post-synaptic mechanisms affecting glutamatergic synaptic transmission, which has elucidated a number of discrete molecular targets for therapeutic intervention. For many of these targets (AMPA, NR2B, $mGlu_2$ and $mGlu_5$), the development of orthosteric ligands has proven extremely difficult, from a chemical, pharmacological or safety perspective; however, targeting allosteric sites of these targets has emerged as a promising alternative [8–12]. Indeed, many recent manuscripts and reviews have detailed the virtues of allosteric modulation [10–15], and many valuable allosteric modulator tool compounds have been developed (Fig. 1, compounds **1**–**9**) to enable key preclinical proof-of-concept studies. Because these have been extensively reviewed recently, we will focus this review on a large body of new data on the M_1 muscarinic acetylcholine receptor (mAChR) and its link to the cholinergic and *N*-methyl-D-aspartate (NMDA) hypofunction hypotheses of schizophrenia, as well as its link to Alzheimer's disease (AD).

A cholinergic hypothesis of schizophrenia

The mAChRs are G-protein-coupled receptors (GPCRs) for the neurotransmitter acetylcholine (ACh) and consist of five different subtypes, termed M_1–M_5. These subtypes are further grouped based on their coupling to signal transduction pathways [16–18]. When stimulated by ACh, M_1, M_3 and M_5 induce release of intracellular calcium stores through the activation of phospholipase C through $G\alpha_q$. M_2 and M_4 couple to $G\alpha_{i/o}$ to regulate adenylyl cyclase and many ion channels (Fig. 2) [19]. Numerous preclinical and clinical studies with nonselective mAChR agonists suggest that activation of mAChRs improves cognitive function in patients suffering from various central nervous system (CNS) disorders, and these studies, along with genetic studies, indicate that M_1 is the mAChR subtype mediating the procognitive effects [20,21]. Furthermore, agents that enhance cholinergic transmission, including acetylcholinesterase (AChE) inhibitors, have established efficacy in improving cognitive function in patients suffering from AD and other memory disorders [22,23]. Over 50 years ago, nonselective muscarinic antagonists, such as scopolamine, were shown to induce many of the symptoms associated with schizophrenia in healthy humans and exacerbate existing symptoms in schizophrenia patients. During this time, muscarinic agonists were shown to be moderately effective as neuroleptic agents, which gave rise to a cholinergic hypothesis of schizophrenia, decades before the now prevalent dopamine hyperfunction hypothesis. A large body of clinical, preclinical, postmortem, genetic and brain-imaging studies provides strong support for involvement of the cholinergic system in the pathophysiology

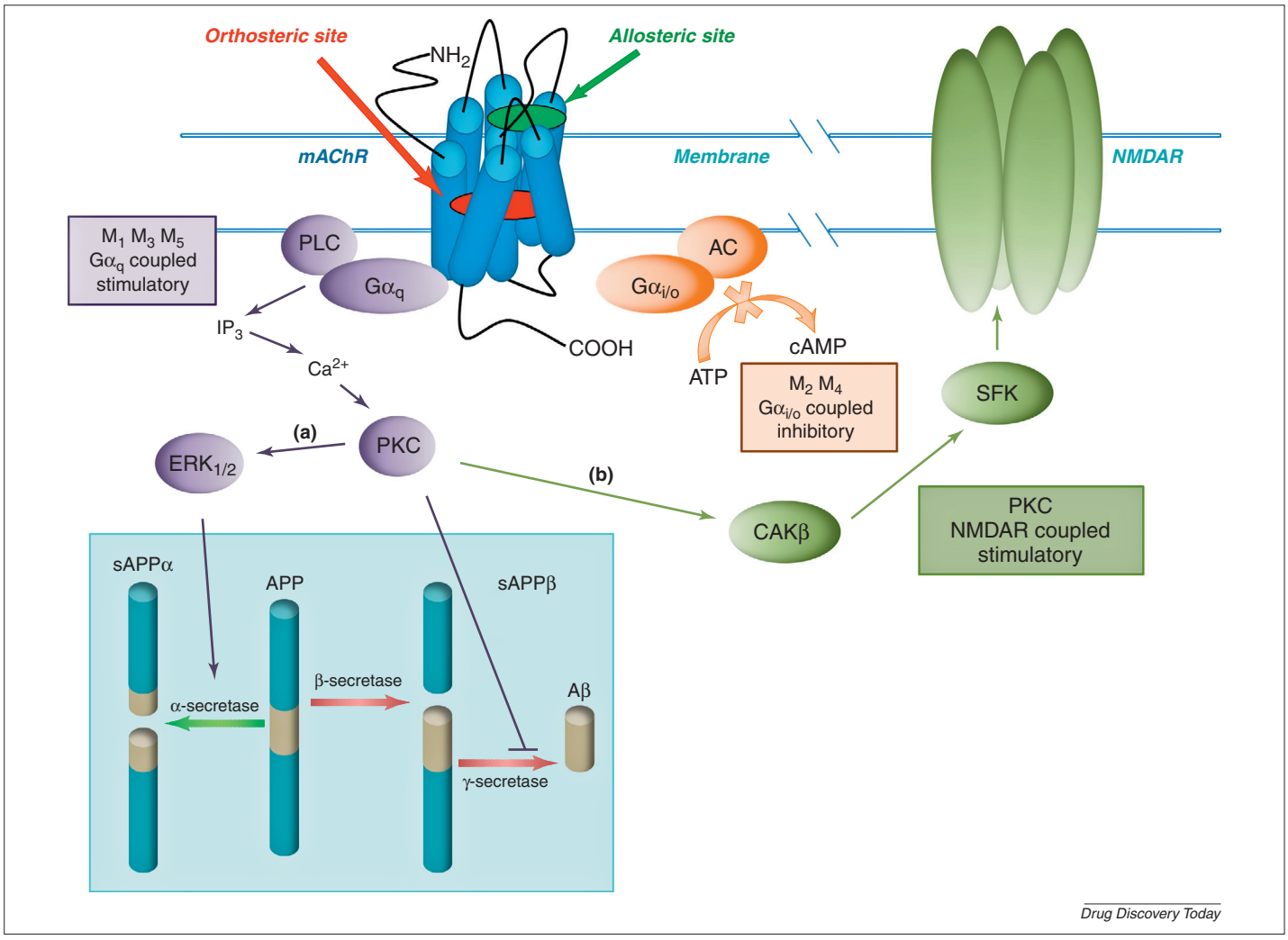

FIGURE 2

Schematic illustration of the muscarinic acetylcholine receptor subtypes M_1–M_5. The seven transmembrane domains of the family A G-protein-coupled receptors (GPCRs) are highlighted in blue. The orthosteric binding site is indicated in red. A putative allosteric site is illustrated in green and not indicative of a singular or unique site. The downstream effectors indicate M_1 signaling related to the **(a)** amyloidogenic precursor protein (APP) and subsequent secretase activity to generate soluble amyloidogenic precursor protein alpha (sAPPα), soluble amyloidogenic precursor protein beta (sAPPβ) and Aβ. **(b)** NMDAR through the CAKβ and SFK pathway. *Abbreviations*: AC, adenylyl cyclase; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; $ERK_{1/2}$, extracellular signal-regulated kinase; IP_3, inositol triphosphate; PKC, protein kinase C; PLC, phospholipase C, CAKβ, cell adhesion kinase β; SFK, Src family kinases.

of schizophrenia. Receptor protein and mRNA levels of M_1 have been shown to be decreased in frontal cortex of schizophrenic patients, which led to the characterization of a subpopulation of schizophrenic patients referred to as muscarinic receptor deficient schizophrenics (MRDS) [24]. In addition, circulating antibodies against M_1 have been found in the serum of schizophrenics, suggesting a link between the immune system and M_1 in schizophrenics. Interestingly, it is unclear if the antipsychotic efficacy of mAChR activation is caused by direct muscarinic effects alone, or through modulatory effects on the dopaminergic system and other neurotransmitter systems.

M_1 activation and the NMDA receptor hypofunction hypothesis of schizophrenia

NMDA receptors have an important role in the regulation of circuits that are crucial for normal cognitive and executive functions and that are disrupted in schizophrenia and other psychotic disorders [25]. Competitive and noncompetitive antagonists of the NMDA receptor, such as ketamine and phencyclidine (PCP), can induce a psychotic state that closely resembles that seen in schizophrenic patients [26–28]. Furthermore, co-agonists at the NMDA receptor, such as glycine and D-cycloserine, produce improvements in the symptoms of schizophrenic patients and the glycine transporter 1 (GlyT1) inhibitor RG-1678 recently provided robust efficacy against negative symptoms in schizophrenia patients [29]. Thus, a large number of clinical and animal studies have led to the hypothesis that potentiation of NMDA receptor neurotransmission might help to normalize the imbalances in neural circuitry associated with schizophrenia and provide antipsychotic action and improvements for the negative and cognitive symptoms [30,31]. One of the most prominent effects of M_1 activation in the hippocampus and other forebrain regions is the potentiation of NMDA receptor currents [31]. Many studies have also confirmed that M_1 is colocalized with the NR1 subunit of the NMDA receptor

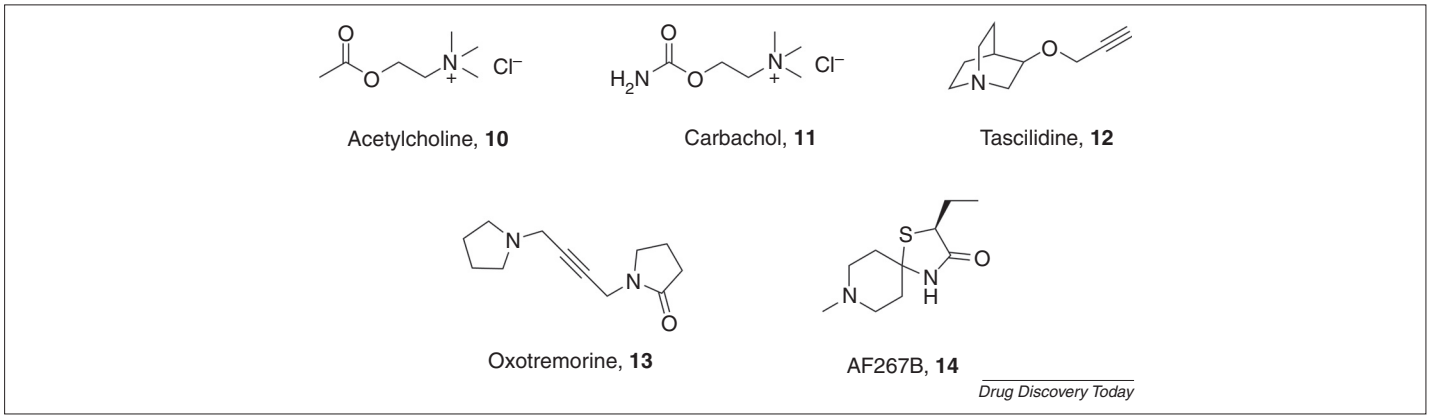

FIGURE 3

Orthosteric agonists of the M_1 mAChR.

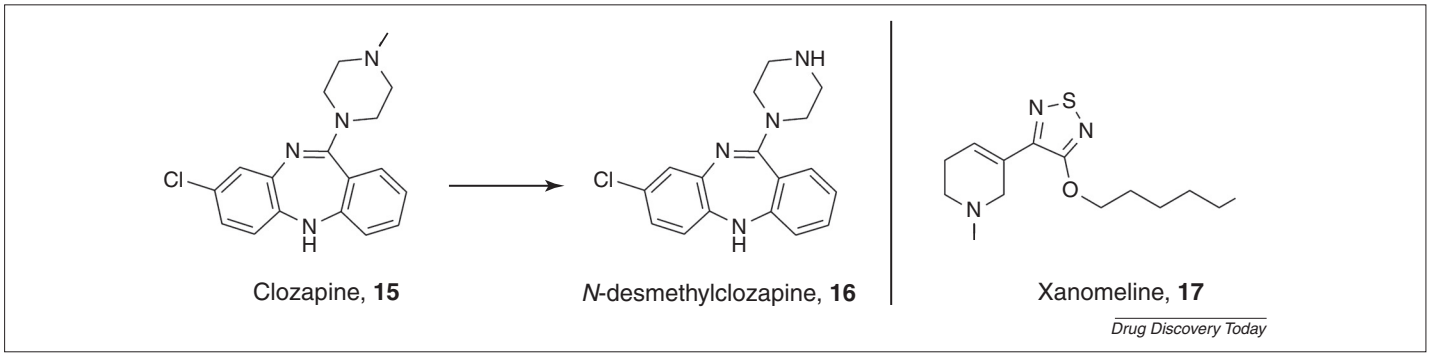

FIGURE 4

M_1 agonists for the treatment of schizophrenia.

in CA1 pyramidal cells in the hippocampus and other cortical regions. Therefore, it is proposed that M_1-induced potentiation of NMDA receptor function could play an important part in the therapeutic efficacy of mAChR activation in psychotic disorders [31]. Additional strong support comes from *N*-desmethylclozapine (NDMC) [32], which is an M_1 allosteric agonist that potentiates NMDA receptor currents in CA1 pyramidal cells in the hippocampus, and further supports the view that selective activation of M_1 by allosteric agonism or potentiation compliments the 'dopamine hyperfunction hypothesis' and the 'NMDA receptor hypofunction hypothesis' of schizophrenia [32,33]. In addition, atypical antipsychotics and muscarinic agonists are efficacious in behavioral models where deficits have been induced by dopamine agonists and NMDA receptor antagonists [34–36]. The development of novel therapeutic agents for schizophrenia that induce selective M_1 activation offers new hope to patients; to address the cognitive, negative and positive symptom clusters while complementing existing treatment strategies. Although muscarinic receptors are expressed throughout the body, M_1 has attracted much attention owing to its expression levels in the brain, in particular its localization in the cortex, striatum and hippocampus, implicating this receptor in the regulation of signals that deal with cognition, movement and memory. For this reason, drug discovery efforts have tried to fulfill the need for a truly selective cohort of muscarinic modulators (Fig. 3, examples of muscarinic agonists **10**–**14**). However, the high sequence homology of the orthosteric site among this family of receptors has proven a difficult hurdle to overcome. As mentioned previously, targeting allosteric sites has rejuvenated the field, providing highly subtype-selective tools and a novel approach to modulate M_1 with the promise to address multiple symptom clusters of schizophrenia.

Clozapine and NDMC

Support for the cholinergic hypothesis of schizophrenia can be found in the clinical evidence generated from the atypical antipsychotic agent clozapine. Clozapine (Fig. 4, **15**) is very effective as an antipsychotic treatment but, as a result of its side effects, is typically a drug of last resort for schizophrenics that do not respond to other treatments (see: http://www.nimh.nih.gov/health/publications/mental-health-medications/what-medications-are-used-to-treat-schizophrenia.shtml). The efficacy of clozapine is partially attributed to its major metabolite, NDMC (Fig. 4, **16**), which is an M_1 allosteric agonist (EC_{50} = 115 nM), as well as the classical D_2 antagonism of the parent [32,33]. It is probable that this combination of activity sets clozapine apart as a clinically effective treatment for schizophrenics through modulation of glutamatergic and muscarinic neurotransmission.

Xanomeline

In 1997, Bodick *et al.* reported on the results of a large-scale clinical trial for the effect of xanomeline (Fig. 4, **17**) on cognitive impairments and behavioral disturbances in AD patients [37]. In this study, the purported M_1/M_4-preferring muscarinic agonist xanomeline improved cognitive performance and also had robust

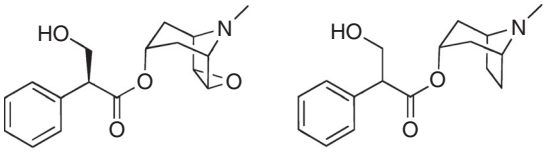

FIGURE 5

Nonselective muscarinic antagonists.

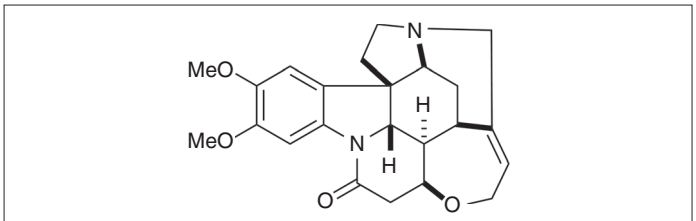

FIGURE 6

Brucine, an M_1-selective positive allosteric modulator.

therapeutic effects on psychotic symptoms and behavioral disturbances associated with AD, such as delusions, vocal outbursts and hallucinations. These improvements in psychotic symptoms prompted scientists at Lilly to evaluate xanomeline in a small clinical trial for its effects on schizophrenia [38,39].

Typical and atypical antipsychotics work to increase dopamine release in the prefrontal cortex through induction of c-Fos expression. Xanomeline showed similar effects to normal antipsychotic agents olanzapine and clozapine by inducing c-Fos expression in the same brain regions and increased dopamine levels as well [40]. The effects of xanomeline further supported a novel mechanism for the treatment of psychosis and, indeed, the effect of xanomeline could be blocked by a muscarinic antagonist, scopolamine (Fig. 5, **18**) [41]. In 2008, the Phase II clinical trial results for schizophrenia patients receiving xanomeline showed significant improvements in the Brief Psychiatric Rating Scale (BPRS) and the Positive and Negative Syndrome Scale (PANSS) over the placebo group scores [38]. There were robust cognitive improvements as well, with the same patients showing marked improvements in short-term memory function and vocal learning measurements.

Despite the encouraging results, xanomeline is still marred by dose-limiting side effects. In animal models and Phase II and III clinical trials the subjects experienced moderate to severe gastrointestinal (GI) distress, salivation and sweating [42,43], which are attributed to the off-target activity of xanomeline at other mAChRs, specifically M_2 and M_3. It is not surprising that many researchers have endeavored to provide the scientific community with an array of selective muscarinic tools to probe the palliative and potentially disease-modifying effects of M_1 activation.

M_1 and the potential for disease-modifying efficacy in AD

AD is one of the most prevalent neurodegenerative disorders affecting over 26 million people worldwide (data recorded in 2006). It is a disease that predominantly affects the elderly (individuals over 65) resulting in cognitive dysfunction and severe memory loss [44]. Possessing a confounding etiology, AD is characterized by the formation of two types of brain structures: neurofibrillary tangles (from hyperphosphorylated τ proteins) and amyloid plaques (aggregated amyloid-β (Aβ) peptide) [45,46]. The hallmark of the progression of AD is the formation of amyloid plaques through Aβ accumulation, which has led to the investigation of many potential therapies that inhibit the formation of Aβ peptides (Fig. 2; and see: http://www.alz.org).

It has been postulated that increasing M_1 receptor activity could not only provide symptomatic relief but also have disease-modifying outcomes in AD patients by influencing the processing of amyloid precursor protein (APP) [47–49]. APP is known to undergo proteolytic cleavage in two competing pathways: amyloidogenic and nonamyloidogenic [50]. In the amyloidogenic pathway, sequential cleavage of APP by β-secretase and γ-secretase releases the Aβ peptide, the core of amyloid plaques and source of neurotoxicity. In the nonamyloidogenic pathway, APP is cleaved by α-secretase, preventing Aβ peptide generation and forming soluble amyloid precursor protein alpha (sAPPα) [50]. Evidence suggests that activation of M_1 can shunt APP processing through the nonamyloidogenic pathway, producing sAPPα, and deterring the formation of Aβ peptides and, ultimately, slowing the progression of AD [51]. Efforts to elucidate the enzymes responsible for formation of Aβ have revealed β-site APP-cleaving enzyme 1 (BACE1) to be the β-secretase responsible. Studies have conclusively shown that M_1 interacts with BACE1 to regulate its proteosomal degradation and activation of M_1 lowers Aβ levels *in vitro*. M_1 activation was also shown to increase sAPPα formation *in vitro* thereby preventing the formation of Aβ via MAPK- and PKC-dependent pathways [48]. In addition, M_1 activation decreases τ phosphorylation; therefore, M_1 activation affects the major pathological hallmarks of AD [52]. These results were corroborated *in vivo* with the M_1-positive allosteric modulator (PAM) BQCA [51,53–55]. Transgenic Tg2576 mice, which overexpress a familial AD mutant form of the APP, are impaired on compound discrimination reversal learning [56,57]. Treatment of Tg2576 mice with BQCA reverses impairment in compound discrimination and compound discrimination reversal models [51].

Brucine

Given the potential of M_1 as a therapeutic target for the treatment of a variety of disease states, substantial effort has been dedicated to the elucidation of selective allosteric agonists of the M_1 receptor. In 1998, Lazareno *et al.* reported that the natural product brucine (Fig. 6, **20**) was a PAM selective for M_1 over the other muscarinic subtypes [58]. Although brucine only weakly potentiated ACh at micromolar concentrations (<twofold increase in potency), it did establish that allosteric activation of the mAChRs was a valid strategy for obtaining subtype selectivity.

AC-42

In a seminal advancement, Spalding *et al.* disclosed AC-42 (Fig. 7, **21**) as the first allosteric agonist selective for M_1 [59]. AC-42 was shown to stimulate calcium mobilization and inositol monophosphate (IP) accumulation in recombinant hM_1 cell lines. It

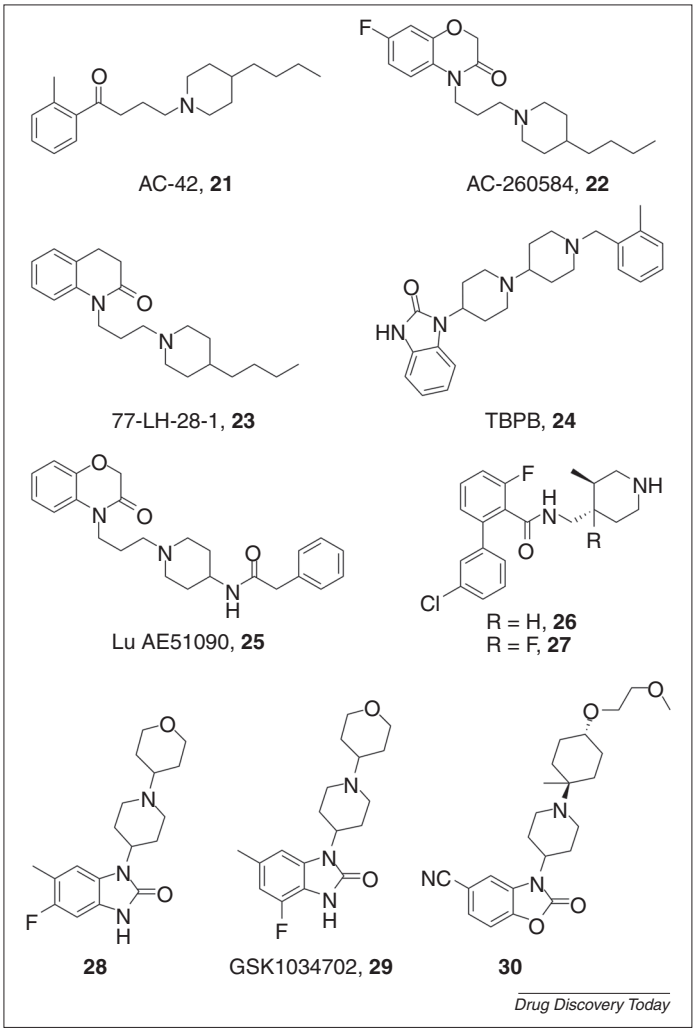

FIGURE 7

Selective allosteric agonists of the M_1 mAChR.

possessed an excellent selectivity profile (>400-fold) for M_1 over the M_2–M_5 receptors. Using a series of chimeric receptors, AC-42 was shown to bind to an allosteric site at transmembrane (TM) domains one and seven, which is distinct from the orthosteric binding site (TM domains three, five and six). However, when it was evaluated in native tissues, AC-42 failed to elicit a response in single-unit cell firing of the CA1 region in the rat hippocampus. Additionally, AC-42 binds to D_2 dopamine and $5HT_{2B}$ biogenic amine receptors, making it a less than ideal tool for studying the role of M_1 in diseases where other biogenic amine receptors might also play a part (i.e. schizophrenia and the dopamine D_2 receptor) [60]. After AC-42 verified that mAChR subtype selectivity could be achieved with allosteric agonists, a number of second-generation agonists were reported in the literature including AC-260584 (**22**) [61], 77-LH-28-1 (**23**) [62] and TBPB (**24**) [63,64] (Fig. 7).

77-LH-28-1

GSK subsequently reported 77-LH-28-1 (**23**), a close structural analog of AC-42, as an allosteric agonist of M_1 with an improved pharmacological profile [62]. In a calcium mobilization assay, 77-LH-28-1 was found to have EC_{50} = 8 nM at M_1. Although approximately an order of magnitude more potent than AC-42 at M_1, subtype selectivity was somewhat eroded (M_2 EC_{50} = 760 nM, M_3 EC_{50} = 159 nM and M_5 EC_{50} = 206 nM). Similar to AC-42, 77-LH-28-1 also displayed activation of the human dopamine D_2 and $5HT_{2B}$ receptors. Pharmacokinetic studies showed that 77-LH-28-1 was rapidly metabolized (T_{max} = 15 min); however, **23** did penetrate the CNS with a B:P of ~4. In a key advance over AC-42, 77-LH-28-1 was able to show efficacy in native rat tissues rather than transfected cell lines. It also stimulated single-unit firing and initiated network oscillations in rat hippocampal CA1 cells, as did the orthosteric agonist carbachol (CCh, **11**; Fig. 3).

TBPB

In 2008, Jones *et al.* reported on 1-(1′-(2-methylbenzyl)-[1,4′-bipiperidin]-4-yl)-1*H*-benzo[*d*]imidazol-2-(3*H*)-one (TBPB, **24**; Fig. 7) as one of the first novel allosteric agonists of the M_1 mAChR [63,64]. TBPB emerged as an unoptimized HTS hit with EC_{50} = 289 nM and displayed an effect at 82% of a maximal CCh response. Through an M_1 receptor Y381A mutation, it was shown that TBPB behaved as an allosteric agonist at the M_1 receptor [Y381A mutation robustly right-shifted a CCh response when

compared to wild type (WT) rM_1; TBPB EC_{50} = 220 nM in WT rM_1; EC_{50} = 97 nM in Y381A rM_1] [63]. TBPB was analyzed for its ability to shift APP processing to the nonamyloidogenic pathway and, indeed, was shown to increase the production of sAPPα. Treatment of PC12 cells with 1 μM TBPB increased sAPPα release by 58% compared to vehicle. Analysis of conditioned media from these cells for $A\beta_{40}$ levels indicated a 61% decrease compared to vehicle control. Both of these effects could be blocked by atropine (Fig. 5, **19**), a nonselective muscarinic antagonist. These results are consistent with the hypothesis that selective activation of M_1 can regulate APP processing and increase sAPPα formation.

Unfortunately, more results from *in vitro* experiments precluded the advance of TBPB as a lead compound. TBPB showed appreciable levels of D_2 antagonism (IC_{50} = 2.6 μM), which compromised its utility as a novel antipsychotic. Also, TBPB robustly antagonized an ACh EC_{80} response at M_2–M_5 [65]. These results demonstrated that TBPB possesses a two-site binding profile (two-site binding: a ligand that binds to a high-affinity allosteric site at low concentrations and binds to a low-affinity orthosteric site at higher concentrations). Although this antagonist activity was only seen at higher concentrations than required for agonist activity at M_1, these data revealed that TBPB was not an M_1-selective ligand. SAR studies were undertaken to determine if TBPB could be further optimized to remove the D_2 and M_2–M_5 antagonism in this series. After several hundred compounds were synthesized and tested, the original screening hit could not be improved. All efforts led to a decrease in M_1 efficacy or increases or decreases in M_2–M_5 agonism or antagonism, D_2 antagonism or completely inactive compounds [49,66,67]. This ultimately led to the discontinuation of TBPB as a lead candidate for M_1 activation.

Lu AE51090

Following the disclosure of TBPB, Lundbeck reported a related allosteric agonist: Lu AE51090 [68] (Fig. 7, **25**). Beginning from an HTS campaign that identified two hits possessing EC_{50}s of 13 and 130 nM at hM_1, parallel synthesis was used to probe the SAR around the eastern and western amide regions, with Lu AE51090 emerging as the lead compound. Lu AE51090 provided high selectivity for hM_1; no activity was observed for hM_2–hM_5. Screening against a panel of 69 GPCRs identified the adrenergic α_{1A} receptor as the only major off-target liability. Its DMPK properties were evaluated, and Lu AE51090 was found to possess a good free fraction (f_u) in rat and human plasma and to have moderate CNS exposure with a B:P ratio of 0.20 in rat. Compound **25** suffered from high clearance and low oral bioavailability; however, it exhibited efficacy in a dose-dependent manner in an *in vivo* model of working memory (delayed alternation Y-maze in mice).

GlaxoSmithKline muscarinic agonists

GlaxoSmithKline has disclosed two separate series of M_1-selective allosteric agonists arising from screening of their corporate libraries [69–71]. Rescreening compounds originally designed for their M_3 antagonist program against M_1 yielded an initial hit that displayed M_1 selectivity, albeit with EC_{50} = 250 nM. Iterative library synthesis yielded compounds **26** and **27** (Fig. 7), where potencies were improved to EC_{50} = 0.8 nM and 10 nM, respectively. These compounds were found to be pan-antagonists of M_2–M_5 (predominant interactions: **26** M_3 IC_{50} = 40 nM; **27** M_2 IC_{50} = 2.5 μM). However, **26** exhibited clean ancillary pharmacology in a CEREP panel. Compounds **26** and **27** were found to have good oral bioavailability in rat, possessed a $t_{1/2}$ of 2.3 hours and 3.0 hours, respectively, and penetrated the CNS with B:P ratios of 0.9 and 0.3, respectively.

A second series was reported arising from a virtual screen of their corporate compound library against the pharmacophore of AC-42. Initial optimization efforts led to the discovery of compound **28**, which, although bearing significant structural similarity to TBPB, was found to exhibit divergent muscarinic activity. Compound **28** was found to be a weak agonist at M_2–M_5 receptors, in addition to κ-opiod receptor binding in the CEREP selectivity panel [72]. Compound **28** was evaluated in native tissues and found to increase the firing rate of hippocampal CA1 cells. Additionally, the compound displayed *in vivo* efficacy in a dose-dependent reversal of scopolamine-induced amnesia in a rat model. However, compound **28** was found to have limited exposure to the CNS, indicating the possible role of efflux transporters. GSK1034702 (Fig. 7, **29**), which emerged from this series, is currently in clinical trials as a positron emission tomography (PET) tracer and has been shown to improve episodic memory in humans in the nicotine abstinence model of cognitive dysfunction [73,74]. Subsequent lead optimization endeavors in this series identified compounds exemplified by **30** [71] (Fig. 7). These compounds maintained selectivity for M_1, although weak pan-agonism of M_2–M_5 was observed. Compound **30** was also found to be effective in a novel object recognition (NOR) model of cognition in rats.

VU0357017 and VU0364572

In an effort to expand the diversity of novel M_1 activators, our laboratories initiated work on compounds such as VU0177548 (**31**) and VU0184670 (**32**) [10,65,75,76] (Fig. 8). Beginning from a HTS of the 65,000 member MLPCN library, two hits: VU0177548 (**31**) and VU0207811 (**33**), were identified as allosteric agonists selective for M_1 over M_2–M_5. Employing an iterative parallel synthesis screening approach, optimization of the HTS hits was met with steep SAR. Changes to the western amide, introduction of fluorine onto the piperidine ring, amine or amide alkylation, alternative chain lengths and introduction of basic heterocycles on the western amide abolished M_1 activity. However, subtle changes to the aryl amide were tolerated, and **32** and VU0357017 (**34**) were identified as lead compounds with M_1 agonist activity (EC_{50} = 152 nM and 198 nM, respectively, in a high-expressing rat M_1 CHO cell line) [65]. Compounds **32** and **34** maintained selectivity at M_2–M_5 (EC_{50} >30 μM). A putative allosteric binding site, located on extracellular loop three, was identified via site-directed mutagenesis studies. Schild analysis through treatment with **19** and competition binding experiments with [^{3}H]-*N*-methylscopolamine led to **34** being initially identified as an allosteric agonist. In addition to **34** displaying selectivity for M_1 over the other muscarinic subtypes, it also exhibited selectivity in a panel of 68 GPCRs (exhibited binding selectivity <50% radioligand displacement at 10 μM), including biogenic amine receptors. However, it did show weak functional D_2 antagonism (IC_{50} = 4.5 μM). Finally, **34** possessed a desirable drug metabolism and pharmacokinetic (DMPK) profile; it was CNS penetrant (B:P = 4:1), low to moderately cleared

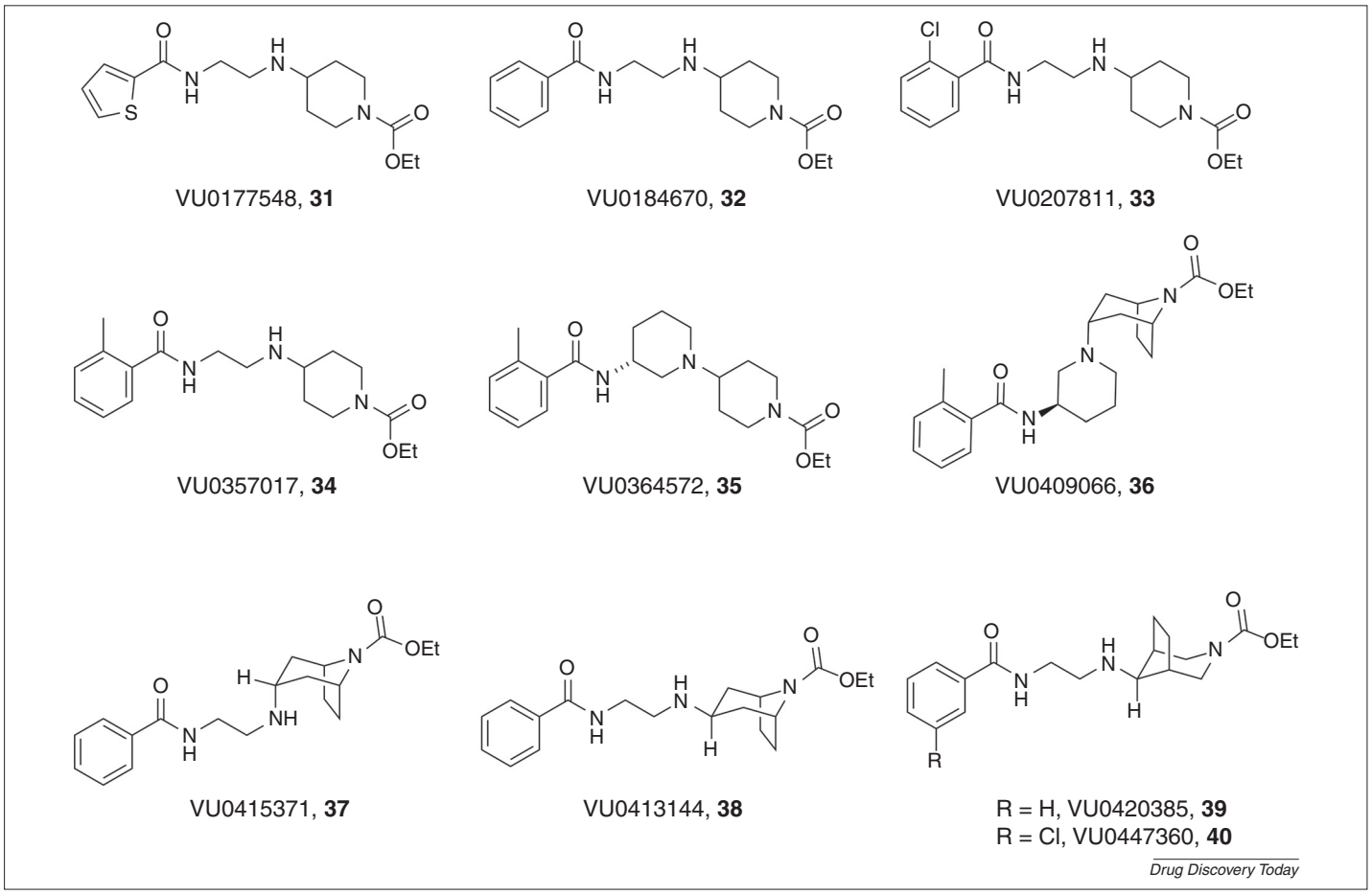

FIGURE 8

M_1-selective allosteric agonists from the Vanderbilt Center for Neuroscience Drug Discovery.

(Cl_{obs} = 13.8 mL/min/kg; $t_{1/2}$ = 1.1 hours), orally bioavailable and, as a result of its high aqueous solubility, it could be dosed in saline.

Further optimization efforts explored introducing cyclic constraints into the ethyl linker of **34**. Again, steep SAR was encountered; however, replacement of the ethyl linker with a three-amino piperidine resulted in a novel class of M_1 agonists, with the lead structure VU0364572 (**35**) [77] (Fig. 8). Evaluation of each enantiomer showed that the *R*-enantiomer was active (EC_{50} = 110 nM in high-expressing cell lines), whereas the *S*-enantiomer was inactive, illustrating the first example of enantioselective activation of M_1. Compound **35** maintained selectivity for M_2–M_5 in a functional calcium mobilization assay, and was selective when screened against a panel of 68 GPCRs (<30% displacement at 10 μM). Furthermore, the functional D_2 antagonism displayed by **34** was ablated in **35**. Compound **35** was also found to have good free fraction, no cytochrome P450 (CYP450) inhibition, low to moderate clearance (Cl_{obs} = 14.5 mL/min/kg; $t_{1/2}$ = 45 min), oral bioavailability and to be CNS penetrant (B:P = 1.5). Whereas calcium assays were originally conducted in high-expressing cell lines, tetracycline-inducible rat M_1 and human M_1 cell lines were developed with expression levels similar to native tissues to determine if selectivity was still present. In these cell lines, the EC_{50}s of **34** and **35** were attenuated (EC_{50} = 16 μM and EC_{50} = 2.3 μM, respectively) [78]. This change in activity as a function of differing receptor reserve raised the possibility that **34** and **35** could act as full agonists or weak partial agonists.

Compounds **34** and **35** were tested for their ability to affect APP processing. A 2 μM concentration of **34** showed the same increase in sAPPα as a 10 μM CCh concentration in a stably expressed tetracycline repressor protein hM_1 (TREx293-hM_1) cell line. Compound **35** showed a robust increase in the formation of sAPPα, in this case a threefold increase (normalized to a 10 μM CCh response, in TREx293-hM_1 cell line) [78]. Once again, these findings support the hypothesis that activation of M_1 can regulate APP processing and increase sAPPα formation, and could therefore have a disease-modifying role in the treatment of AD.

Tropane modifications to VU0357017

Our continued efforts to introduce cyclic constraints to improve upon **32** led to the development of a potent series of tropane derivatives. Replacement of the eastern piperidine ring of **35** with a tropane scaffold resulted in VU0409066, a potent M_1 agonist (Fig. 8, **36**; hM_1 EC_{50} = 59 nM) [79]. Similarly, replacement of the eastern piperidine of **32** with a tropane scaffold afforded the endo- (VU0415371, **37**) and exo- (VU0413144, **38**) isomers, which possessed an EC_{50} of 110 nM and 970 nM, respectively. Flat SAR was again encountered in the exo-tropane series, with many different

amide modifications showing little to no improvement in potency relative to **38**. Moving the bicyclic bridge of the tropane scaffold afforded compounds VU0420385 (**39**) and VU0447360 (**40**), representing the most potent agonists in this system (EC_{50} = 92 nM and 47 nM, respectively). This novel series of M_1 agonists was also tested for ability to enhance the release of sAPPα processing. In all cases, these compounds (**36**–**40** at 2 μM) stimulated the release of sAPPα to the same extent as a 10 μM dose of CCh (TREx293-hM_1 cell line). These experiments continue to support the belief that activation of M_1 is potentially a disease-modifying treatment of AD.

Although much effort was spent to determine tractable SAR, the tropane replacements in this series possessed some blemishes. Compound **36** was analyzed for its PK properties and was found to have high clearance (Cl_{obs} = 189 mL/min/kg; V_{ss} = 11.8 l/kg, $t_{1/2}$ = 46 min) and good oral bioavailability (%*F* = 70). Unfortunately, **36** had a poor selectivity profile, lacking in muscarinic subtype selectivity (hM_2 EC_{50} = 1.8 μM; hM_5 EC_{50} = 3.7 μM; weak agonist at hM_3 and hM_4) [79]. Other structural modifications did not improve their muscarinic selectivity profile, exemplified by **37**. This compound displayed antagonist activity at the other muscarinic subtypes (hM_2–hM_5 weak to mid micromolar antagonists; Table 1) reminiscent of the ancillary pharmacology associated with TBPB. This lack of selectivity in this series raised concerns that compounds with higher potency would engender poorer selectivity across the other subtypes. Initially, changes to the bridgehead location to deliver alternative tropanes **39** and **40** were promising. Both compounds displayed improved hM_1 potencies; however, the off-target activity was mixed. Compound **39** was a weak antagonist at hM_3 and hM_4 but showed partial agonist activity at hM_5 (hM_5 EC_{50} = 2.8 μM). Similarly, **40** was a weak antagonist at hM_4 but showed partial agonist activity at hM_5 (hM_5 EC_{50} = 4.2 μM).

These modifications to provide potent tropane agonists brought into focus the inherent challenges in working with this class of agonists. Although high selectivity could be achieved for the M_1 receptor through an allosteric interaction at lower concentrations, **34**, **35** and later analogs resulted in orthosteric interactions at higher concentrations and a loss of muscarinic subtype selectivity for more-potent compounds within the same series (similar two-site binding profiles observed with TBPB, **34** and **35**) [80]. Although the loss of subtype selectivity and dependence on receptor reserve can preclude their development into strong lead candidates, there remains inherent value in the study of their *in vitro* pharmacology and electrophysiological effects, because muscarinic receptor density is not uniform throughout the CNS.

Signal differentiation

Characterization of novel allosteric agonists has brought to light interesting differential effects in the downstream signaling pathways of orthosteric and allosteric M_1 agonists. AC-260584 (Fig. 7, **22**), TBPB (**24**) and CCh (**11**) were found to be functionally active in calcium efflux and extracellular regulated signal kinase ($ERK_{1/2}$) phosphorylation assays, which are downstream responses in the $G\alpha_q$ signaling pathway [61,81]. A second $G\alpha_q$-independent pathway acts to regulate the response of M_1 by recruitment of arrestin proteins, the most important of which is thought to be β-arrestin. In addition to their role in receptor desensitization and endocytosis, arrestins have also been found to play a part in chemotaxis, stress fiber formation and protein synthesis. When cells were treated with CCh, β-arrestin was recruited to the surface of the cell within 5 min, with additional binding studies showing significant degradation (~25%) of the M_1 receptor after 24 hours. Furthermore, cells pretreated with CCh showed almost no response to a subsequent CCh challenge. Allosteric modulators **22** and **24**, however, showed no significant change in arrestin localization after 5 min. Upon incubating overnight, TBPB only achieved 20% CCh maximum arrestin recruitment, whereas **22** showed a delayed but robust response with 80% CCh arrestin recruitment. Both allosteric agonists showed no significant degradation of M_1 and pretreatment of cells with either agonist did not desensitize a subsequent CCh challenge. Allosteric agonists exhibiting this pharmacological profile can avoid desensitization to treatment with prolonged exposure. These results serve to highlight the complexity associated with developing M_1 allosteric agonists, as well as the need for additional studies to determine ligand-biased signaling in other scaffolds. Similarly, **34** and **35** failed to promote β-arrestin recruitment.

It is known that induction of calcium release, $ERK_{1/2}$ phosphorylation and β-arrestin recruitment are all activated post-M_1 stimulation, eliciting responses to induce a range of physiological effects [82]. Therefore, differential activation of some M_1-mediated responses will have impact in determining the therapeutic potential of novel M_1 agonists. The pharmacologies of **34** and **35** were further characterized in cellular assays examining ERK phosphorylation and β-arrestin recruitment. Although both compounds induced a robust response in calcium assays, only compound **35** induced robust $ERK_{1/2}$ phosphorylation, and neither compound recruited β-arrestin [78]. When the responses of these two agonists were examined in an inducible cell line they were found to show reduced potency in lower expressing cell lines, behaving as weak partial agonists. On the basis of these observations, it was postulated that **34** and **35** could show differential effects in M_1-containing CNS regions thought to be important for *in vivo* therapeutic effects. To characterize their properties further, CCh **11**, **34** and **35** were tested for their ability to enhance long-term potentiation (LTP) and long-term depression (LTD) in the CA1 region of rat hippocampal slices, where M_1 is the predominant subtype expressed (60% of total mAChR expression). All three compounds were found to potentiate NMDA receptor currents in hippocampal pyramidal cells and significantly enhance LTP, a response thought to be mediated exclusively by the M_1 mAChR. Additionally, high concentrations of CCh (50 μM, effect not observed at 30 μM) and **35** robustly potentiated LTD, whereas **34** did not. This was one of the first results in native tissues that demonstrated that all M_1 activation is not equal.

Following these studies, **34** and **35** were examined in tissues with lower M_1 receptor density (low receptor reserve). In rat striatal medium spiny neurons (MSNs) M_1 activation is believed to be responsible for locomotor activity through M_1 agonism. Compunds **34** and **35** showed a significant, albeit weak, increase in the firing rate of MSNs; however, both compounds showed no effect in the depolarization of mouse prefrontal cortex (mPFC) pyramidal cells [78]. Extending these findings into *in vivo*

TABLE 1

Summary of muscarinic acetylcholine receptor (mAChR) ligands reported to be selective for M_1

Name	Institution, year	M_1	M_2–M_5	Ancillary	DMPK	*In vitro*	*In vivo*
AC-42 (21)[a]	Acadia, 2002	320 nM	M_5 EC_{50} = 6.93 μM	hD_2 K_i = 20 nM, $5HT_{2B}$ K_i = 450 nM	Data not available	No effects in native tissue	Data not available
AC-260584 (22)[a]	Acadia, 2008	41 nM	M_2 IC_{50} >10 μM, M_3 EC_{50} = 5.9 μM, M_4 IC_{50} >10 μM, M_5 EC_{50} = 1.0 μM	hD_2 K_i = 50 nM, $5HT_{2B}$ K_i = 1.59 μM	High clearance, 26% oral bioavailability	Promotes $G\alpha_q$-mediated signaling, reduced activation of arrestin signaling	Increases $ERK_{1/2}$ phosphorylation in mouse hippocampus; improvement in NOR mouse model
77-LH-28-1 (23)[a]	GSK, 2008	8 nM	M_2 EC_{50} >765 nM, M_3 EC_{50} = 159 nM, M_4 EC_{50} >10 μM, M_5 EC_{50} = 206 nM	hD_2 K_i = 60 nM, $5HT_{2B}$ K_i = 950 nM	B:P = 4, rapidly cleared, subcutaneous admin. optimal	Stimulate CA1 cell firing and gamma frequency oscillations in rat hippocampus	Enhances NMDA-mediated neuronal excitation in hippocampus
TBPB (24)	Merck, Vanderbilt, 2006	289 nM	M_2 IC_{50} = 1.1 μM, M_3 IC_{50} = 3.0 μM, M_4 IC_{50} = 415 nM, M_5 IC_{50} = 10 μM	hD_2 IC_{50} = 2.6 μM	Data not available	Promotes $G\alpha_q$-mediated signaling, reduced activation of arrestin signaling, enhances sAPPα, potentiates NMDA receptor currents	Reverses amphetamine hyperlocomotion in rat model
VU0357017 (34)	Vanderbilt, 2008	198 nM (16 μM)[b]	M_2–M_5 >30 μM	<50% 68 GPCRs, functional D_2 antagonist	Low-moderate clearance, CNS penetrant, dosable in saline	Stimulates $G\alpha_q$-signaling, little effect on arrestin signaling; efficacious in hippocampus, little effect in other brain regions	Effective in contextual fear conditioning model; inactive in spatial memory and AHL reversal
VU0364572 (35)	Vanderbilt, 2011	110 nM (2.3 μM)[b]	M_2–M_5 >30 μM	<30% 68 GPCRs, no D_2 antagonism	Low-moderate clearance, CNS penetrant, oral bioavailable	Stimulates $G\alpha_q$-signaling, little effect on arrestin signaling; efficacious in hippocampus, little effect in other brain regions	Effective in contextual fear conditioning and spatial memory; inactive in AHL reversal
VU0409066 (36)	Vanderbilt, 2012	59 nM	M_2 EC_{50} = 1.8 μM, M_3 EC_{50} >10 μM, M_4 EC_{50} >10 μM, M_5 EC_{50} = 3.7 μM	Data not available	High clearance, $t_{1/2}$ = 46 min, orally bioavailable	Enhance sAPPα	Data not available
VU0415371 (37)	Vanderbilt, 2012	110 nM	M_2 IC_{50} >10 μM, M_3 IC_{50} >10 μM, M_4 IC_{50} = 3.1 μM, M_5 IC_{50} = 4.9 μM	Data not available	Data not available	Enhance sAPPα	Data not available
VU0413144 (38)	Vanderbilt, 2012	970 nM	M_2–M_5 >30 μM	Data not available	Data not available	Enhance sAPPα	Data not available
VU0420385 (39)	Vanderbilt, 2012	92 nM	M_3 IC_{50} >10 μM, M_4 IC_{50} >10 μM, M_5 EC_{50} = 2.8 μM	Data not available	Data not available	Enhance sAPPα	Data not available
VU0447360 (40)	Vanderbilt, 2012	47 nM	M_4 IC_{50} >10 μM, M_5 EC_{50} = 4.2 μM	Data not available	Data not available	Enhance sAPPα	Data not available
NDMC (16)[a]		115 nM	M_2 EC_{50} = 295 nM, M_3 EC_{50} = 31 nM, M_4 EC_{50} = 1.23 μM, M_5 EC_{50} = 50 nM	hD_2 K_i = 180 nM, $5HT_{2C}$ K_i = 5 nM, $5HT_{2B}$ K_i = 4 nM	Data not available	Data not available	Data not available

Lu AE51090 (25)	Lundbeck, 2010	61 nM	M_2 K_i = 2.2 μM, M_3 K_i = 7.0 μM, M_4 K_i = 6.9 μM, M_5 K_i = 8.9 μM	hα_{1A} K_i = 260 nM, hα_{1B} K_i = 910 nM, hH_1 K_i = 780 nM	High clearance, low oral bioavailability, moderate B:P	Data not available	Observable dose-dependent improvement in learning and memory in mouse Y-maze task
26	GSK, 2010	0.8 nM	M_2 IC_{50} = 200 nM, M_3 IC_{50} = 40 nM, M_4 IC_{50} = 158 nM, M_5 IC_{50} = 500 nM	No inhibition in CEREP panel	Good brain exposure (AUC = 1655 ng h/g), $t_{1/2}$ = 2.3 hours, B:P = 0.9	Data not available	Data not available
27	GSK, 2010	10 nM	M_2 IC_{50} = 2.5 μM, M_3 IC_{50} = 5.0 μM, M_4 IC_{50} = 3.2 μM, M_5 IC_{50} = 6.3 μM	Not determined	Oral bioavailability (F = 57%), brain exposure (AUC = 2221 ng h/g), $t_{1/2}$ = 3.0 hours, Cl = 35 mL/min/kg	Data not available	Data not available
28	GSK, 2010	8 nM	M_2 EC_{50} = 630 nM, M_3 EC_{50} = 2.5 nM, M_4 EC_{50} = 500 nM, M_5 EC_{50} = 790 nM	hERG IC_{50} = 12 μM, κ-OR 53% inhibition at 1 μM, hD_2 K_i = 264 nM, 5HT_{2C} K_i = 52 nM, 5HT_{2B} K_i = 12 nM	Cl_i <0.7 ml/min/kg, oral bioavailability (F = 49%), B:P = 0.6	Data not available	Increases cell firing of hippocampal CA1 cells; dose-dependent reversal of scopolamine model
30	GSK, 2010	10 nM	M_2 EC_{50} = 2.5 μM, M_3 EC_{50} = 4.0 μM, M_4 EC_{50} = 790 nM, M_5 EC_{50} = 1.0 μM	CEREP panel sigma-receptor (75% inhibition at 10 μM)	Cl = 23 mL/min/kg, free concentration brain = 261 nM, free concentration blood = 265 nM	Data not available	Dose-dependent improvement of novel object recognition of temporal induced memory deficit in rat
Xanomeline[a] (17)	Eli Lilly, 1994	0.3 nM	M_2 EC_{50} = 93 nM, M_3 EC_{50} = 5 nM, M_4 EC_{50} = 52 nM, M_5 EC_{50} = 42 nM	hD_2 K_i = 264 nM, 5HT_{2C} K_i = 52 nM, 5HT_{2B} K_i = 12 nM	3–7% oral bioavailabilty, $t_{1/2}$ = 32 min (rat)	Enhance sAPPα	Reverses amphetamine hyperlocomotion in rat model; reverses apomorphine-induced deficit in PPI in rat model; effective in conditioned emotional response in rat model (anxiety); did not induce catalepsy in rats; advanced to Phase II and Phase III clinical trials

Abbreviations: AHL, amphetamine-induced hyperlocomotion; CNS, central nervous system; DMPK, drug metabolism and pharmacokinetics; GPCR, G-protein-coupled receptor; NMDA, *N*-methyl-D-aspartate; NOR, novel object recognition; sAPPα, soluble amyloid precursor protein alpha.

[a] Ancillary data taken from Ref. [49]; values differ somewhat from original report.

[b] EC_{50} values reported are from TREx293 hM_1 cell line with low receptor reserve. These data were taken from Ref. [67].

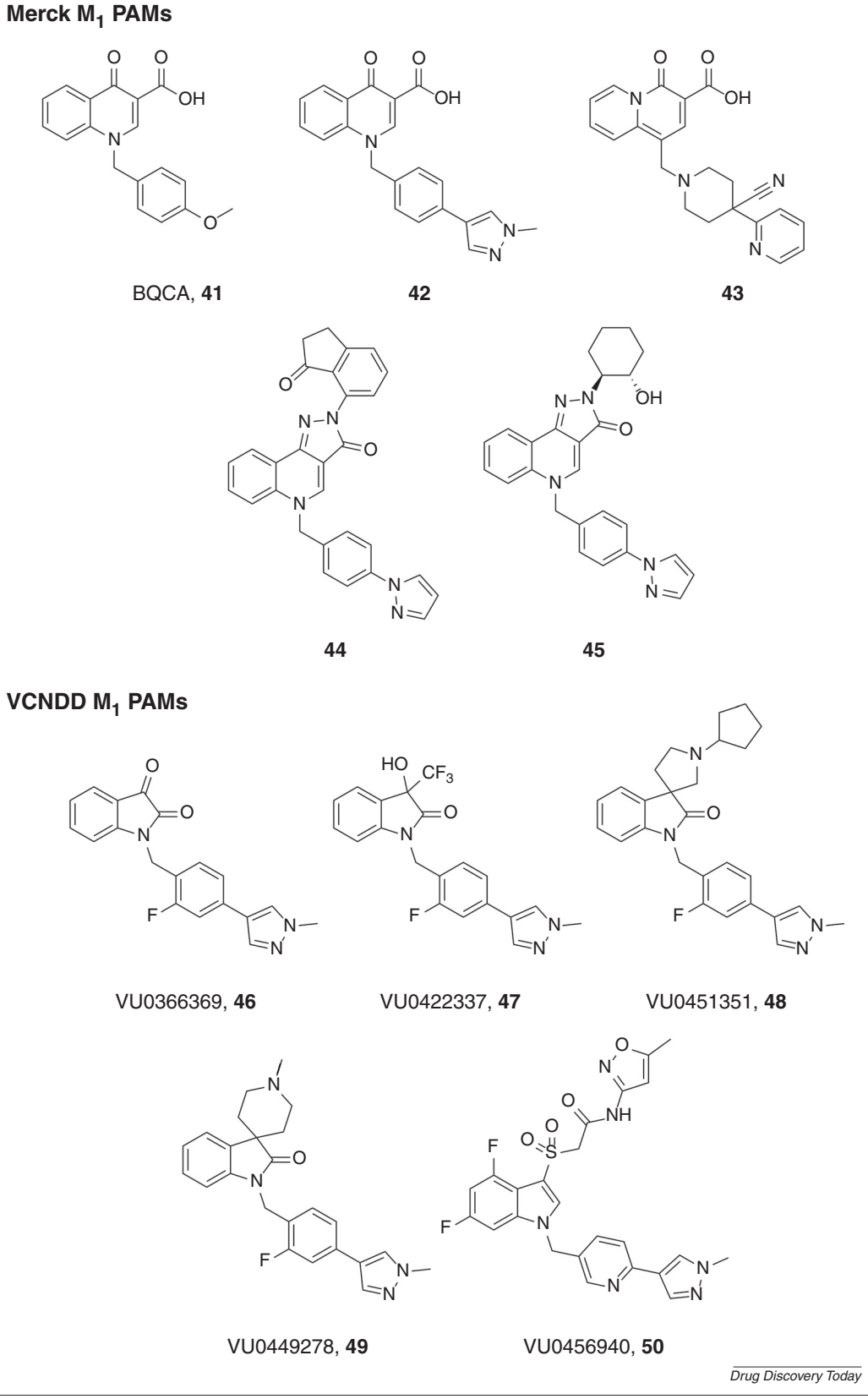

FIGURE 9

M_1-selective positive allosteric modulators from Merck and Vanderbilt Center for Neuroscience Drug Discovery.

systems, **34** and **35** were evaluated in two assays assessing hippocampal-dependent learning and a separate striatal-dependent antipsychotic assay. In the hippocampal-dependent assays, **34** and **35** showed robust responses in contextual fear conditioning, yet only **35** showed efficacy in a Morris water maze test to probe spatial learning. Both compounds showed limited effects on the striatum, which is implicated in antipsychotic affects associated with the M_1 receptor. In an amphetamine-induced hyperlocomotion (AHL) assay, neither **34** nor **35** showed efficacy. These data support the hypothesis that relying on a single assay (in this case: potency in calcium mobilization) to advance chemical lead optimization might not deliver compounds with the desired *in vivo* CNS action. These data illustrate the importance of advancing key compounds by assaying in multiple signaling pathways and under conditions where receptor reserve is known, because these 'agonists' display receptor reserve-dependent and, hence, brain-region-dependent pharmacology. This phenomenon will make it difficult to achieve the necessary selectivity versus M_3 in humans (high M_3 receptor reserve in the GI tract) to avoid GI side effects noted for orthosteric agonists [83].

M_1 PAMs as a way forward

Challenges exemplified in this review serve to highlight the difficulty in the development of truly selective M_1 agonists owing to the high conservation of the orthosteric binding site among this family of receptors. In addition, the two-site binding profile possessed by many of these agonists often results in pan-muscarinic activation or antagonism in low receptor reserve systems [80]. To avoid these hurdles, targeting ligands that bind exclusively to a less-conserved allosteric site, which is topologically distinct from the orthosteric site, imparts a level of subtype selectivity not observed with two-site binding agonists. Allosteric ligands possess several modes of pharmacology, including allosteric agonism, PAMs and negative allosteric modulators (NAMs) [14,84]. PAMs are characterized by eliciting an increase in the efficacy or affinity of a native orthosteric agonist, such as ACh, as a result of a change in conformation of the receptor, but possess no intrinsic pharmacology alone. PAMs have offered advantages over classical agonists by conferring greater subtype and receptor selectivity. Also, by operating in conjunction with physiological signaling conditions, they offer an exquisite level of temporal selectivity not seen with traditional muscarinic activators.

A proof-of-concept M_1 PAM, benzylquinolone carboxylic acid (BQCA, **41**; Fig. 9) was discovered by scientists at Merck Laboratories. BQCA is a potent and highly selective M_1 PAM (hM_1 EC_{50} = 840 nM, 129-fold leftward shift of the ACh CRC; hM_2–hM_5 inactive) with acceptable pharmacokinetics and CNS exposure [53–55]. It was shown to have no competitive interactions with the orthosteric binding site (determined through mutagenesis studies) and increased M_1 affinity for CCh. BQCA increased spontaneous excitatory postsynaptic currents (sEPSCs) in the mPFC and induced a robust inward current. These effects were absent in brain slices from M_1 knockout mice. BQCA regulated the nonamyloidogenic pathway for APP processing, increasing the release of sAPPα in the presence of a 50 nM CCh dose (displayed no activity in the absence of CCh) and restored discrimination reversal learning in a transgenic mouse model of AD. Both of these effects provide further support that M_1 PAMs can potentially be disease modifying in a similar manner to M_1 agonists. Moreover, BQCA was efficacious in reversing AHL [54], which led Merck to pursue this series actively through extensive chemical lead optimization delivering **42**–**45** [85–90].

Researchers at our laboratories recently reported on VU0456940 (Fig. 9, **50**), a potent M_1 PAM with excellent selectivity (hM_1 EC_{50} = 340 nM, 14-fold leftward shift of the ACh CRC; hM_2–hM_5 inactive), derived from a weak HTS hit [52,91]. Further development of this series of M_1 PAM was precluded owing to problems with high clearance and moderate CYP450 inhibition, but **50** was tested in native tissues for its ability to potentiate M_1. Compound **50** potentiated the excitation of a subthreshold concentration of CCh in MSNs. Also, **50** shifted APP processing and engaged the nonamyloidogenic pathway inducing the release of sAPPα in the presence of a 100 nM CCh dose (displayed no activity in the absence of CCh) [52]. A structurally distinct series arose from an M_1, M_3, M_5 PAM, which, through chemical optimization efforts, afforded several subseries (Fig. 9, **46**–**49**) of highly M_1-selective PAMs [92–94]. The development of novel M_1 PAMs related to **46**–**49** are currently underway.

Concluding remarks

Following the promising clinical efficacy of the orthosteric agonist xanomeline for the treatment of psychosis and cognitive deficits in schizophrenia and AD patients, tremendous effort has been dedicated to finding suitable therapeutics to target mAChRs, with subtype selectivity at the orthosteric site representing the major hurdle in these efforts. With the disclosure of AC-42, targeting less-conserved allosteric sites has become the paradigm for engaging the mAChRs. A number of allosteric agonists have subsequently been reported with improved potency, muscarinic selectivity, attenuated ancillary pharmacology and desirable pharmacokinetic profiles. These compounds have aided in further understanding the role of the M_1 receptor in cognitive functions. Numerous compounds have been shown to potentiate the NMDA receptor, CA1 firing in hippocampal cells and enhance soluble APP processing, perhaps offering a disease-modifying treatment for AD, as well as showing efficacy in multiple rodent cognition models. ^{13}C-labeled compound **29** has entered clinical trials as a PET tracer. However, despite these advances, the development of M_1 allosteric agonists possessing suitable profiles to advance through clinical trials has remained an unmet challenge. Many of the reported compounds exhibit binding to an orthosteric and an allosteric site, display poor selectivity or display a wide range of pharmacology based on receptor reserve *in vitro* and *in vivo*. A promising strategy that has emerged for addressing this challenge is the use of mAChR subtype-selective PAMs to potentiate the effect of ACh. PAMs such as these exhibit lower receptor desensitization, have less ancillary pharmacology (and are therefore more selective) and are generally not subject to issues associated with receptor reserve; however, PAMs are not a panacea because M_1 PAMs must still be critically evaluated for ligand-biased signaling (β-arrestin, ERK, among others) and across safety species (rat, dog, nonhuman primate) to ensure no variation in potency or efficacy. Despite the challenges and caveats, the past five years have witnessed a revolution in muscarinic drug discovery efforts. Currently, Merck

& Co. has advanced M_1 PAM MK-7622 into Phase II clinical trials as an adjunct therapy to donepezil in patients with AD (http://clinicaltrials.gov/ct2/show/NCT01852110). This illustrates that we are closer than ever before to assessing the efficacy of a selective M_1 activator in humans for the treatment of schizophrenia and AD.

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