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Synthesis and biological evaluation of isoxazoline derivatives as potent M_1 muscarinic acetylcholine receptor agonists

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ABSTRACT

A series of azacyclic compounds substituted with isoxazole and 5-substituted isoxazolines were synthesized as acyclic modifications of the oxime class M_1 mAch receptor agonist. Among them, 3-(tetrahydropyrin-3-yl)-5-(2-pyrrolodin-1-yl)isoxazoline compound **4f** displayed potent and selective M_1 mAch receptor agonist activity in the functional calcium mobilization assay (EC_{50} = 31 nM). Introduction of 2-pyrrolidinone and 3-tetrahydropyridine groups are pivotal to the high potency. Moreover, **4f** was found to facilitate non-amyloidogenic amyloid precursor protein (APP) processing by significantly increasing ERK1/2 phosphorylation and sAPPα secretion, known disease-modifying effects related to M_1 mAChR agonists in Alzheimer's disease (AD).

Muscarinic acetylcholine (Ach) receptors (mAChRs) play important roles in mediating the action of the neurotransmitter Ach in the peripheral and central nervous system (CNS).[1–3] Five mAchR subtypes (M_1–M_5) have been cloned and are G-protein-coupled receptors. The M_1, M_3, and M_5 mAChRs are coupled to the $G\alpha_{q/11}$ proteins and activate phospholipase C and increase the resulting intracellular calcium release during phosphoinositide turnover. The M_2 and M_4 mAChRs are preferentially coupled to an inhibitory $G\alpha$ ($G\alpha_{i/o}$) protein. They inhibit adenylate cyclase activity and lead to decreased cyclic AMP formation.[3,4] The predominant type of mAChRs in the CNS is the M_1 subtype, and the M_1 mAChR is located in the cerebral cortex and hippocampus.[5] Both of these brain areas are known to be important for cognition, learning and memory and the development of amyloid plaques in Alzheimer's disease (AD). Notably, M_1 receptors have been implicated in various important disorders including Alzheimer's disease (AD), cognitive dysfunction, Sjögren's syndrome, schizophrenia and Parkinson's disease.[6,7]

Extensive studies indicate that M_1 AChR has a vital role in modulating three major pathological hallmarks of AD, which are pre-synaptic cholinergic hypofunction, extracellular amyloid plaques, and intracellular neurofibrillary tau tangles.[3,8–10]

Data from mAChR antagonist[11] and knockout mice[6,12–14] suggest that mAChRs play a central role in cognitive functions, and many M_1 mAChR agonists[9,15,16] showed significant efficacy in restoring cognitive dysfunction in pre-clinical models and clinical trials including oxotremorine, milameline, xanomeline, sabcomeline, cevimeline (AF102B), and talsaclidine, as shown in Figure 1. However, most of these compounds have failed in clinical trials due to low efficacy in cognitive improvement or intolerable cholinergic side effects most likely due to the relatively low central M_1 subtype selectivity of these orthostatic ligands.[17]

In recent years, studies with selective M_1 allosteric agonists, such as AC-42 analogues, TBPB, *N*-desmethylclozapine and ML071 analogues, and M_1 positive allosteric modulators (PAM), such as BQCA, ML137 and ML169, have demonstrated that selective activation of M_1 produces efficacy in preclinical models of cognitive enhancement.[15,18]

In addition, M_1 mAChR activation has been shown to promote the nonamyloidogenic processing of amyloid precursor protein (APP) via activation of α-secretase to elevate soluble sAPPα secretion, preclude the formation of aggregable Aβ and reduced Aβ-induced toxicity[19] via activation of PKC and subsequent mediation of ERK1/2[20] or ADAM17.[21] The ERK1/2 signaling pathway is known to play a critical role in APP processing, brain development, learning and memory, cognition, and neuronal plasticity[22,23] Activation of M_1 mAChR can also reduce the generation of Aβ by

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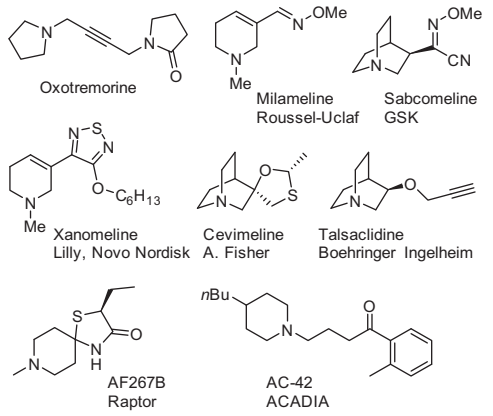
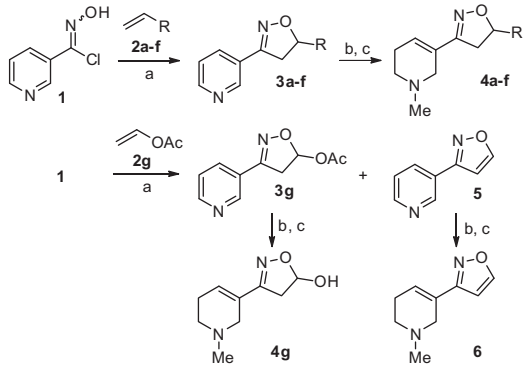


Figure 1. Chemical structures of representative M_1 agonists.



Scheme 1. Reagents and reaction conditions: (a) Et_3N, CH_2Cl_2; (b) CH_3I, acetone, 0 °C; (c) $NaBH_4$, H_2O/EtOH (1:1), 0 °C.

inhibition of the amyloidogenic processing of APP via inhibition of the γ-secretase pathway,[24] and mediation of proteasomal degradation of β-secretase-1 (BACE1).[25]

Furthermore, activation of M_1 mAchR decreases tau hyperphosphorylation via increased PKC leading to inhibition of GSK-3β, a physiological kinase known to be increased and associated with neurofibrillary tangles (NTF) and Aβ-induced neurotoxicity in AD.[13,26]

Remarkably, M_1 mAChR agonists, such as AF267B, were found to reduce cortical and hippocampal levels of Aβ,[3] and cevimeline and talsaclidine significantly decreased cerebro spinal fluid (CSF) Aβ level in AD patients.[27,28] No allosteric M_1 mAchR agonists have been reported to be effective at decreasing Aβ levels in in vivo studies. To the best of our knowledge, M_1 allosteric agonists alone (TBPB, ML071) and M_1 PAMs (BQCA, ML169) co-dosed with the orthosteric agonist carbachol (CCh) elicited an increase in sAPPα production in vitro.[8,29,30] Treatment with the selective M_1 AChR agonist AF267B was shown to reduce both Aβ and tau pathologies in the cortex and hippocampus and to reverse the cognitive deficits in triple transgenic (3xTg-AD) mice.[13]

Compounds with an oxime moiety,[15,31] such as sabcomeline[32] and milameline,[33] exhibited potent activity as M_1 mAChR agonists. We are also interested in the 2-pyrrolidinone compounds, such as pan-mAChR agonist oxotremorine derivatives[15,34] and piracetam family nootropics.[16,35] Inspired by the oxime and 2-pyrrolidinone compounds, we designed new isoxazole and isoxazoline compounds as cyclic oxime derivatives by modification of oxime class M_1 mAChR agonists to discover new potent and selective M_1 mAChR agonists possessing disease-modifying effects in AD.

Here, we described the synthesis of *N*-methyl 3-(5-substituted isoxazolin-3-yl and isoxazol-3-yl)tetrahydropyridine compounds, and their biological evaluation against five types of mAChRs using a functional calcium mobilization assay. Furthermore, we examined the effect of potent M_1 mAChR agonists in ERK1/2 signal pathway and APP processing (ERK1/2 phosphorylation and sAβPPα release) related to the disease-modifying effects in AD.

The synthesis of *N*-methyl 3-(5-substituted isoxazolin-3-yl and isoxazol-3-yl)tetrahydropyridine compounds **4** and **6** is shown in Scheme 1.[36] The isoxazoline compounds were first described in our published patent applications.[37] Dipolar cycloaddition of 3-pyridyl nitrile oxide, generated in situ from 3-pyridine hydroximoyl chloride **1** in the presence of triethylamine,[38] with various dipolarophiles **2a**–**f** produced 3-(pyridin-3-yl)isoxazoline derivatives **3a**–**f** in good yields as shown in Table 1. When vinyl acetate was used as a dipolarophile, both the corresponding isoxazoline **3g** and isoxazole **5** were generated by subsequent elimination in 44% and 31% yield, respectively. N-methylation of pyridines **3a**–**g** and **5** followed by sodium borohydride reduction of the corresponding *N*-methyl pyridinium salts provided tetrahydropyridine compounds **4a**–**g** and **6** successfully.

In addition to tetrahydropyridine compounds, several pyrrolidine and piperidine compounds substituted with 5-(2-pyrrolodin-1-yl)isoxazoline were prepared as shown in Scheme 2.[37] One-pot 1,3-dipolar cycloaddition of the appropriate aldoximes (**5a**–**e**) was performed using *N*-vinyl-2-pyrrolidinone (**7**) in the presence of sodium hypochlorite to provide isoxazoline derivatives **8a**–**e** bearing a 2-pyrrolidinone group at the C-5 position in 9–31% yields.

The synthesized compounds were evaluated in vitro against M_1–M_5 muscarinic acetylcholine receptor agonist activity in HEK293 cells expressing each mAChR subtype via calcium mobilization assay using a Functional Drug Screening System 6000 (FDSS6000), 96-well calcium dye-based fluorescence plate reader (Hamamatsu Photonics, Japan).[39] In the M_1 mAChR antagonist experiments, the calcium response was measured after adding a final concentration of 100 nM acetylcholine into the HEK cells pre-incubated with the test compounds. The M_1 selective antagonist pirenzepine, mAChR pan-agonist oxotremorine and pilocarpine, and endogenous mAChR agonist acetylcholine were used as references. The preliminary screening was performed against the M_1 mAChR subtype to measure the fluorescence-based calcium response at final concentrations of 10 μM of the compounds and determine the percentage of the maximum response obtained at a 100 nM concentration of acetylcholine. The compounds showing M_1 agonist activity were further examined for muscarinic receptor subtype selectivity against the other muscarinic receptors. The agonist activity of the human M_2 or M_4 receptors could be determined by measuring the calcium mobilization of the HEK293 cell lines co-expressed as the receptors with the $G\alpha_{15}$ subunit by the FDSS6000 system.[40]

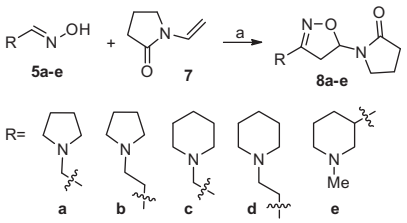
The results are summarized in Table 1. The M_1 agonist activity of the tested tetrahydropyridine compounds **4a**–**g** was found to be highly dependent on the substituent at 5-position of the isoxazoline ring. Isoxazoline compounds **4c** and **4d** were substituted with methylthiomethyl and nitrile, respectively, at the C-5 position and exhibited high agonist activity with significantly improved M_1-mediated calcium responses (EC_{50} = 5.68 and 7.54 μM, respectively). Additionally, compounds **4c** and **4d** displayed significant selectivity for M_1 over the other muscarinic subtypes at concentrations of 10 μM. However, 5-hydroxymethyl (**4a**) and 5-ethoxy (**4b**) isoxazoline compounds showed weak M_1 antagonist activity, and **4e** with a large 5-phenylthio substituent also revealed moderate M_1 antagonist activity.

Although 5-hydroxyisoxazoline derivative **4g** exhibited low M_1 agonist activity, isoxazole compound **6** showed good M_1 agonist efficacy (95.8% Max ACh response) and a submicromolar EC_{50} value (0.78 μM) with higher selectivity than the M_2 and M_4 subtypes.

Table 1
Effects of **4f** and other pyrrolidonyl isoxazoline compounds on M_1 muscarinic acetylcholine receptors using a FDSS6000 system

Entry	R	Yield		EC_{50} (μM)	% effect (or % inhibition) at 10[a] μM				
		3/5	4	*h*M_1	*h*M_1	*h*M_2	*h*M_3	*h*M_4	*h*M_5
4a	$-CH_2OH$	76	94		1.7 ± 0.3 (12.3±0.3)				
4b	$-OCH_2CH_3$	81	69		2.4 ± 0.3 (34.0±1.8)				
4c	$-CH_2SCH_3$	72	28	5.68 ± 1.62	61.3 ± 2.4	13.8 ± 1.8	18.8 ± 0.5	3.4 ± 0.1	23.8 ± 0.9
4d	–CN	78	31	7.54 ± 0.11	58.8 ± 1.0	17.5 ± 1.3	9.5 ± 0.9	7.4 ± 0.7	14.7 ± 0.8
4e	–SPh	57	46		2.3 ± 0.6 (49.4 ± 0.2)				
4f		73	69	0.031 ± 0.0004	99.5 ± 2.5	25.5 ± 3.7	56.6 ± 2.2	15.7 ± 0.2	56.3 ± 1.6
4g	–OH	44	82	17.32 ± 0.05	36.9 ± 1.1				
6	—	31	26	0.78 ± 0.04	95.8 ± 2.0	21.4 ± 1.2	40.5 ± 0.8	7.5 ± 1.0	38.4 ± 0.6
7a–e	—	—	—		2.2∼8.1 (20.7∼47.0)				
Pirenzepine					(96.7 ± 0.4)				
Milameline[b]				0.021	83.0	30.7	58.6	41.7	55.3
Oxotremorine				0.0016 ± 0.00001	108.1 ± 3.9	112.8 ± 3.7	88.8 ± 4.3	92.1 ± 2.2	82.8 ± 4.2
Pilocarpine				0.0175 ± 0.0041	102.0 ± 1.7	73.3 ± 1.1	76.5 ± 1.0	61.5 ± 5.8	62.2 ± 0.6
ACh				0.0061 ± 0.0004	100 ± 6.8	99.3 ± 2.1	100 ± 2.6	110.3 ± 8.5	97.3 ± 0.4

[a] Compounds were tested in M_1 mAChR/HEK293 cells. The responses are expressed as a percentage of the maximum response obtained at a 100 nM concentration of ACh. The values given in parenthesis represent the percent inhibition obtained after the addition of Ach (100 nM) to the pre-incubated cells with each compound. The data are represented as the mean ± SEM (Standard Error of the Mean) of three independent experiments.
[b] The agonist assay was conducted ($n = 2$) on a FLIPR™ instrument at Eurofins Pharma Discovery Services (Dundee, Scotland).



Scheme 2. Reagents and reaction conditions: (a) NaOCl, $CHCl_3$, 0 °C to rt, 11% (**8a**), 13% (**8b**), 9% (**8c**), 45% (**8d**), and 31% (**8e**).

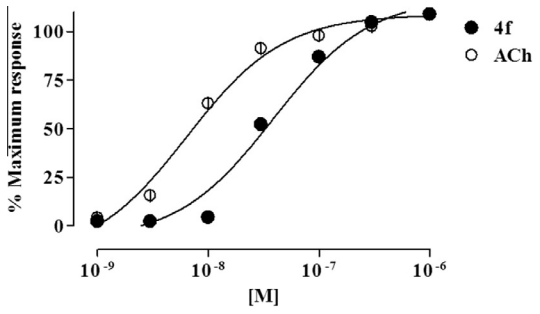


Figure 2. Dose-dependent response of Ach and **4f**. HEK293 cells stably expressing the M_1R and loaded with Fluo-4-AM. The cells were treated with increasing concentrations of the compound and the changes in the intracellular Ca^{2+} responses were measured using a FDSS6000 system. The responses of the compounds were expressed as the percentage of the maximum response obtained at a 100 nM concentration of ACh.

The pyrrolidin-2-one substituted isoxazoline compound **4f** showed potent M_1 agonist activity (EC_{50} = 31 nM) and robust efficacy (99.5% of the maximum ACh response at 10 μM) against M_1 AChR (Fig. 2). The 2-pyrrolidone derivative **4f** proved to be an M_1 selective agonist showing weak pan agonist activity across the other M_{2-5} subtypes, up to 56.6% at a concentration of 10 μM. Among the selected known agonists, milameline showed similar subtype selectivity with **4f** and **6** except M_4 receptor, and oxotremorine and pilocarpine showed potent and broad agonist activity against all mAChRs as reported previously. Compound **4f** was also found to be a potent orthosteric M_1 agonist because **4f** activated M_1 receptor directly with a similar maximum calcium response to the endogenous ligand acetylcholine, and did not enhance the activity of Ach in the Allosteric Profiler™ assay conducted at Eurofins Pharma Discovery Services (Dundee, Scotland).

However, replacement of the tetrahydropyridine ring in **4f** with several linked pyrrolidine and piperidine rings (**8a–d**) was found to destroy the agonist activity and displayed weak M_1 antagonist activity. Interestingly, piperidine compound **8e**, where the double bond in the tetrahydropyridine ring at **4f** was reduced, was found to act as a moderate M_1 antagonist. These results suggest that both 2-pyrrolidone and tetrahydropyridine rings are crucial components of agonist activity in this series.

In order to evaluate the contribution of **4f** to the high activation of M_1 AChR and subtype selectivity, docking studies were performed using homology model of M_1 AChR receptor generated based on human M_2 agonist-occupied active conformation (PDB code: 4MQS)[41] using Modeller9v8 implemented DiscoveryStudio ver 4.0. Docking studies revealed that *S*-isomer of **4f** is a favorable to form hydrogen bond with Asn382$^{6.52}$ and showed higher binding affinity (*G* score, −10.23) to active conformation of M_1 AChR binding with agonist than its enantiomer (*G* score, −9.97), and the resulting binding mode was shown in the Figure 3 (see also Supplementary material). Like M_1 agonist iperoxo[41], D105$^{3.32}$ and N382$^{6.52}$ has a central role in receptor binding of **4f** in the active M_1 orthosteric binding pocket with N382$^{6.52}$ forming hydrogen bond with carbonyl group of 2-pyrrolidinone while D105$^{3.32}$ engages in a charge-charge interaction with the amine moiety of the tetrahydropyridine ring. Milameline did not show the hydrogen bonding with N382$^{6.52}$ in docking studies unlike oxotremorine, **4f** and iperoxo. Cation–π interactions of the amine with Y106$^{3.33}$, Y381$^{6.51}$, Y404$^{7.39}$, Y408$^{7.43}$ forms aromatic lid over the ligand. However, compound **4f** did not show any preference to M_1 orthosteric binding site on the analogous M_{1-5} AChR docking studies because highly conserved orthosteric binding pockets are identical in all five muscarinic receptor subtypes with the same structural homology.

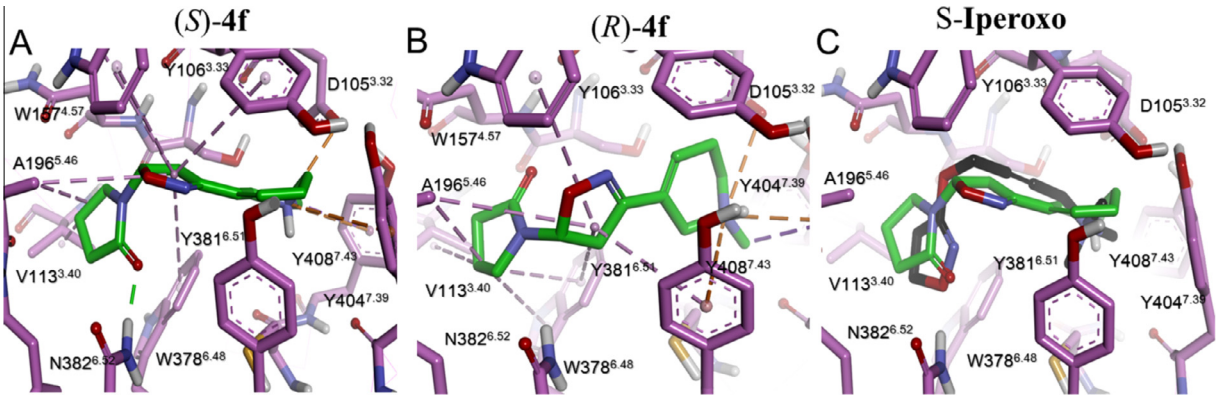


Figure 3. Docked binding mode of compound (*S*)-**4f** (A), (*R*)-**4f** (B) in orthosteric site of M_1 AChR subtype, and superimposition of (*S*)-**4f** and iperoxo (black) with interaction residues (C). The dot lines are represented a hydrogen bond (green), charge interaction (orange), hydrophobic (magenta) and π–cation interaction (violet). The Glide SP program is used for molecular docking.

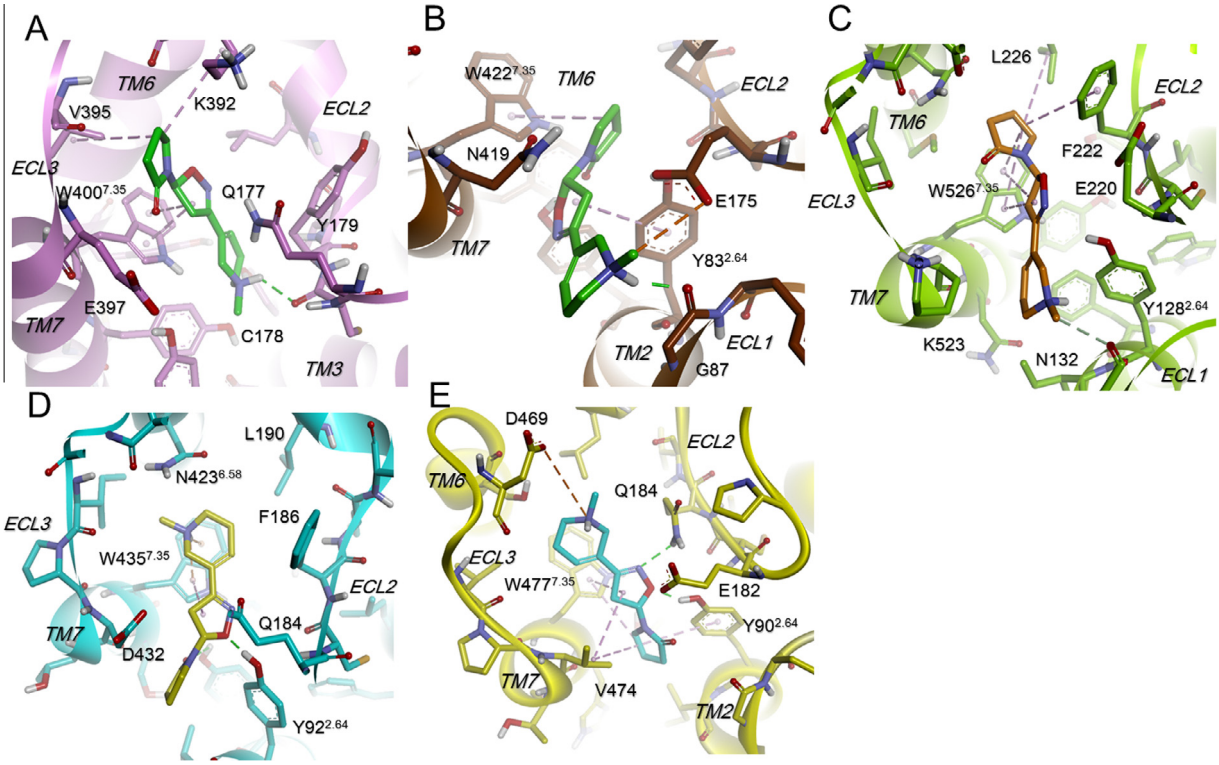


Figure 4. Docked binding mode of compound (*S*)-**4f** in allosteric sites of M_1 (A), M_2 (B), M_3 (C) M_4 (D) and M_5 (E) AChR subtypes. The dot lines are described in Figure 3.

For further evaluating the M_1 subtype selectivity of **4f**, docking studies were performed using homology model of M_{1-5} muscarinic receptors in the allosteric binding sites of active conformations located in the extracellular vestibule. Allosteric binding sites are less conserved region among the receptors, and mapped to residues located at the entrance to the orthosteric binding pocket by separation with aromatic lid. Docking studies showed that the ligands located between extracellular loops 2 (ECL2) and the end of transmembrane 6 and 7 (TM6 and TM7) with small variation of binding modes, and the results for M_{1-5} receptors were shown in Figure 4 (see also Supplementary material). The most important interactions of **4f** in the M_1 receptor are stacking interaction of isoxazoline ring with W400$^{7.35}$ and hydrogen bonding of ammonium hydrogen in the tetrahydropyridine ring with C178. Also, the 2-pyrrolidinone ring of **4f** showed hydrophobic interactions with V395 and K392, and the carbonyl oxygen showed stacking interactions with the indole ring of W400$^{7.35}$. The results of docking experiments with the **4f**, **6**, oxotremorine and milameline to the M_{1-5} receptors are summarized in Table 2. Total energy of the ligand complex with M_2 and M_3 receptors are less stable than that

Table 2
Binding energies in allosteric sites of mACh receptors[a]

Compd	M_1	M_2	M_3	M_4	M_5
4f	−7.912	−5.01161	−6.966	−6.561	−5.417
6	−6.022	−4.54339	−5.6117	−4.548	−3.868
Oxotremorine	−7.018	−4.80955	−6.981	−4.471	−3.889
Milameline	−4.640	−4.16119	−4.541	−4.157	−4.332

[a] Glide *G* score were calculated in Glide.

of M_1, and energy minimized ligand positioned inversely in M_4 and M_5 receptors. These results correlated well with their corresponding subtype selectivities obtained from in vitro mAChR agonist activities. Although compound **4f** has perfectly same binding mode in orthosteric sites among the subtypes, its binding energies in allosteric sites showed moderate correlation with experimental results.

Next, the effect of **4f** on M_1-induced ERK1/2 activation was investigated to evaluate the downstream response of M_1 activation.[20] The endogenous agonist acetylcholine increased the phosphorylation of ERK1/2 by stimulating the human M_1 mACh receptor stably expressed in HEK293 cells to reach the peak of the relative ratio of p-ERK1/2 versus ERK1/2 twice 2 min after acetylcholine treatment (100 μM) in a time-dependent manner (Fig. 5A). The treatment of **4f** activated ERK1/2 phosphorylation via the human M_1 receptor in a concentration-dependent manner. Compound **4f** showed 1.3, 1.7 and 1.8 times larger relative ratios of p-ERK1/2 versus ERK1/2 than 100 μM of acetylcholine after 2 min at doses of 1, 10, and 100 μM, respectively (Fig. 5B).

The treatment of acetylcholine (100 μM) increased the sAPPα release in human SH-SY5Y neuroblastoma cells to the maximum level after 1 h, which was 3.4 times greater than that of the initial time as shown in Figure 6A. Treatment with **4f** enhanced the release of soluble sAPPα in a dose-dependent manner at lower concentrations than Ach (Fig. 6B). The sAPPα releases induced by **4f** after 1 h were 1.5, 2.5 and 3.4 times greater at 0.01, 0.1, and

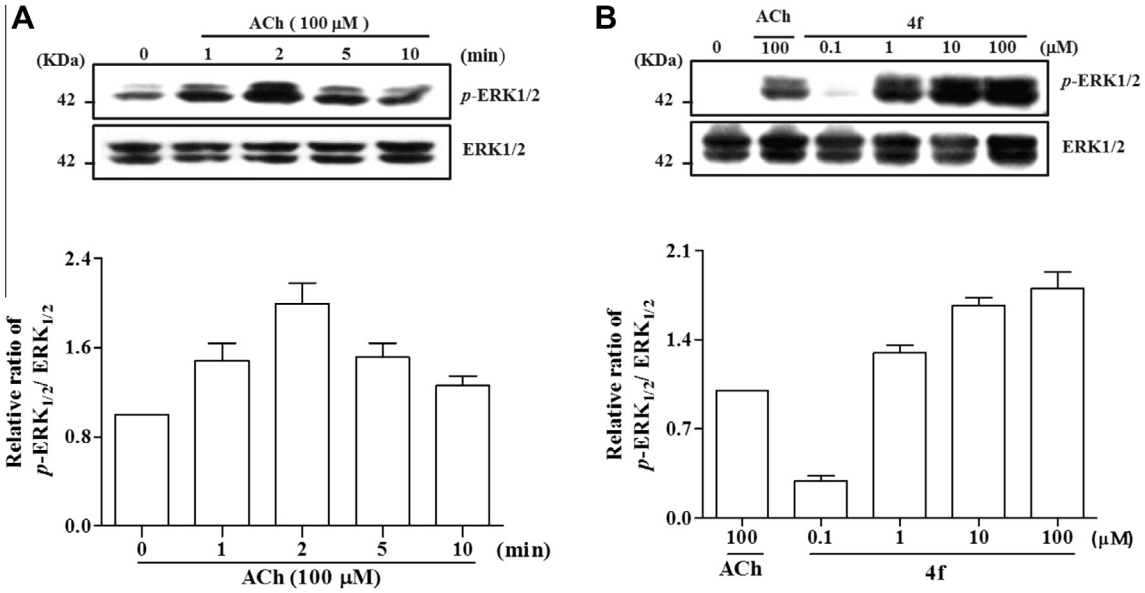


Figure 5. Effect of **4f** on ERK phosphorylation in M_1R/HEK293 cells. M_1R/HEK293 cells were treated with 100 μM of ACh for the indicated time (A) and treated with the indicated concentration (0.1–100 μM) of **4f** for 2 min (B). Western blots of the M_1R/KEK293 cells were probed with anti-pERK1/2 and anti-total ERK1/2 antibodies. The data were obtained from three independent experiments.

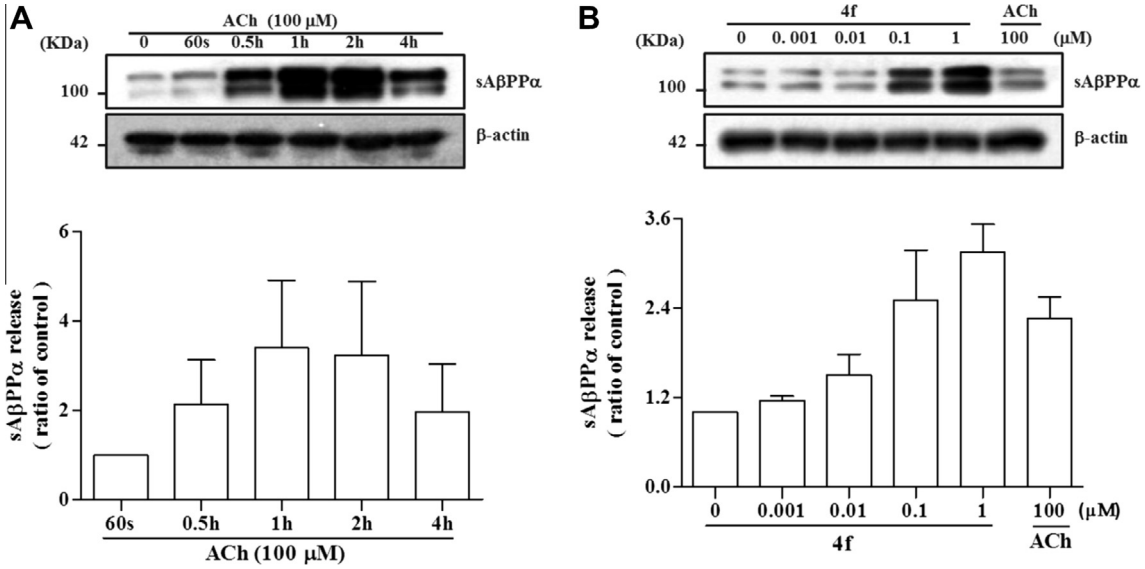


Figure 6. Increase in the sAβPPα release due to treatment with **4f**. (A) SH-SY5Y cells were treated with ACh (100 μM) at the indicated time intervals. (B) The SH-SY5Y cells were treated with the indicated concentrations of **4f** for 1 h, and the culture medium was then subjected to immunoblotting to detect sAβPPα. The data were obtained from three independent experiments.

1 μM, respectively, than acetylcholine at 100 μM. The results showed that **4f** also shifted APP processing toward a nonamyloidogenic pathway similar to some other previously described orthosteric and allosteric M_1 agonists.

In conclusion, a series of azacyclic compounds possessing 5-substituted isoxazoline groups were synthesized, and their functional mACh receptor agonist activities were evaluated. Among them, compound **4f**, a tetrahydropyridine compound substituted with 5-(2-pyrrolodin-1-yl)isoxazoline at the 3 position, showed potent and selective M_1 mACh receptor agonist activity in the functional calcium mobilization assay. The results demonstrated that the 2-pyrrolidone group substituted at the 5-position of the isoxazoline markedly improved the agonist activity, and the 3-tetrahydropyridine scaffold is crucial for the activity. Furthermore, **4f** was also found to have a disease-modifying role in Alzheimer's disease, such as an increase in ERK1/2 phosphorylation and sAPPα secretion, which are known as downstream processes and reduce the β-amyloid level by shifting APP processing toward the nonamyloidogenic pathway. Further modifications of **4f** to improve potency, efficacy, and subtype selectivity against M_1 mACh receptors are currently investigated and will be reported shortly.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2015.02.012.

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36. *General procedure for the preparation of compounds **4a–f***: To a solution of 3-pyridine hydroximoyl chloride **1** (1.0 g, 6.39 mmol) in dichloromethane (20 mL) was added the dipolarophile **2a–f** (2.0 equiv) and triethylamine (1.1 mL, 1.3 equiv). The reaction mixture was stirred at room temperature for 1 h and then was extracted with chloroform three times. The combined organic layers were washed with saturated sodium carbonate solution twice and water, and dried over anhydrous Na_2SO_4. Removal of solvent under reduced pressure followed by column chromatography (hexanes/EtOAc = 1:1, or $CHCl_3$/MeOH = 50 ~ 15:1) gave the corresponding 3-isoxazolylpyridines **3a–f** (57–81% yield). To a solution of 3-isoxazolylpyridine **3** (5.83 mmol) in acetone (15 mL) was added methyl iodide (3 equiv) at 0 °C. The reaction mixture was stirred for 3 h at the same temperature, filtered, and dried under vacuum to afford pyridinium salt. To the pyridinium salt (4.0 mmol) in a mixture of ethanol and H_2O (1:1, 30 mL) was added $NaBH_4$ (454 mg, 3 equiv) at 0 °C. After stirred for 1 h at the same temperature, the reaction mixture was concentrated to remove ethanol. To the residue, water was added and the mixture was extracted with chloroform (15 mL) three times. The combined organic layers were washed with brine and then water, dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was purified by flash chromatography on silica gel ($CHCl_3$:MeOH, 10:1 ~ 5:1) to give the desired compounds **4a–f** (28–94% yield): 1-[3-(1-Methyl-1,2,3,6-tetrahydro-pyridin-3-yl)-4,5-dihydro-isoxazol-5yl]-pyrrolidin-2-one (**4f**): 73% yield; ^{1}H NMR ($CDCl_3$) δ: 6.46 (dd, J = 3.35, 9.80 Hz, 1H), 6.30–6.01 (m, 1H), 3.27–3.11 (m, 4H), 2.86–2.83 (m, 1H), 2.52–2.30 (m, 9H), 1.95–1.92 (m, 2H); ^{13}C NMR ($CDCl_3$) δ: 175.3, 155.3, 130.9 127.8, 81.4, 53.4, 51.2, 45.7, 41.4, 36.3, 31.0, 26.7, 17.7; HRMS (ESI-TOF) m/z: $[M+Na]^+$ calcd for $C_{13}H_{19}N_3NaO_2$: 272.1375. Found: 272.1385.
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