Among the muscarinic acetylcholine receptor (mAChR) subtypes, the M₄ receptor has been investigated as a promising drug target for the treatment of schizophrenia. These investigations have been based on findings from M₄-deficient mice studies as well as on the results of a clinical trial that used xanomeline, an M₁/M₄ mAChRs-preferring agonist. Both orthosteric agonists and positive allosteric modulators of M₄ mAChR have been reported as promising ligands that not only have antipsychotic effects, but can also improve cognitive impairment and motor dysfunction. However, challenges remain due to the high homology of the orthosteric binding site among all muscarinic receptors. In this review, we summarize our approach to the identification of M₄ mAChR activators, orthosteric agonists, and positive allosteric modulators based on M₄ mAChR structural information and structure–activity relationship studies. These findings indicate that selective M₄ mAChR activators are promising potential therapeutic agents for several central nervous system conditions.

Key words M₄ muscarinic acetylcholine receptor; selective activator; orthosteric agonist; positive allosteric modulator

1. Introduction

Neural responses to neurotransmitter signals play a crucial role in the regulation of various functions in the central nervous system (CNS). Acetylcholine (ACh) is one of the most important neurotransmitters and is synthesized by choline acetyltransferase from choline and acetyl CoAs. It is well-known that ACh system plays a crucial role on higher brain function.1,2) Aside from its role in the brain, ACh is involved in a number of regulatory mechanisms in the sympathetic and parasympathetic nerve systems. These findings promoted ACh as a pivotal target in a number of drug discovery studies and subsequently led to the identification of several CNS-acting drugs. Among them, ACh esterase inhibitors (AChEIs), such as donepezil, galantamine, and rivastigmine, are currently used for the treatment of cognitive dysfunction associated with Alzheimer’s disease (AD). However, although AChEIs can ameliorate cognitive symptoms in patients with AD, they have limited clinical utility because of unwanted side effects and their limited efficacy in treating behavioral and psychological symptoms of dementia (BPSD).3–8) Direct and subtype-selective activation of ACh receptors are therefore considered to be better approaches to treat cognitive impairment and BPSD in AD.9)

Muscarinic ACh receptors (mAChRs) belong to the rhodopsin-like G-protein-coupled receptor (GPCR) family10–13) and are classified into five subtypes (Fig. 1). M₁, M₄, and M₅ mAChRs are predominantly expressed in the brain, while M₂ and M₃ mAChRs are highly distributed in peripheral tissues.12,14) Among these subtypes, M₁ and M₄ mAChRs are thought to be promising targets for the treatment of AD and schizophrenia. These assumptions are supported by knockout mice studies, as well as by results from clinical trials of xanomeline, an M₁/M₄ mAChRs-preferring agonist. It is believed that identification of M₄ mAChR-selective agonists or M₁/M₄ mAChRs-dual activators could enhance the safety profile of mAChR ligands. In the early 2000s, a number of M₁ mAChR-selective agonists were reported and proceeded to clinical development, but the identification of M₄ mAChRs-dual or M₄ mAChR-selective activators remains challenging because of high sequence similarity of the orthosteric ACh binding site among mAChR subtypes.15) In this review, we present recent findings on a novel M₄ mAChR-selective activator as a promising therapeutic agent for CNS diseases.

2. Pharmacological Benefits of M₄ mAChR Activation

The benefits of M₄ mAChR activation for psychotic symptoms in CNS diseases are supported by a number of clinical and preclinical studies.16) Indeed, xanomeline, an M₁/M₄ mAChRs-preferring agonist, has been shown to improve cognition as well as behavioral and psychological symptoms in...
patients with AD, although it caused adverse gastrointestinal effects in a dose-dependent manner.\(^7\) In addition, clinical studies with xanomeline in patients with schizophrenia suggested that activation of \(M_4\) mAChRs is effective in the treatment of positive, negative, and cognitive symptoms of this disorder.\(^8,9\) In preclinical studies, xanomeline has been shown to exhibit potent efficacies in behavioral tasks associated with psychosis (i.e., conditioned avoidance, apomorphine-induced climbing, amphetamine-induced hyperactivity, prepulse inhibition (PPI) deficit, and psychotic-like behaviors) in rodents and non-human primates.\(^20-23\) Using pharmacological magnetic resonance imaging, xanomeline suppressed the \(N\)-methyl-\(d\)-aspartate (NMDA) blocker ketamine-induced activation on blood oxygen level-dependent signal across several brain regions including the association, motor, and primary sensory cortices in rats.\(^24\) Furthermore, \(M_4\) mAChR knockout mice studies have highlighted the potential of \(M_4\) mAChR activation in the treatment of psychosis. Mice lacking \(M_4\) mAChR showed increased basal locomotor activity and PPI deficits.\(^25,26\) Dopamine release, induced by amphetamine or another NMDA receptor antagonist phencyclidine, is elevated in the nucleus accumbens of \(M_4\) mAChR knockout mice, suggesting a role for \(M_4\) mAChR in preventing hyperexcitability in midbrain dopamine neurons. Woolley \textit{et al.} reported that the effect of xanomeline on amphetamine-induced locomotor hyperactivity is marginally attenuated in \(M_4\) mAChR knockout mice, but is canceled in \(M_4\) mAChR knockout mice.\(^27\) Furthermore, Dencker \textit{et al.} have shown that xanomeline does not have antipsychotic-like effects in mice that lack the \(M_4\) mAChR in \(D_1\) dopamine receptor-expressing cells.\(^28\)

As expected, \(M_4\) mAChR activators have also been found to possess antipsychotic effects in behavioral tests for psychosis. These compounds reversed psychostimulant- or NMDA receptor antagonist phencyclidine-induced hyperactivity, as well as psychostimulant-induced disruption of PPI\(^29-38\) in rodents. In a rat electroencephalogram study, the \(M_4\) mAChR positive allosteric modulator (PAM) VU0467154, like clozapine, reversed MK-801-induced elevations in high frequency gamma power, which is consistent with their antipsychotic activities.\(^39\) Interestingly, VU0467154 induced state-dependent alterations in sleep architecture and arousal. It brought about delayed rapid eye movement sleep onset, increased cumulative duration of total and non-rapid eye movement sleep, and increased arousal during waking periods. These results suggest that \(M_4\) mAChR PAMs may lack the adverse sedative effects that are commonly observed with antipsychotics.

Until recently, it was generally believed that \(M_4\) and/or \(M_1\) mAChR activation was associated with antipsychotic efficacy, while \(M_1\), but not \(M_4\), mAChR activation was associated with enhancing cognitive function.\(^36\) However, recent preclinical work suggests that \(M_4\) mAChR also have an important role in cognitive function.\(^39\) The \(M_4\) mAChR PAM VU0152100 improves memory in a rat object recognition task. In addition, VU0467154 was shown to reverse MK-801-induced learning and memory deficits in a touch screen pairwise visual discrimination task and a fear conditioning experiment in mice.\(^41\) Importantly, the beneficial effects of VU0467154 on associative learning are absent in \(M_4\) mAChR knockout mice, which indicates that these cognitive effects are mediated by the \(M_4\) mAChR.

Currently, \(M_4\) mAChR activators are believed to have therapeutic potentials for motor dysfunction in neurological diseases. Pancani \textit{et al.} reported that chronic administration of VU0467154 beginning at a pre-symptomatic age (2 months old) could prevent the appearance of deficits in both glutamatergic and dopaminergic neurotransmissions in 5 months old YAC128 mice models of Huntington’s disease.\(^42\) Motor coordination deficits and locomotor hypoactivity were also prevented by chronic treatment in YAC128 mice. Additionally, Shen \textit{et al.} reported that the \(M_4\) mAChR PAM VU10010 blocks aberrant long-term potentiation in direct-pathway spiny projection neurons in an L-3,4-dihydroxyphenylalanine (L-DOPA)-induced dyskinesia (LID) mice model.\(^43\) Because giant cholinergic interneurons have dense terminal fields that overlap those of dopaminergic neurons, they promote \(M_4\) mAChR suppression of dopamine \(D_1\) receptor signaling through adenylyl cyclase,\(^44,45\) leading to the modulation of motor functions. Actually, acute treatment with two other \(M_4\) mAChR PAMs, VU0467154 or VU0476406, was found to attenuate LID behaviors in mice and rhesus monkeys. It is worth mentioning that these beneficial effects of \(M_4\) mAChR PAMs on LID behaviors do not compromise the symptomatic benefits of L-DOPA treatment.\(^43\)

![Fig. 2. Structures of mAChR Orthosteric Agonists](image-url)
3. M₄ mAChR Agonists

A number of orthosteric mAChR activators have already been reported and used clinically (Fig. 2). The representative orthosteric M₄ mAChR agonist xanomeline has been shown to improve the three major symptoms (positive symptoms, negative symptoms, and cognitive impairment) of schizophrenia. Although xanomeline is reported as an M₁/M₄ mAChRs-prefering agonist, our calcium mobilization assay revealed that xanomeline exhibits non-selective activation of mAChRs in Chinese hamster ovary cells that express human M₁–M₅ mAChRs.³⁰

Based on the reported clinical benefits and potential risks of xanomeline, we launched an exploratory program aimed at discovering selective M₁ and M₄ mAChRs-dual agonists. A high-throughput screening (HTS) campaign of our chemical library led to the discovery of lead scaffolds. Using previously reported information,⁴⁶,⁴⁷ we devised a hypothetical pharmacophore for allosteric M₄ mAChR agonistic activity and hybridized it with HTS hit compounds (Fig. 3).

Hybridization of the hit compound 1 and the M₄ mAChR pharmacophore led to the preferable second lead compound 2, which showed higher M₁ and M₄ mAChR agonistic activity relative to 1. However, compound 2 also exhibited agonistic activity for M₂ and M₃ mAChRs. Replacement from a benzene to a pyridine ring attenuated M₃ mAChR agonistic activity. In addition, optimization of the substituents at the nitrogen atom gave the M₁ and M₄ mAChRs-selective agonist 3. Compound 3 displayed strong M₁ (91% at 1 µM) and M₄ (134% at 1 µM) mAChRs activation with weak or negligible activation of M₂ (27% at 10 µM), M₃ (3% at 10 µM), and M₅ (3% at 10 µM) mAChRs in a calcium mobilization assay. In addition, compound 3 showed moderate human ether-a-go-go related gene (hERG) inhibition (IC₅₀=2.9 µM)⁴⁸ (Fig. 4).

However, pharmacokinetic (PK) studies of this compound revealed low bioavailability (BA) (2.6% at 2.5 mg/kg, per os (p.o.)) and rapid clearance (CL) (49.3 mL/min/kg) in rats. An analysis of compound 2 metabolites in urine revealed dealkylation. We then turned our attention to the newly fused ring moiety. Hybridization of compound 3 and the HTS hit compound 4 led to the 7-azaindoline derivative 5. Although compound 5 maintained high mAChR subtype selectivity (M₁: 55%, M₂: 13%, M₃: 5%, M₄: 77% at 1 µM, M₅: 11% at 10 µM), it also showed strong hERG inhibition (IC₅₀=0.23 µM). Eventually, conversion to the N,N'-dimethyl urea moiety provided compound 6 with good M₁ and M₄ subtype selectivity (M₁: 76%, M₂: 10%, M₃: 2%, M₄: 118% at 0.3 µM, M₅: 11% at 10 µM) and ameliorated hERG inhibition (IC₅₀>10 µM). In addition, compound 6 showed improved BA (51.1% at 2.5 mg/kg, p.o. and CL (28.3 mL/min/kg), and good blood–brain barrier penetration (brain/blood ratio=0.7)²⁹ (Fig. 5).

Our search also led to the identification of M₁ and M₄ mAChRs partial agonists; i.e., N-substituted oxindole derivatives. In this case, the high versatility M₄ mAChR agonist pharmacophore was first incorporated into the HTS hit compound 7, and then the ring size of the amine linker was modified to give the lead compound 8, which had good selectivity for M₁ and M₄ over the M₂, M₃, and M₅ mAChRs. Replacement of the piperidine moiety with tropane, a bulky amine ring, led to the discovery of compound 9, which partially but selectively activated M₁ (EC₅₀=12 nM, IA=60%) and...
M₄ (EC₅₀ = 29 nM, IA = 42%) mAChRs over M₂, M₃, and M₅ mAChRs. In addition, compound 9 (3 µM) showed negligible binding to 68 off-target proteins, including a broad range of GPCRs, ion channels, enzymes, and transporters. Finally, compound 9 exhibited potent CNS penetration (brain/blood ratio = 2.0) in rats.

In addition to the discovery of an M₄ mAChR partial agonist, dihydroquinazolinone derivatives were identified as selective M₁ full and M₄ mAChRs partial agonists (compound 10: M₁: 81%, M₂: 1%, M₃: 2%, M₄: 49% at 0.3 µM, M₅: 3% at 30 µM).

Finally, we succeeded in identifying a selective M₄ mAChR agonist. The introduction of a methanesulfonyl group into the 7-azaindoline scaffold resulted in a decrease in M₁ mAChR agonistic activity, while M₄ mAChR agonistic activity was maintained. Further optimization around the N-carbethoxypiperidine, by introducing a methyl group at the 4-position, led to compound 11, which displayed high M₄ mAChR selectivity (M₁: 5%, M₂: 3%, M₃: 4%, M₄: 94% at 0.3 µM, M₅: 3% at 30 µM). (Fig. 6).

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Using our promising pharmacophore for M₄ mAChR activation, we could identify various M₁/M₄ and M₄ mAChR agonists. Recently, researchers at Heptares Therapeutics reported a unique pharmacophore that helped to identify selective M₁/M₄ mAChRs and M₄ mAChR activators using the Alphascreen Surefire phospho-ERK1/2 assay. Other selective mAChR agonists may be identified by optimizing the hetero ring moiety in compound 11, or even by modifying the pharmacophore for M₄ mAChR activation.

4. M₄ mAChR Positive Allosteric Modulators

Allosteric modulators bind to the allosteric site of a GPCR instead of the orthosteric site, which has theoretical advantages. Generally, PAMs display high target selectivity due to higher sequence divergence in the allosteric sites compared with the orthosteric site of the receptor. In addition, PAMs do not induce independent agonistic activity, indicating that they generally possess a good safety profile.

In nature, thiochrome, an oxidation product and metabolite of thiamine (vitamin B₁), acts as an M₄ mAChR PAM. Thiochrome enhances the affinity of ACh by 5-fold at the M₄ mAChR (Fig. 8).

Eli Lily researchers also reported LY2033298, as a potent M₄ mAChR PAM that selectively enhances the ACh response at the human M₄ mAChR. Szabo et al., on the other hand, reported on the structure–activity relationship (SAR) of the thieno[2,3-b]pyridine core of LY2033298. They found that the allosteric properties of LY2033298 progressively decrease when the O- and N-alkyl chains are substituted by groups greater than two carbon atoms. Although a chlorine atom was tolerated, it significantly decreased M₄ mAChR positive modulation. Conversely, acylation of the primary amine completely abolished activity (Fig. 9).

Eli Lily researchers also reported LY2119620, an M₂ and M₄ mAChRs-dual PAM built from a thieno[2,3-b]pyridine core and a cyclopropyl amide scaffold. The introduction of an N-methyl acetylpiperazine moiety into LY2033298 led to...
LY2119620, with a potentiated muscarinic agonist iperoxo at both M₂ and M₄ mAChRs (Fig. 10).

Conversely, VU10010 was reported by Shirey et al., who based their research on LY2033298 and used cheminformatics and medicinal chemistry as an initial approach. They performed a search of the chemical database of ChemBridge Corporation and picked up 232 compounds containing a core structure similar to that of LY2033298. According to the SAR of the selected compounds, a dimethyl substitution on both the pyridine ring and the primary amine was preferable. After optimization of the substituents on the amide, the para-chlorobenzyl VU10010 was identified as a compound that binds to the allosteric site of M₄ mAChR and selectively potentiates (47-fold) M₄ mAChR-Ach concentration with an EC₅₀ value in the 400 nM range. However, VU10010 showed unfavorable physiochemical properties, including high lipophilicity (Log P ca. 4.5), poor solubility, and P-glycoprotein efflux, which prevented its use in vivo studies. As a result of a SAR study, functionalized benzyl amides, as well as pyridyl methyl congeners, were found to be well tolerated, providing selective M₄ mAChR PAMs with EC₅₀ values ranging from 380 nM to 3.7 μM, and shifts in the Ach dose-response curve ranging from 8.6- to 70-fold. The identified compounds; i.e., VU0152099 (rat M₄ EC₅₀ = 0.4 μM, 29.7-fold shift in Ach) and VU0152100 (rat M₄ EC₅₀ = 0.38 μM, 70.1-fold shift in Ach), had improved Log P values (3.65 and 3.6, respectively).

Following administration of these compounds (56.6 mg/kg intraperitoneally (i.p.)) to rats, the area under the curve (AUC) brain/AUC plasma ratio for VU0152099 was 0.39±0.01, whereas that for VU0152100 was 0.86±0.08. Further optimization of VU0152099 and VU0152100 produced ML173 (human M₄ EC₅₀ = 500 nM, rat M₄ EC₅₀ = 900 nM) and VU0448088 (human M₄ EC₅₀ = 77 nM, rat M₄ EC₅₀ = 176 nM) as selective human and rat M₄ mAChR PAMs (Fig. 11).

Wood et al. described 5-amino-thieno[2,3-c]pyridazine as a new chemotype that expands the 3-amino-thieno[2,3-b]pyridine core chemical diversity and improves on some of the remaining issues, including a steep SAR, species differences, and insufficient pharmacokinetic properties. The identified VU0467154 showed strong and selective M₄ mAChR positive allosteric modulation with a good drug metabolism and pharmacokinetics (DMPK) profile. However, this compound displayed different M₄ mAChR modulating activity between humans and rats (35 times less potent activity for the human M₄ mAChR). Further optimization on the benzene ring led to the identification of the preclinical candidate, VU0467485/AZ13713945. This compound showed improved M₄ mAChR modulating activity between humans
and rats (human M₄ EC₅₀ = 78.8 nM, rat M₄ EC₅₀ = 26.6 nM) and an attractive DMPK profile in rats (CL = 29 mL/min/kg, F = 79%, brain/blood ratio = 0.31 at 1 mg/kg iv and 3 mg/kg p.o.). VU0467485/AZ13713945 also showed clean CYP450 inhibition (3A4, 2D6, 2C9, 1A2 IC₅₀ > 30 µM in human hepatic microsomes), appropriate induction (3A4, 1A2, 2B6 EC₅₀ > 50 µM, Eₘₐₓ ≤ 1.0 in cryopreserved human hepatocytes), and weak hERG inhibition (IC₅₀ = 11 µM) or significant off-target activity (200 targets in an internal AZ/Cerep panel). Moreover, Tarr et al. have recently reported a substitution of the benzyl linker with a 3-amino azetidine moiety, leading to VU6000918. This compound also showed an excellent M₄ mAChR potency (human M₄ EC₅₀ = 19 nM) and DMPK profile in rats (CL = 16 mL/min/kg, F = 38%, brain/blood ratio = 0.77). VU6000918 also demonstrated robust efficacy in a rat amphetamine-induced hyperlocomotion reversal model (minimum efficacious dose = 0.3 mg/kg) (Fig. 12).

Other chemotypes have also been reported in the literature; VU0409524, with a benzoazole scaffold and human M₄ EC₅₀ of 13 µM, was reported by Salovich et al. In addition, compounds with a thieno[2,3-d]pyrimidine core or a 6-fluoroquinazoline core, such as VU6002703 (human M₄ EC₅₀ = 0.60 µM) and VU6003130 (human M₄ EC₅₀ = 0.6 µM) were described by Wood et al.

Despite being useful for SAR studies, these compounds have limited design for titration curves because they yield apparently “flat” SAR profiles when compound activity relies on a single parameter. Huynh et al. reported useful information on parameters for the investigation of allosteric modulator activity. These parameters are the affinity of the allosteric ligand for the free receptor (KB); the cooperativity factors that define the magnitude and direction of the allosteric ligand’s effect on the orthosteric ligand’s affinity (α) and/or downstream efficacy (β); and the intrinsic agonist efficacy of the allosteric ligand (τB). It is worth mentioning that all synthesized compounds that demonstrate PAM activity of the M₄ mAChR also exhibit intrinsic activation (τB) at the allosteric site in their own right.

Well-designed M₄ mAChR PAMs have been reported by researchers at Vanderbilt University. As mentioned above, their effort led to an improvement in the benefits of M₄ mAChR allosteric modulators. It is therefore expected that further work will validate the efficacy and safety of M₄ mAChR PAMs in humans.

5. Structure of M₄ mAChR

Until recently, only the crystal structures of M₂ and M₃ mAChRs have been reported. However, Thal et al. recently reported the crystal structures of M₁ and M₄ mAChRs bound to the inverse agonist tiotropium. In their work, the intracellular loop 3 of M₄ mAChR was replaced with a minimal T4 lysozyme fusion to aid crystallization. It was also necessary to remove the first 21 residues of the amino terminus from the M₄ mAChR to improve diffraction. This led to the determination of the M₄ mAChR structure with a resolution of 2.6 Å. Overall, the structures of the M₄ mAChRs are similar to the previously identified structures of inactive M₁ and M₃ mAChRs, with similar positioning of the seven-transmembrane bundle and root mean squared deviations of 0.6–0.9 Å. In M₁ mAChR, the rotameric change of D112 resulted in pointing D112 away from the tiotropium and is accompanied by slight movements of Y439 and Y443, allowing the formation of a hydrogen bond network between D112 and S85, W108, Y439, and Y443, which is
distinct from the M₁, M₂, and M₄ mAChR structures.

Furthermore, Thal et al. revealed the similarity of the orthosteric binding site and the difference of the allosteric biding site between M₁–M₄ mAChRs subtypes owing to differences in amino acid composition. The alanine mutations of M₄ mAChR are believed to be fundamental for interaction with the selective M₄ PAM LY2033298. Specifically, W345 at the top of TM7, Y113, Y146, and Y493, which form the roof of the orthosteric site, as well as Y89, W108, and L109, are all considered to contribute to the PAM binding pocket. These findings are helpful in the search for novel M₄ mAChR activators.

6. Conclusion

Based on the interaction between M₄ mAChR and dopamine D₁ receptors or other targets, an M₄ mAChR activator has the potential to be a therapeutic agent for psychotic symptoms and cognitive dysfunction in schizophrenia and AD, as well as for motor dysfunction in Parkinson’s disease and Huntington’s disease. Potent, selective, and brain penetrant M₄ mAChR orthosteric agonists and M₄ mAChR positive allosteric modulators have already been reported as promising ligands that display antipsychotic effects and are beneficial for cognitive and motor dysfunctions. Finally, revelation of the structure of the M₄ mAChR will help us to understand the SAR of ligands and thus identify novel M₄ mAChR activators for clinical use.

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Conflict of Interest The authors are employees of Sumitomo Dainippon Pharma Co., Ltd.

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