# Structural Modifications to Tetrahydropyridine-3-carboxylate Esters en Route to the Discovery of M₅-Preferring Muscarinic Receptor Orthosteric Antagonists

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S Supporting Information

ABSTRACT: The M₅ muscarinic acetylcholine receptor is suggested to be a potential pharmacotherapeutic target for the treatment of drug abuse. We describe herein the discovery of a series of M₅-preferring orthosteric antagonists based on the scaffold of 1,2,5,6-tetrahydropyridine-3-carboxylic acid. Compound **56**, the most selective compound in this series, possesses an 11-fold selectivity for the M₅ over M₁ receptor and shows little activity at M₂−M₄. This compound, although exhibiting modest affinity (K_i = 2.24 μM) for the [^{3}H]N-methylscopolamine binding site on the M₅ receptor, is potent (IC₅₀ = 0.45 nM) in inhibiting oxotremorine-evoked [^{3}H]DA release from rat striatal slices. Further, a homology model of human M₅ receptor based on the crystal structure of the rat M₃ receptor was constructed, and docking studies of compounds **28** and **56** were performed in an attempt to understand the possible binding mode of these novel analogues to the receptor.
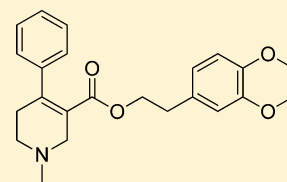
INTRODUCTION

Muscarinic acetylcholine receptors (mAcChRs) are G-protein-coupled receptors (GPCRs) activated by the endogenous neurotransmitter acetylcholine (ACh) and the natural alkaloid muscarine. Upon ACh activation, these receptors regulate a variety of central and peripheral physiological functions such as cognition, movement, sleep, cardiovascular function, smooth-muscle contractility, and glandular secretion.[1−4] Thus, mAcChRs have emerged as important therapeutic targets for many diseases, such as Alzheimer's disease, Parkinson's disease, psychosis, pain, asthma, diabetes, and smooth-muscle disorders.[3,4] Five mAcChR subtypes (M₁−M₅) have been identified.[2] Each of the five mAcChR subtypes has a defined distribution pattern within specific brain regions and peripheral tissues and mediates distinct physiological functions.[1−4] To avoid side effects, selectivity at specific mAcChR subtypes is often a major focus of discovery of mAcChR agonists and antagonists as therapeutic agents.

The M₅ mAcChR was the last subtype to be cloned.[2,5] A growing body of evidence suggests that this subtype is a potential target for the discovery of treatments for drug abuse.[6] The rewarding effects of abused drugs are believed to be mediated by the mesolimbic dopamine (DA) pathway, which projects from the midbrain ventral tegmental area (VTA) to the nucleus accumbens.[7−10] M₅ mAcChRs are the only muscarinic subtype localized to VTA and that modulate DA release from VTA DA neurons[11−17] Consistent with M₅ mAcChR modulation of mesolimbic DA transmission, behavioral studies using M₅ knockout mice show a reduction in reward and withdrawal responses following repeated morphine or cocaine administration, as well as a reduction in the rate of cocaine self-administration.[18−21]

Furthermore, microinfusion into VTA of an antisense oligonucleotide targeting M₅ receptor mRNA inhibits brain stimulation reward in rats[15] and microinfusion into the VTA of the nonselective mAcChR antagonist, scopolamine (**1**, Figure 1), reduces cocaine-facilitated DA release in nucleus accumbens.[17] Microinfusion of the nonselective mAcChR antagonist atropine (**2**, Figure 1) into the VTA in rats dose-dependently inhibits morphine-induced conditioned place preference.[22] Taken together, these studies suggest that discovery of subtype-selective M₅ mAcChR antagonists may provide novel treatments for drug abuse. Importantly, mice lacking M₅ receptors exhibit no difference from their wild-type littermates in various behavioral and pharmacologic tests,[18,23] suggesting that centrally active M₅ receptor antagonists will be well tolerated.

Subtype-selective M₅ receptor antagonists also will be useful pharmacological tools in defining the physiological functions of this receptor. Currently, little information is available on the effects of selective activation or inhibition of M₅ receptors. Recent success in identifying M₅-selective positive allosteric modulators may provide new information in this regard.[24−26] Nevertheless, no subtype-selective orthosteric M₅ antagonists are available to date. Unlike allosteric binding sites, which are usually more divergent across subtypes, orthosteric ACh binding sites across the five mAcChR subtypes have been shown to be highly conserved at the amino acid sequence level (73−83% identity).[5] As such, selectivity among the mAcChR subtypes is likely based on conformational dissimilarities rather than upon
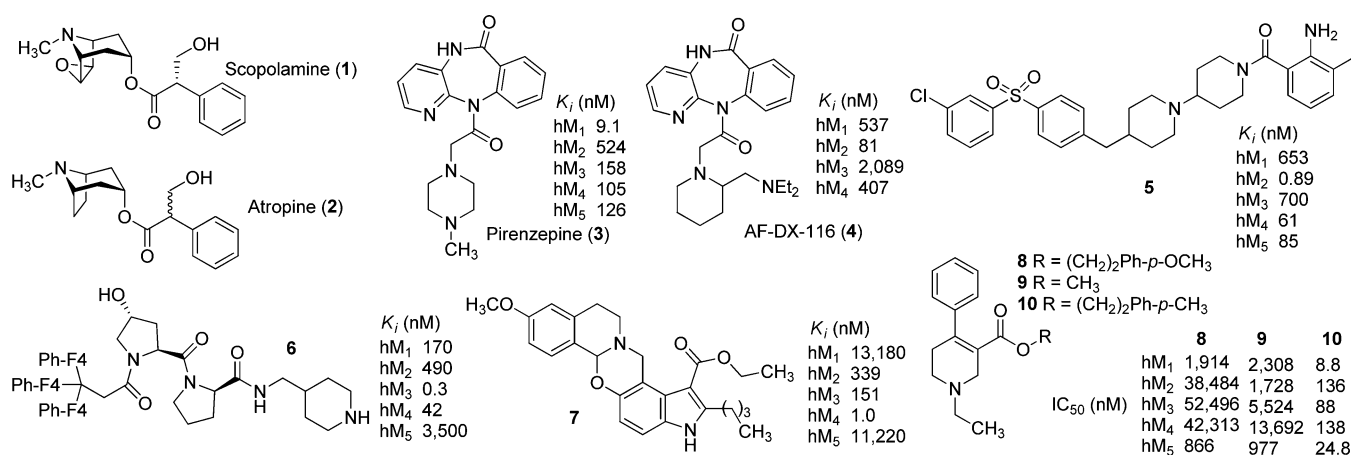
Figure 1. Structures of selected mAcChR antagonists and their literature reported mAcChR affinity data.

single amino acid residues.[27−29] In fact, small molecule ortho-steric antagonists with preference for an individual subtype have been reported for M_1−M_4 but not M_5 (e.g., compound **3** for M_1,[30] **5** for M_2,[31] **6** for M_3,[32] and **7** for M_4,[29] Figure 1), indicating the existence of sufficient differences among the agonist recognition sites on the five mAcChR subtypes allowing targeting of selective compounds to these sites.

Radioligand binding assays using membranes from recombinant cells expressing human mAcChRs (hM_1−hM_5) show that compound **8** (1-ethyl-4-phenyl-1,2,5,6-tetrahydropyridine-3-carboxylic acid 4-methoxyphenethyl ester, Figure 1) preferentially binds to the M_5 receptor (subtype selectivity: M_1/M_5 = 2.2-fold, M_2/M_5 = 44-fold, M_3/M_5 = 60-fold, M_4/M_5 = 48-fold, Figure 1).[33] Thus, the current exploration of the structure−activity relationship (SAR) regarding M_5 selective orthosteric ligands is based on compound **8**. To our best knowledge, compound **8** and its analogue **9** (Figure 1) are the only orthosteric small molecules reported to exhibit preference for M_5 receptors.[33]

The current structural modification strategy is provided in Figure 2. The 1,2,5,6-tetrahydropyridine-3-caboxylate core structure
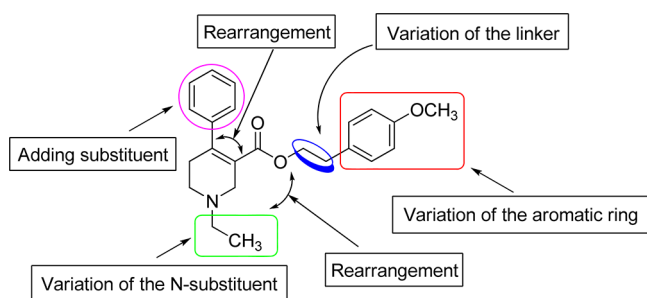

Figure 2. General structural modification strategy.

was retained while modifying each part of the "appending" pharmacophores in a stepwise manner. These "appending" pharmacophores were to be rearranged around the core structure in compound **8** (Figure 2). Herein, we report the synthesis, pharmacological evaluation, and SAR analysis of this new series of mAcChR antagonists. Additionally, a homology model of hM_5 mAcChR was constructed based on the crystal structure of the rat M_3 mAcChR. Preliminary docking experiments were performed using this model in an attempt to understand the structural basis of the interaction between these ligands and the receptor.
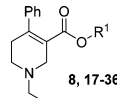
RESULTS AND DISCUSSION

Synthesis. The general synthetic methods for analogues **17−36** (Table 1) with modifications on the p-methoxyphenethyl moiety of compound **8** are depicted in Scheme 1. Transformation of commercially available ethyl 1-benzyl-4-oxo-3-piperidinecarboxylate (**11**) into compound **12** was achieved by initial conversion of **11** to N-BOC protected compound via a two-step process,[34] followed by triflation under diisopropylamine and trifluoromethanesulfonic anhydride.[35] Suzuki coupling of **12** with phenylboronic acid afforded compound **13**. Removal of BOC in **13** by TFA afforded compound **14**, which was then N-ethylated to form compound **15**. Compound **15** was subjected to ester hydrolysis under basic conditions to afford carboxylic acid **16**. Treatment of **16** under a Steglich esterification condition with appropriate alcohols afforded **8** and its analogues **17−36**. The synthetic route for analogues with variations on the N-ethyl moiety of compound **8** was initially devised to introduce the N-substituent at the last step. Accordingly, compound **13** was converted to compound **38** in a sequence similar to that for compound **15** to **8** (Scheme 1). However, deprotection of **38** to form **39** using various de-BOC methods (TFA/CH$_2$Cl$_2$, HCl/EtOAc, AcCl/MeOH, or TsOH/CH$_2$Cl$_2$) resulted in complex mixtures. Attempts to purify compound **39** by silica gel column chromatography failed. Impure intermediate **39** (obtained from TFA treatment) was employed to provide N-Me, N-n-Pr, N-n-Bu, or N-p-methoxybenzyl analogues via reductive amination, but only N-p-methoxybenzyl analogue (compound **40**) was obtained in pure form after preparative TLC purification. Alternatively, a similar route to that for analogues **17−36** was applied to prepare analogues **42−59** from compound **14** (Scheme 1, Table 2).

Analogues **61−69** (Table 2) were synthesized in a similar manner to analogues **42−59** from triflate **12**. Phenyl ring substituted phenylboronic acids were used instead of phenylboronic acid as the Suzuki coupling partners (Scheme 2).

Analogues **70−73** were prepared by N-alkylation of compound **14** with appropriate alkyl halides (Scheme 3). Analogues **74−76** were prepared by reductive amination of compound **14** with an N-sulfonated or N-acylated 4-piperidinone using sodium triacetoxyborohydride (Scheme 3).

The syntheses of analogues **84** and **85** were initially attempted by applying a route similar to that used for the synthesis of their regioisomers in Scheme 1 (analogues **56** and **45**, respectively), starting from ethyl 1-benzyl-3-oxo-4-piperidinecarboxylate hydrochloride (**77**) (Scheme 4). However, de-BOC reaction of

Table 1. Structures and Binding Affinity for Scopolamine (1), 8, and 17−36 at the hM$_1$ and hM$_5$ mAcChRs^a

compd	R^1	K_i, nM		
		hM$_1$	hM$_5$	hM$_1$/hM$_5$
1	-	7.5	17.6	0.4
8		170	420	0.4
17		1,730	1,250	1.4
18		470	150	3.1
19		3,060	3,610	0.8
20		3,290	470	7.0
21		>100,000	>100,000	-
22		200	290	0.7
23		100	190	0.5
24		560	410	1.4
25		240	370	0.6
26		810	440	1.8
27		>100,000	>100,000	-
28		1,390	230	6.0
29		200	280	0.7
30		60	120	0.5
31		11,700	2,460	4.8
32		>100,000	>100,000	-
33		>100,000	>100,000	-
34		410	760	0.5
35		370	140	2.6
36		140	190	0.7

^{a}At least three independent experiments with samples evaluated in duplicate were performed to obtain the K_i value.

compound **78** failed to yield the desired intermediate **79**. As an alternative, analogues **84** and **85** were synthesized starting from 3-bromoisonicotinic acid (**80**). Ethyl esterification of **80** followed by Suzuki coupling with phenylboronic acid afforded

compound **81**, which was treated with methyl iodide to yield the corresponding quaternary pyridinium salt **82**. Reduction of **82** with sodium borohydride gave 1,2,5,6-tetrahydropyridine derivative **83**, which underwent ester hydrolysis and subsequent esterification with appropriate alcohols to afford analogues **84** and **85**.

Synthesis of analogue **89** was achieved by initial Suzuki coupling of 4-bromopyridine with 2-(ethoxycarbonyl)phenyl-boronic acid, followed by a route similar to that for analogues **84** and **85** from compound **81** (Scheme 5).

Radioligand Binding Assay. Receptor affinities were determined by measuring inhibition of the binding of [^{3}H]N-methylscopolamine (NMS), an orthosteric antagonist probe, to membranes from Chinese hamster ovary (CHO) cells individually expressing human hM$_1$−hM$_5$ mAcChR receptors. Compound **1** (scopolamine) and compound **8** were used as reference compounds. Methods for these binding assays are described briefly in the Experimental Section. All synthesized analogues were evaluated first at hM$_1$ and hM$_5$ subtypes (Table 1 and 2, Scheme 3−5). Selectivity for M$_5$ over M$_1$ is important because antagonism of CNS M$_1$ receptors has been suggested to result in cognitive deficits[36] and to increase DA release in striatum.[37] Binding affinity for analogues that exhibited binding preference for M$_5$ over M$_1$ was determined at each of the five mAcChR subtypes (Table 3).

Identification of compound **8** was the starting point. Compound **8** was an analogue of the 1-ethyl-4-phenyltetrahydropyridine-3-carboxylic acid scaffold aimed at identifying M$_1$ selective antagonists. Compound **8** was reported to have a 2.2-fold preference for M$_5$ over M$_1$ and to be more than 40-fold selective for M$_5$ over M$_2$−M$_4$ mAcChR subtypes (Figure 1).[33] However, in the current study, compound **8** showed no selectivity for M$_5$ over M$_1$−M$_4$ (Table 3). Preliminary SAR from the previous report[33] suggested that small changes on the p-methoxyphenyl ring of **8** afforded remarkably different binding affinity and selectivity profiles at mAcChR subtypes. For example, when the methoxy group on the phenyl ring of **8** was replaced by a methyl group, the resulting analogue **10** bound preferentially to the M$_1$ receptor, while binding affinity at the M$_5$ receptor increased 35-fold, when compared to compound **8** (Figure 1).[33]

Results in Table 1 summarize the modifications to the p-methoxyphenethyl moiety of compound **8** evaluated in the current study and the influence of these modifications on binding affinity and selectivity for M$_1$ and M$_5$ receptors. Results from para-, meta-, and ortho-methoxy analogues **8**, **17**, and **18**, respectively, indicate that the meta-substitution was favorable to M$_5$ selectivity. Compared to **8** (K_i = 420 nM) and **17** (K_i = 1250 nM), meta-analogue (**18**, K_i = 150 nM) exhibited a small 3- and 8-fold, respectively, increase in affinity at M$_5$ over M$_1$. Replacement of the ethylene link in the p-methoxyphenethyl moiety by a methylene (compound **19**, K_i = 3610 nM) or a methylethylene link (compound **21**, K_i > 100 μM) significantly decreased binding potency at M$_5$ receptor, whereas replacement with a propylene link (compound **20**, K_i = 470 nM) retained binding affinity at M$_5$ and markedly increased selectivity (7-fold) over M$_1$ receptors. Interestingly, there were no marked differences in M$_5$ affinities when the 4-methoxy was replaced with another para-substitution group, including halogens (compounds **23**−**25**) and nitro group (compound **26**), indicating modification at this position is somewhat tolerated. However, compound **27**, in which a 4-methylsulfonyl group was attached to the phenyl ring, exhibited little affinity for either M$_1$ or M$_5$ receptors. Compounds **28** and **31**, wherein both 3- and 4-positions of the phenyl ring were substituted, not

Scheme 1. Synthesis of Compounds 8, 17−36, 40, and 42−59[a]

[a]Reagents and conditions: (a) H$_2$, 10% Pd/C (5 w/w%), EtOH; (b) (BOC)$_2$O, Et$_3$N, CH$_2$Cl$_2$; (c) Tf$_2$O, i-Pr$_2$NH, CH$_2$Cl$_2$, −78 °C; (d) PhB(OH)$_2$, Pd(PPh$_4$)$_3$, Na$_2$CO$_3$ (2.0 M), THF, 65 °C; (e) TFA/CH$_2$Cl$_2$ (1:1); (f) EtI, K$_2$CO$_3$, EtOH; (g) 10% KOH (aq)/EtOH (1:1); (h) alcohols, EDCI, DMAP, CH$_2$Cl$_2$; (i) p-methoxybenzaldehyde, NaBH(OAc)$_3$, HOAc, THF; (j) aldehydes, NaBH$_3$CN, EtOH; (k) alcohols, 2,4,6-trichlorobenzoyl chloride, DMAP, Et$_3$N, THF.

only retained affinity at the M$_5$ receptor (K$_i$ of 230 and 2460 nM, respectively) but also exhibited preference for this subtype (6.0- and 4.8-fold M$_5$/M$_1$, respectively). Trisubstitution of the phenyl ring in compounds 32 and 33 revealed that this modification was detrimental to binding. Finally, replacement of the 4-methoxyphenyl ring with a heterocyclic ring, including pyridyl (compounds 34 and 35) and thiophenyl (compound 36) rings, resulted in retention of binding affinity at both M$_1$ and M$_5$ receptors.

In parallel with the modification on the p-methoxyphenethyl group, the N-ethyl moiety of compound 8 (Table 2) also was altered. Replacement of the ethyl group by a methyl group (compound 42) resulted in an 8-fold and 14-fold increase in binding affinity at M$_1$ and M$_5$ receptors, respectively. Increasing the size of the substituted group from ethyl to n-propyl (compound 43), n-butyl (compound 44), or 4-methoxybenzyl group (compound 40) eliminated affinity for these two receptors. Similar results were observed for N-methyl, N-n-propyl, and N-n-butyl analogues (compounds 45, 46, and 47, respectively) of compound 28. Interestingly, the M$_1$/M$_5$ selectivity of two N-methyl containing analogues, compound 42 (0.7-fold) and 45 (4.8-fold), were similar to their corresponding N-ethyl analogues, 8 (0.4-fold) and 28 (6.0-fold), respectively.

On the basis of the above SAR data, our focus changed to analogues bearing an N-methyl group in lieu of N-ethyl group, and the SAR was extended regarding the substitution group of the carboxylic ester moiety (Table 2). Replacement of the ethyl link in compound 45 by a methylene (compound 49) or propylene (compound 50) link resulted in a decrease in binding affinity at both M$_1$ and M$_5$ receptors and also resulted in a decrease in selectivity for M$_5$ over M$_1$ receptors. SAR among compounds 45, 49, and 50 was inconsistent with previous SAR among compounds 8, 19, and 20, indicating an unpredictable nature of this component of the structure. On the other hand, analogues containing substituents at the meta-position or both the meta- and para-position of the ester phenyl ring (compounds 53−56)

consistently exhibited a binding preference at M$_5$ over M$_1$ receptor. Compound 56 was identified as the most selective (11-fold for M$_1$/M$_5$) M$_5$ compound.

On the basis of compounds 45 and 56, SAR was further extended by introduction of an electron-withdrawing (fluoro) or electron-donating (methoxy) group onto the phenyl ring on the C4 of the tetrahydropyridine core (compounds 61−69, Table 2). In general, these analogues exhibited decreased or completely abolished activity at either M$_1$ or M$_5$ receptor when compared to compounds 45 and 56, suggesting that this component of the molecule is less tolerant to structural modification.

Further SAR exploration was focused on previous hypotheses that spatial rearrangement or reorientation of the pharmacophore elements in mAcChR ligands would alter affinity and selectivity profiles.[38] For example, rearrangement of the amino group in M$_1$ preferring antagonist 3 (pirenzepine) afforded M$_2$ preferring antagonist 4 (Figure 1).[39] To test this hypothesis with respect to the current analogues, we conducted three types of pharmacophoric "rearrangements". First, transposition of the substituted group on the ester functionality and the ethyl group on the N atom in compound 8 and analogues afforded compounds 70−73 (Scheme 3). An N-acylated or N-sulfonated piperidine-4-yl group was also introduced to the N atom of the tetrahydropyridine ring (compounds 74−76, Scheme 3). The N-substituents were selected because they are present in potent mAcChR orthosteric antagonists such as darifenacin (90), zamifenacin (91), and compounds 92 and 93[40] (Figure 3). Surprisingly, none of these new analogues displayed any activity at either the M$_1$ or M$_5$ receptor, indicating a different receptor binding mode for the basic N atom in compound 8 and its analogues compared with compounds 90−93.

Second, the ester group on C3 and the phenyl group on C4 of the 1,2,5,6-tetrahydropyridine ring in compounds 56 and 45 were transposed to produce compounds 84 and 85 (Scheme 4), respectively. Both compounds 84 (M$_1$/M$_5$ = 2.4) and 85 (M$_1$/M$_5$ = 2.1) displayed reduced selectivity for M$_5$ over M$_1$

**Table 2. Structures and Binding Affinity for Analogues 40, 42−59, and 61−69 at the hM$_1$ and hM$_5$ mAcChRs^a**

compd	R^1	R^2	R^3	K_i, nM hM$_1$	hM$_5$	hM$_1$/hM$_5$
40	(4-OCH$_3$-benzyl chain)	4-MeO-Bn	Ph	>100,000	>100,000	-
42	ibid.	Me	Ph	20	30	0.7
43	ibid.	nPr	Ph	>100,000	>100,000	-
44	ibid.	nBu	Ph	>100,000	>100,000	-
45	(dihydrobenzofuran chain)	Me	Ph	290	60	4.8
46	ibid.	nPr	Ph	>100,000	>100,000	-
47	ibid.	nBu	Ph	>100,000	>100,000	-
48	(4-OCH$_3$-phenyl butyl chain)	Me	Ph	5,400	1,160	4.7
49	(benzofuran chain)	Me	Ph	>100,000	>100,000	-
50	(dihydrobenzofuran chain)	Me	Ph	920	630	1.5
51	(4-SMe-phenyl chain)	Me	Ph	340	190	1.8
52	(4-SO$_2$Me-phenyl chain)	Me	Ph	510	110	4.6
53	(3-Me-phenyl chain)	Me	Ph	880	280	3.1
54	(3-F-phenyl chain)	Me	Ph	330	80	4.1
55	(4-OCH$_3$-3-Me-phenyl chain)	Me	Ph	2,700	740	3.6
56	(3,4-di-OCH$_3$-phenyl chain)	Me	Ph	25,300	2,240	11.3
57	(naphthyl chain)	Me	Ph	24,400	5,040	4.8
58	(1-OCH$_3$-phenyl chain)	Me	Ph	>100,000	>100,000	-
59	(O-4-OCH$_3$-phenyl chain)	Me	Ph	6,190	3,070	2.0
61	(dihydrobenzofuran chain)	Et	4-F-Ph	7,900	2,350	3.4
62	ibid.	Me	3-F-Ph	>100,000	>100,000	-
63	ibid.	Me	2-F-Ph	5,270	740	7.0
64	ibid.	Me	3-MeO-Ph	2,430	2,680	0.9
65	ibid.	Me	2-MeO-Ph	8,350	2,410	3.5
66	(3,4-di-OCH$_3$-phenyl chain)	Me	3-F-Ph	27,000	3,340	8.1
67	ibid.	Me	2-F-Ph	>100,000	>100,000	-
68	ibid.	Me	3-MeO-Ph	>100,000	>100,000	-
69	ibid.	Me	2-MeO-Ph	>100,000	>100,000	-

^{a}At least three independent experiments with samples evaluated in duplicate were performed to obtain the K_i value.

receptors when compared to their corresponding position isomers, **56** and **45**, respectively. Last, the ester functionality in compound **45** was moved from C3 of the tetrahydropyridine ring to the phenyl ring on C4 (compound **89**, Scheme 5). This "parallel" shift resulted in a complete loss of binding affinity at both M$_1$ and M$_5$ receptors.

Scheme 2. Synthesis of Compounds 61–69[a]
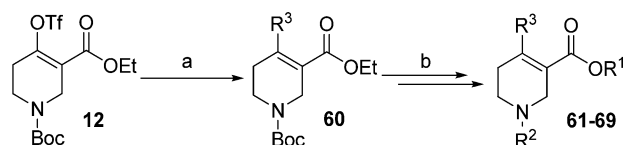
[a]Reagents and conditions: (a) substituted phenylboronic acids, Pd(PPh$_4$)$_3$, Na$_2$CO$_3$ (2.0 M), THF, 65 °C; (b) same as from compound **13** to **42** in Scheme 1.

Scheme 3. Synthesis of Compounds 70–76[a]
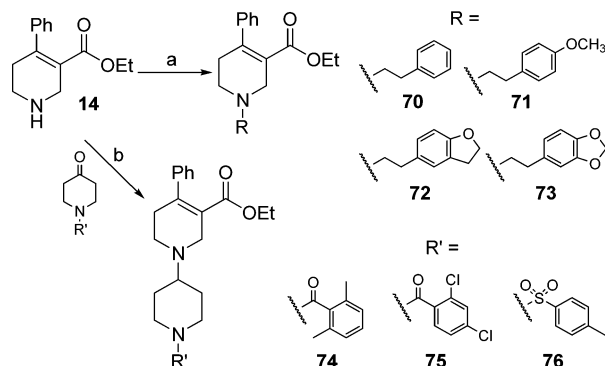
70–76, not active at hM$_1$ and hM$_5$ (K_i >100,000 nM)

[a]Reagents and conditions: (a) alkyl bromides, KI, K$_2$CO$_3$, CH$_3$CN, reflux; (b) NaBH(OAc)$_3$, HOAc, THF, 65 °C for **74**, rt for **75** and **76**.

Scheme 4. Synthesis of Compounds 84 and 85[a]
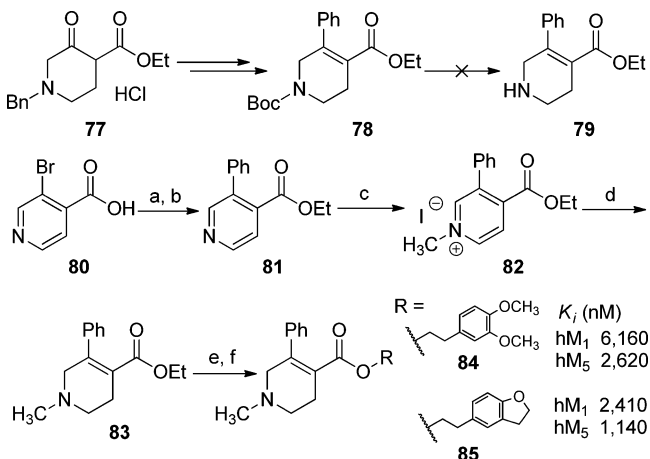
[a]Reagents and conditions: (a) EtOH, H$_2$SO$_4$ (conc), reflux; (b) PhB(OH)$_2$, Pd(PPh$_4$)$_3$, Na$_2$CO$_3$ (2.0 M), THF, 65 °C; (c) MeI, acetone; (d) NaBH$_4$, EtOH; (e) 10% KOH (H$_2$O)/EtOH (1:1); (f) alcohols, EDCI, DMAP, CH$_2$Cl$_2$.

At saturation concentrations, all analogues except for those with K_i > 100 000 nM exhibited complete inhibition (maximal inhibition I_{max} = 100%, data not shown) of the binding of the orthosteric antagonist [³H]NMS at mAcChRs, which is consistent with an orthosteric mechanism of action. Selected compounds, including **20**, **28**, **45**, **56**, **57**, **63**, and **66**, that exhibited binding preference for hM$_5$ over hM$_1$ mAcChRs were also evaluated for affinity at M$_2$, M$_3$, and M$_4$ mAcChR subtypes (Table 3). Results showed that five of these seven compounds exhibited good selectivity for M$_5$ over M$_2$, M$_3$, and M$_4$ receptors. The most selective compound, **56** (11-fold for M$_5$

Scheme 5. Synthesis of Compound 89[a]

K_i (nM)
hM$_1$ >100,000
hM$_5$ >100,000
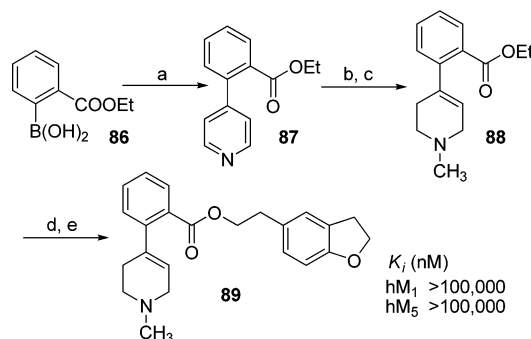
[a]Reagents and conditions: (a) 4-bromopyridine, Pd(PPh$_3$)$_4$, Na$_2$CO$_3$ (2.0 M), THF, 65 °C; (b) MeI, acetone; (c) NaBH$_4$, EtOH; (d) 10% KOH (H$_2$O)/EtOH (1:1); (e) 2-(2,3-dihydrobenzofuran-5-yl)-ethanol, EDCI, DMAP, CH$_2$Cl$_2$.

over M$_1$ receptor), had no affinity (K_i > 100 μM) at the M$_2$, M$_3$, and M$_4$ mAcChR subtypes. Compound **28** exhibited 6-fold, >435-fold, 135-fold, and 46-fold for M$_5$ over M$_1$, M$_2$, M$_3$, and M$_4$, respectively. Although slightly less selective for the M$_5$ over the M$_1$ receptor, it exhibited higher affinity at M$_5$ when compared to compound **56**.

Inhibition of Oxotremorine-Evoked Striatal [³H]DA Release. In vitro functional assays for mAcChR antagonists measure the ability of molecules to block mAcChR agonist-induced receptor activation at recombinant mAcChR subtypes expressed in cells.[41] Pharmacological studies of M$_5$ receptors using mouse basilar artery have also been reported.[42] However, these recombinant and native M$_5$ receptors functional assays are far removed from a potential role for M$_5$ receptors in cocaine and opiate addiction. Studies have shown that oxotremorine, a nonselective mAcChR agonist, concentration-dependently increases [³H]DA release from striatal slices prepared from wild-type mice and that oxotremorine-evoked striatal [³H]DA release was reduced significantly in M$_5$ receptor knockout mice.[18,43] We hypothesized that an M$_5$ receptor selective antagonist would also reduce oxotremorine-mediated rat striatal [³H]DA release. Current results show that oxotremorine evokes [³H]DA release from rat striatal slices and that scopolamine inhibits this effect in a concentration-dependent manner (Figure 4). These results support the contention that this functional assay probes native M$_5$ receptors. Furthermore, this functional assay is highly relevant to the underlying dopaminergic mechanisms involved in drug reward and abuse.

Results revealed that compound **56** inhibited (IC$_{50}$ = 0.45 nM) oxotremorine (100 μM) evoked [³H]DA release from rat striatal slices (Figure 5). Unlike scopolamine (1 μM), which completely inhibits oxotremorine-mediated [³H]DA release from rat striatal slices (Figure 4), compound **56** produced maximal inhibition (I_{max}) of only 48% of the oxotremorine-evoked [³H]DA release (Figure 5). These current results are consistent with previous reports that ~50% of oxotremorine-evoked [³H]DA release from striatal slices was eliminated in M$_5$ knockout mice compared to wild-type mice,[18] indicating that other mAcChR subtype(s) also mediate oxotremorine-evoked striatal DA release. In agreement with this hypothesis, studies using mAcChR knockout mice suggested that M$_3$ and M$_4$ receptors were also involved in mediating striatal DA release.[43] The observations that both compound **56** and the deletion of the M$_5$ receptor resulted in similar effects on oxotremorine-evoked striatal [³H]DA release, together with the

Table 3. Binding Affinity and Selectivity for Selected Analogues at the hM$_1$−hM$_5$ mAcChRs[a]

	K_i, nM				
compd	hM$_5$	hM$_1$ (M$_1$/M$_5$)[b]	hM$_2$ (M$_2$/M$_5$)[b]	hM$_3$ (M$_3$/M$_5$)[b]	hM$_4$ (M$_4$/M$_5$)[b]
1	17.6	7.5 (0.4)	9.5 (0.5)	6.5 (0.4)	36.9 (2.1)
8	420	170 (0.4)	1100 (2.6)	1370 (3.3)	1730 (4.1)
20	470	3290 (7.0)	2030 (4.3)	4070 (8.7)	11500 (24)
28	230	1390 (6.0)	>100000 (>435)	31100 (135)	10500 (46)
45	60	290 (4.8)	1170 (20)	2840 (47)	1890 (32)
56	2240	25300 (11)	>100000 (>45)	>100000 (>45)	>100000 (>45)
57	5040	24400 (4.8)	>100000 (>20)	>100000 (>20)	>100000 (>20)
63	740	5270 (7.1)	540 (0.7)	4890 (6.6)	5740 (7.8)
66	3340	27000 (8.0)	>100000 (>30)	>100000 (>30)	>100000 (>30)

[a]At least three independent experiments with samples evaluated in duplicate were performed to obtain the K_i value. [b]Numbers in the parentheses are the ratios of binding affinity between M$_5$ and the respective subtype.
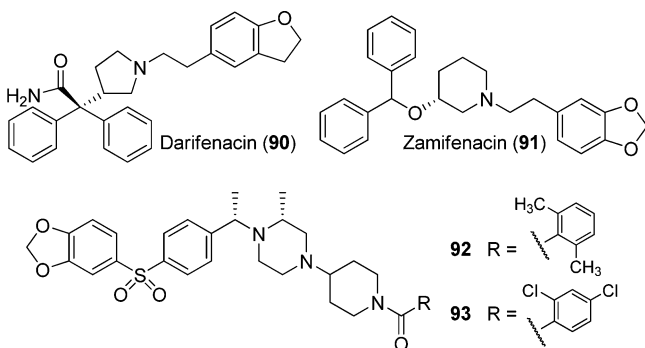
Figure 3. Structures of darifenacin (90), zamifenacin (91), and compounds 92 and 93.

Figure 5. Compound 56 inhibits oxotremorine (100 μM) evoked [^{3}H]DA release from rat striatal slices (data are expressed as the mean ± SEM, n = 4).

selective binding of 56 to M$_5$ over M$_3$ and M$_4$ receptors, strongly suggest that 56 interacts with M$_5$ receptors to inhibit muscarinic agonist-induced striatal DA release.

It is noteworthy that compound 56 (IC$_{50}$ = 0.45 nM) appears more potent than scopolamine in inhibiting oxotremorine-evoked [^{3}H]DA release from rat striatal slices, although its [^{3}H]NMS binding affinity on the M$_5$ receptor is 127-fold less than scopolamine. One explanation of the lack of correlation between binding and function is that the [^{3}H]NMS binding assay was performed on a single receptor in a recombinant system but the [^{3}H]DA release assay was on heterogeneous brain slices. Compound 56 may potently act at other sites that also inhibit [^{3}H]DA release. Alternatively, recent studies on the crystal structure of the rat M$_3$ receptor with antagonist tiotropium bound to the orthosteric binding site suggested that tiotropium binds transiently to an allosteric site en route to the orthosteric binding pocket.[44] Compound 56 could have a similar allosteric interaction with the receptor, causing the inhibition of oxotremorine-evoked striatal [^{3}H]DA release. However, further pharmacological studies are needed to elucidate the mechanism.

Binding Mode for M$_5$ mAcChRs for Lead Compounds 28 and 56. To study the interaction of our compounds with M$_5$ mAcChR in atomic detail, homology modeling and molecular docking operations were performed. Compounds 28 and 56 were selected for these studies, since both analogues preferencially bind to M$_5$ mAcChR and thus can be considered current lead compounds. Importantly, compounds 28 and 56 have an order of magnitude difference in binding affinities at M$_5$ receptor (K_i of 230 nM vs 2240 nM, respectively). This moderate difference in affinity between 28 and 56 is ideal for testing the reliability of our homology models. The structural model of the human M$_5$ mAcChR was constructed based on
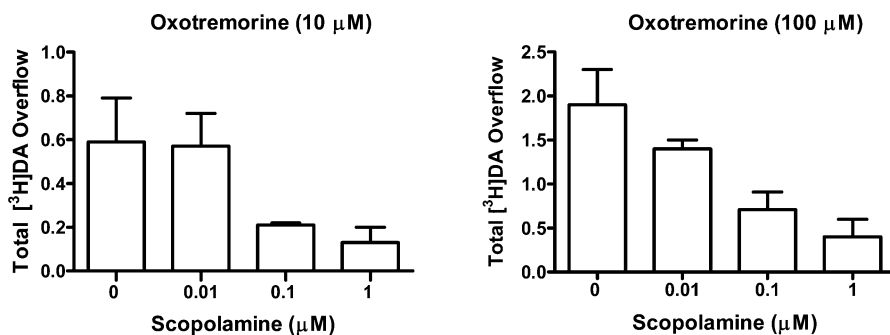
Figure 4. Scopolamine (0.01−1 μM) inhibits oxotremorine (10 and 100 μM) evoked [^{3}H]DA release from rat striatal slices (data are expressed as the mean ± SEM, n = 3).

the newly available X-ray crystal structure of the rat M$_3$ mAcChR with antagonist tiotropium bound to the orthosteric binding site.[44] Compounds **28** and **56** were docked into possible binding sites among the transmembrane (TM) helices of the M$_5$ mAcChR. Binding structures were selected from the docking results and subjected to energy minimization. In accordance with the minimized binding structures, the binding site for compounds **28** and **56** at the M$_5$ mAcChR is the orthosteric site located near the extracellular end of TM3, TM5, TM6, and TM7. As shown in Figure 6, TM2 and TM4 are also
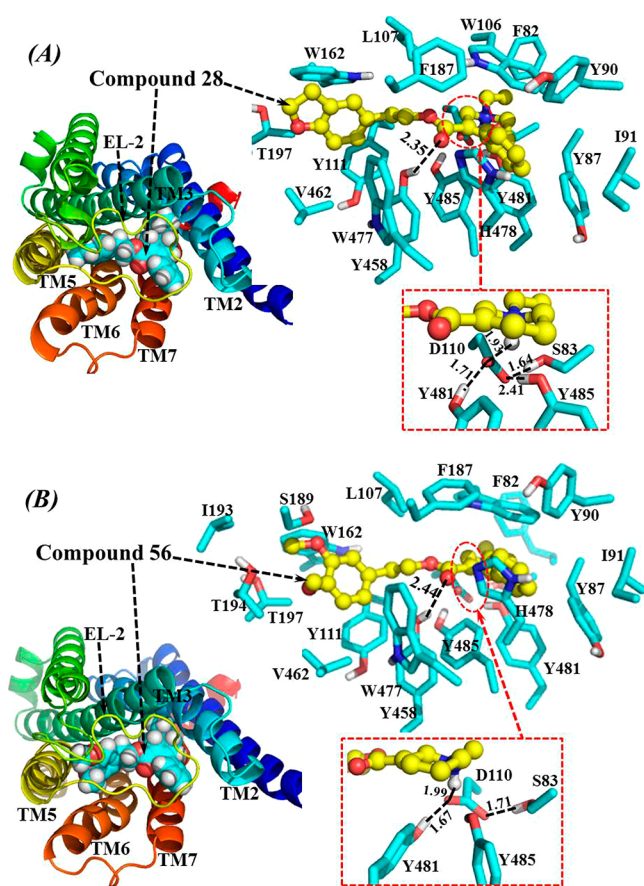
Figure 6. Homology model of human M$_5$ mAcChR based on the X-ray crystal structure of rat M$_3$ mAcChR (PDB entry of 4DAJ at 3.4 Å resolution, A chain). (A) Top view of the energy-minimized binding structure of M$_5$ mAcChR−**28** complex. (B) Top view on the energy-minimized binding structure of M$_5$ mAcChR−**56** complex. The receptor proteins in (A) and (B) are represented as ribbons in rainbow color, and compounds **28** and **56** are shown as spheres (left panel) or ball-and-stick (right panel). Residues within 5 Å of compound **28** and **56** are labeled and shown as sticks. **28** and **56** have very similar hydrogen bonding interactions with the protein, including interactions between the D110 side chain and the cationic heads of the compounds, interactions between the carbonyl oxygen atoms of the compounds and the Y458 side chain, and the interactions among side chains of D110, S83, Y481, and Y485. These hydrogen bonding interactions are shown as dashed lines along with the labeled distances.

partially involved in the formation of the antagonist-binding site. In addition, the antagonist-binding site is partially covered by extracellular loop 2 (EL-2). As depicted in Figure 6A, compound **28** is orientated horizontally inside the binding pocket. The cationic head of compound **28** is anchored around the negatively charged side chain of residue D110 of TM3, interacting

through electrostatic attraction and strong hydrogen bonding. Meanwhile, residue D110 is also hydrogen-bonded with the side chain of S83 from TM2 and with the side chains of Y481 and Y485 from TM7. The cationic head of compound **28** is in close contact with residues W106 and L107 from TM3. The phenyl group on C4 of the tetrahydropyridine ring is closely packed with Y87, Y90, and I91 from TM2, with W106 from TM3, with F187 from EL-2, and with H478 from TM7. The ethyl group at the cationic head of compound **28** makes contact with the aromatic side chain of residue F82 from TM2. The carbonyl oxygen of compound **28** is weakly hydrogen-bonded with the hydroxyl group at the side chain of Y458 from TM6. The tail group (2,3-dihydrobenzofuran-5-ethyl) of compound **28** is packed in parallel with the underneath Y111 from TM3 and packed perpendicularly with the side chain of W162 from TM4. The tail group of compound **28** is also surrounded by T197 from TM5 and by V462 from TM6.

As depicted in Figure 6B, the binding mode of the M$_5$ mAcChR with compound **56** is essentially the same as that with compound **28**. One difference between the binding of **56** and **28** to the M$_5$ mAcChR is that residues I193 and T194 from TM5 are both within 5 Å of compound **56** (Figure 6B), while these two residues have no contacts with compound **28** (Figure 6A). Another difference is the distance of hydrogen bonding formed by the side chain of residue D110 with the cationic headgroup of the antagonist. As shown in Figure 6, the distance between the hydrogen bonding between residue D110 and the cationic head of compound **28** is shorter than that of the hydrogen bonding between residue D110 and the cationic head of compound **56** (1.93 Å vs 1.99 Å, respectively), indicating a stronger bond with **28** than with **56** to the protein. These structural differences, especially the difference in hydrogen bonding distance, may contribute to the difference in binding affinity as represented by the experimentally measured values of K_i (230 nM for **28** vs 2240 nM for **56**) for these two lead compounds (Table 3). The modeled binding structures helped to qualitatively understand the observed SAR and provided clues to design a valuable virtual library of new analogues for computational screening.

■ SUMMARY

Starting from compound **8** as a lead structure, we have identified the first M$_5$-preferring antagonists through a systematic structural modification strategy. The greatest mAcChR selectivity and potency shifts came from the modification of the substituents on the ester group. Replacing the N-Et group on the tetrahydropyridine ring with an N-Me group generally resulted in a significant increase in mAcChR binding affinity while maintaining mAcChR subtype-selectivity profile. This preliminary SAR study provides a basis for further discovery of potent and selective M$_5$ ligands. In addition, we have successfully established a functional assay for M$_5$ receptors using oxotremorine-evoked DA release from superfused rat striatal slices. One of our lead compounds, **56**, demonstrated inhibition of oxotremorine-mediated striatal [3H]DA release, with a maximal inhibition of ∼50%. This result is similar to the effects of M$_5$ knockout on striatal [3H]DA release, providing validation for the current assay. Further, we have constructed a homology model of human M$_5$ based on the newly available crystal structure of the rat M$_3$ receptor. Docking studies performed on compounds **28** and **56** revealed that both possibly interact with the orthosteric binding site on the M$_5$ receptor, which is consistent with the current results from the [3H]NMS binding assay, indicating an orthosteric mechanism of action for these new analogues. We are building and validating homology

models for the other four mAcChR subtypes, and these will be used for virtual library screening. The predictability of these models regarding mAcChR subtype selectivity remains to be tested.

EXPERIMENTAL SECTION

Chemistry. All purchased reagents and solvents were used without further purification unless otherwise noted. All reactions sensitive to air and/or moisture were carried out under argon atmosphere in oven-dried glassware. Flash column chromatography was carried out using $32-63$ μm, 60 Å (230−400 mesh) silica gel. Analytical thin layer chromatography was carried out on glass plates precoated with 250 μm silica gel 60 F_{254}. NMR spectra were recorded in $CDCl_3$ on a Varian 300 or 500 MHz spectrometer, and chemical shifts are reported in ppm relative to tetramethylsilane as the internal standard. Coupling constants are reported in hertz (Hz). Mass spectra were recorded on a JEOL JMS-700T MStation. GC−mass spectra were recorded on an Agilent 6890 GC incorporating an Agilent 7683 autosampler and an Agilent 5973 MSD. Elemental analyses were carried out on a COSTECH elemental combustion system and are within ±0.4% of theory. All final compounds for biological testing were prepared as salts in ≥95% purity, in accord with results from combustion analysis. A detailed description of synthetic methodologies as well as analytical and spectroscopic data for all described compounds is included in the Supporting Information.

Binding Assay. Analogue binding affinities for the five mAcChR subtypes were determined in assays evaluating inhibition of [3H]NMS binding to membranes from CHO-K1 cells expressing one of the recombinant hM_1−hM_5 mAcChRs. The CHO-K1 cell lines expressing the five subtypes of muscarinic receptors were obtained as a gift from Dr. Tom Bonner of National Institute of Mental Health (NIMH). Cells were grown at 37 °C with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 4 mM glutamine, 50 ng/mL Geneticin G418 and 1% Pen−Strep. Cells were harvested at 70−90% confluency. To obtain cell membranes, cells were scraped into ice cold 50 mM Tris-HCl (pH 7.4), sonicated for 30 s, and centrifuged (48000g for 30 min). Pellets were resuspended in 1.5 mL of ice-cold 50 mM Tris-HCl buffer, sonicated for 30 s, and stored at −80 °C until assay. Assays were performed using 96-well plates. Membrane aliquots (100 μL) containing 10−40 μg of protein were added to wells containing 1 nM to100 μM of test compound (25 μL), 0.3 nM [3H]NMS (25 μL, scopolamine methyl chloride [N-methyl-3H], specific activity 82 Ci/mmol; Perkin-Elmer/NEN, Boston, MA), and buffer (50 mM Tris-HCl, pH 7.4, 125 μL) for a total volume of 250 μL. Atropine (1 mM) was used to determine nonspecific binding. Samples were incubated for 120 min at 25 °C with constant agitation. Reactions were terminated by rapid filtration onto GF/B filters using a Filtermate harvester (PerkinElmer Life and Analytical Sciences, Boston, MA). Samples were washed three times with 350 μL of ice-cold 50 mM Tris-HCl buffer and dried for 60 min at 50 °C. Subsequently, 40 μL of MicroScint 20 (PerkinElmer Life and Analytical Sciences, Waltham, MA) was added to each well and radioactivity bound determined using liquid scintillation spectrometry. IC_{50} values were obtained and K_i values calculated using the equation of Cheng and Prusoff.[45]

Inhibition of Oxotremorine-Evoked [3H]DA Release Assay. Assays were performed according to previously published methods[46,47] with minor modifications. Striata were dissected and coronal slices (500 μm, 4−6 mg) obtained with a McIlwain chopper. Slices were incubated for 30 min in Krebs' buffer (in mM: 108 NaCl, 4.7 KCl, 1.2 $MgCl_2$, 1 NaH_2PO_4, 1.3 $CaCl_2$, 11.1 glucose, 25 $NaHCO_3$, 0.11 L-ascorbic acid, and 0.004 disodium EDTA, pH 7.4, saturated with 95% O_2/5% CO_2) in a metabolic shaker at 34 °C. Slices were incubated with 0.1 μM [3H]DA during the latter 30 min of a 60 min incubation period. Each slice was transferred to a superfusion chamber and superfused (0.6 mL/min at 34 °C) for 60 min with Krebs' buffer containing nomifensine (10 μM) and pargyline (10 μM) to inhibit reuptake and prevent metabolism, respectively, ensuring that [3H]-overflow primarily represents [3H]DA rather than [3H]metabolites.[47]

Sample collection began after 60 min of superfusion, when the rate of release was stable. Consecutive 4 min (2.4 mL) samples were collected to determine basal [3H]outflow. Superfusion continued in the absence or presence of a range of analogue concentrations (0.1 nM to 1 mM) for 40 min, followed by 40 min with oxotremorine (100 μM) added to the superfusion buffer. Radioactivity in slices and superfusate samples were determined via liquid scintillation spectroscopy. Data were analyzed by weighted least-squares regression analysis of the sigmoidal concentration−effect curves to obtain IC_{50} values.

Homology Modeling and Molecular Docking. The homology model of human M_5 mAcChR was built based on the X-ray crystal structure of rat M_3 mAcChR (PDB entry of 4DAJ at 3.4 Å resolution, A chain)[44] by using the Protein Modeling module of Discovery Studio (version 2.5.5, Accelrys, Inc., San Diego, CA). The model of human M_5 mAcChR structure was constructed and refined in a very similar manner as in our previous study;[48] i.e., the best sequence alignment was selected based on both the alignment score and the reciprocal positions of the conserved residues among all mAcChR subtypes. The coordinates of the conserved regions were directly transformed from the template structure, while the nonconserved residues were mutated from the template to the corresponding ones in M_5 mAcChR. Structural optimization and energy minimization for the M_5 mAcChR structue were performed using the Amber 11 program suite. The convergence criterion for the energy minization was set to 0.001 kcal mol^{-1} Å^{-1}. On the basis of the optimized M_5 mAcChR structure, the binding mode of the receptor with two lead compounds **28** and **56** was explored through molecular docking using the AutoDock 3.0.5 program.[48] This molecular docking approach was similar to that described in our previous study.[49] A reasonable binding structure of M_5 mAcChR in complex with either compound **28** or **56** was obtained after energy minimization on each complex structure.

ASSOCIATED CONTENT

Ⓢ Supporting Information

Synthetic procedures and analytical data of all compounds prepared. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

VTA, ventral tegmental area; NMS, N-methylscopolamine; CHO, Chinese hamster ovary; TM, transmembrane; EL, extracellular loop

REFERENCES

(1) Caulfield, M. P. Muscarinic receptors—characterization, coupling and function. *Pharmacol. Ther.* **1993**, *58*, 319−379.

(2) Caulfield, M. P.; Birdsall, N. J. International Union of Pharmacology. XVII. Classification of muscarinic acetylcholine receptors. *Pharmacol. Rev.* **1998**, *50*, 279−290.

(3) Wess, J. Muscarinic acetylcholine receptor knockout mice: novel phenotypes and clinical implications. *Annu. Rev. Pharmacol. Toxicol.* **2004**, *44*, 423−450.

(4) Wess, J.; Eglen, R. M.; Gautam, D. Muscarinic acetylcholine receptors: mutant mice provide new insights for drug development. *Nat. Rev. Drug Discovery* 2007, 6, 721–733.

(5) Bonner, T. I.; Young, A. C.; Brann, M. R.; Buckley, N. J. Cloning and expression of the human and rat M$_5$ muscarinic acetylcholine-receptor genes. *Neuron* 1988, 1, 403–410.

(6) Raffa, R. B. The M$_5$ muscarinic receptor as possible target for treatment of drug abuse. *J. Clin. Pharm. Ther.* 2009, 34, 623–629.

(7) Di Chiara, G.; Imperato, A. Drugs abused by humans preferentially increase synaptic dopamine concentrations in the mesolimbic system of freely moving rats. *Proc. Natl. Acad. Sci. U.S.A.* 1988, 85, 5274–5278.

(8) Koob, G. F.; Swerdlow, N. R. The functional output of the mesolimbic dopamine system. *Ann. N.Y. Acad. Sci.* 1988, 537, 216–227.

(9) Koob, G. F.; Nestler, E. J. The neurobiology of drug addiction. *J. Neuropsychiatry Clin. Neurosci.* 1997, 9, 482–497.

(10) Cami, J.; Farre, M. Drug addiction. *N. Engl. J. Med.* 2003, 349, 975–986.

(11) Vilaro, M. T.; Palacios, J. M.; Mengod, G. Localization of M$_5$ muscarinic receptor messenger-RNA in rat-brain examined by in situ hybridization histochemistry. *Neurosci. Lett.* 1990, 114, 154–159.

(12) Yasuda, R. P.; Ciesla, W.; Flores, L. R.; Wall, S. J.; Li, M.; Satkus, S. A.; Weisstein, J. S.; Spagnola, B. V.; Wolfe, B. B. Development of antisera selective for M$_4$ and M$_5$ muscarinic cholinergic receptors—distribution of M$_4$ and M$_5$ receptors in rat-brain. *Mol. Pharmacol.* 1993, 43, 149–157.

(13) Weiner, D. M.; Brann, M. R. Distribution of M$_1$–M$_5$ muscarinic receptor messenger-RNAs in rat-brain. *Trends Pharmacol. Sci.* 1989, 115–115.

(14) Reever, C. M.; FerrariDiLeo, G.; Flynn, D. D. The M$_5$ (m5) receptor subtype: fact or fiction? *Life Sci.* 1997, 60, 1105–1112.

(15) Yeomans, J. S.; Takeuchi, J.; Baptista, M.; Flynn, D. D.; Lepik, K.; Nobrega, J.; Fulton, J.; Ralph, M. R. Brain-stimulation reward thresholds raised by an antisense oligonucleotide for the M$_5$ muscarinic receptor infused near dopamine cells. *J. Neurosci.* 2000, 20, 8861–8867.

(16) Miller, A. D.; Blaha, C. D. Midbrain muscarinic receptor mechanisms underlying regulation of mesoaccumbens and nigrostriatal dopaminergic transmission in the rat. *Eur. J. Neurosci.* 2005, 21, 1837–1846.

(17) Lester, D. B.; Miller, A. D.; Blaha, C. D. Muscarinic receptor blockade in the ventral tegmental area attenuates cocaine enhancement of laterodorsal tegmentum stimulation-evoked accumbens dopamine efflux in the mouse. *Synapse* 2010, 64, 216–223.

(18) Yamada, M.; Lamping, K. G.; Duttaroy, A.; Zhang, W. L.; Cui, Y. H.; Bymaster, F. P.; McKinzie, D. L.; Felder, C. C.; Deng, C. X.; Faraci, F. M.; Wess, J. Cholinergic dilation of cerebral blood vessels is abolished in M$_5$ muscarinic acetylcholine receptor knockout mice. *Proc. Natl. Acad. Sci. U.S.A.* 2001, 98, 14096–14101.

(19) Basile, A. S.; Fedorova, I.; Zapata, A.; Liu, X.; Shippenberg, T.; Duttaroy, A.; Yamada, M.; Wess, J. Deletion of the M$_5$ muscarinic acetylcholine receptor attenuates morphine reinforcement and withdrawal but not morphine analgesia. *Proc. Natl. Acad. Sci. U.S.A.* 2002, 99, 11452–11457.

(20) Fink-Jensen, A.; Sorensen, L.; Bay-Richter, C.; Frikke-Schmidt, H.; Wess, J.; Woldbye, D. P.; Wortwein, G. Involvement of the M$_5$ muscarinic receptor in cocaine and amphetamine induced behaviour: studies in M$_5$ knockout mice backcrossed to the C57BL/6NTac strain. *Schizophr. Res.* 2006, 81, 301–302.

(21) Thomsen, M.; Woldbye, D. P. D.; Wortwein, G.; Fink-Jensen, A.; Wess, J.; Caine, S. B. Reduced cocaine self-administration in muscarinic M-5 acetylcholine receptor-deficient mice. *J. Neurosci.* 2005, 25, 8141–8149.

(22) Rezayof, A.; Nazari-Serenjeh, F.; Zarrindast, M. R.; Sepehri, H.; Delphi, L. Morphine-induced place preference: involvement of cholinergic receptors of the ventral tegmental area. *Eur. J. Pharmacol.* 2007, 562, 92–102.

(23) Yamada, M.; Basile, A. S.; Fedorova, I.; Zhang, W. L.; Duttaroy, A.; Cui, Y. H.; Lamping, K. G.; Faraci, F. M.; Deng, C. X.; Wess, J. Novel insights into M-5 muscarinic acetylcholine receptor function by the use of gene targeting technology. *Life Sci.* 2003, 74, 345–353.

(24) Bridges, T. M.; Marlo, J. E.; Niswender, C. M.; Jones, C. K.; Jadhav, S. B.; Gentry, P. R.; Plumley, H. C.; Weaver, C. D.; Conn, P. J.; Lindsley, C. W. Discovery of the first highly M$_5$-preferring muscarinic acetylcholine receptor ligand, an M$_5$ positive allosteric modulator derived from a series of 5-trifluoromethoxy N-benzyl isatins. *J. Med. Chem.* 2009, 52, 3445–3448.

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

(26) Bridges, T. M.; Kennedy, J. P.; Hopkins, C. R.; Conn, P. J.; Lindsley, C. W. Heterobiaryl and heterobiaryl ether derived M$_5$ positive allosteric modulators. *Bioorg. Med. Chem. Lett.* 2010, 20, 5617–5622.

(27) Liu, J.; Schoneberg, T.; van Rhee, M.; Wess, J. Mutational analysis of the relative orientation of transmembrane helices I and VII in G protein-coupled receptors. *J. Biol. Chem.* 1995, 270, 19532–19539.

(28) Bohme, T. M.; Keim, C.; Dannhardt, G.; Mutschler, E.; Lambrecht, G. Design and pharmacology of quinuclidine derivatives as M$_2$-selective muscarinic receptor ligands. *Bioorg. Med. Chem. Lett.* 2001, 11, 1241–1243.

(29) Bohme, T. M.; Augelli-Szafran, C. E.; Hallak, H.; Pugsley, T.; Serpa, K.; Schwarz, R. D. Synthesis and pharmacology of benzoxazines as highly selective antagonists at M(4) muscarinic receptors. *J. Med. Chem.* 2002, 45, 3094–3102.

(30) Choppin, A.; Stepan, G. J.; Loury, D. N.; Watson, N.; Eglen, R. M. Characterization of the muscarinic receptor in isolated uterus of sham operated and ovariectomized rats. *Br. J. Pharmacol.* 1999, 127, 1551–1558.

(31) Wang, Y.; Chackalamannil, S.; Hu, Z.; Greenlee, W. J.; Clader, J.; Boyle, C. D.; Kaminski, J. J.; Billard, W.; Binch, H., 3rd; Crosby, G.; Ruperto, V.; Duffy, R. A.; Cohen-Williams, M.; Coffin, V. L.; Cox, K. A.; Grotz, D. E.; Lachowicz, J. E. Improving the oral efficacy of CNS drug candidates: discovery of highly orally efficacious piperidinyl piperidine M$_2$ muscarinic receptor antagonists. *J. Med. Chem.* 2002, 45, 5415–5418.

(32) Sagara, Y.; Sagara, T.; Uchiyama, M.; Otsuki, S.; Kimura, T.; Fujikawa, T.; Noguchi, K.; Ohtake, N. Identification of a novel 4-aminomethylpiperidine class of M$_3$ muscarinic receptor antagonists and structural insight into their M$_3$ selectivity. *J. Med. Chem.* 2006, 49, 5653–5663.

(33) Augelli-Szafran, C. E.; Blankley, C. J.; Jaen, J. C.; Moreland, D. W.; Nelson, C. B.; Penvose-Yi, J. R.; Schwarz, R. D.; Thomas, A. J. Identification and characterization of m1 selective muscarinic receptor antagonists. *J. Med. Chem.* 1999, 42, 356–363.

(34) Solymar, M. F., E.; Fulop, F. Enzyme-catalyzed kinetic resolution of piperidine hydroxy esters. *Tetrahedron: Asymmetry* 2004, 15, 3281–3287.

(35) Inokuchi, E.; Narumi, T.; Niida, A.; Kobayashi, K.; Tomita, K.; Oishi, S.; Ohno, H.; Fujii, N. Efficient synthesis of trifluoromethyl and related trisubstituted alkene dipeptide isosteres by palladium-catalyzed carbonylation of amino acid derived allylic carbonates. *J. Org. Chem.* 2008, 73, 3942–3945.

(36) Anagnostaras, S. G.; Murphy, G. G.; Hamilton, S. E.; Mitchell, S. L.; Rahnama, N. P.; Nathanson, N. M.; Silva, A. J. Selective cognitive dysfunction in acetylcholine M1 muscarinic receptor mutant mice. *Nat. Neurosci.* 2003, 6, 51–58.

(37) Gerber, D. J.; Sotnikova, T. D.; Gainetdinov, R. R.; Huang, S. Y.; Caron, M. G.; Tonegawa, S. Hyperactivity, elevated dopaminergic transmission, and response to amphetamine in M$_1$ muscarinic acetylcholine receptor-deficient mice. *Proc. Natl. Acad. Sci. U.S.A.* 2001, 98, 15312–15317.

(38) Naito, R.; Yonetoku, Y.; Okamoto, Y.; Toyoshima, A.; Ikeda, K.; Takeuchi, M. Synthesis and antimuscarinic properties of quinuclidin-3-yl 1,2,3,4-tetrahydroisoquinoline-2-carboxylate derivatives as novel muscarinic receptor antagonists. *J. Med. Chem.* **2005**, *48*, 6597−6606.

(39) Choppin, A.; Eglen, R. M.; Hegde, S. S. Pharmacological characterization of muscarinic receptors in rabbit isolated iris sphincter muscle and urinary bladder smooth muscle. *Br. J. Pharmacol.* **1998**, *124*, 883−888.

(40) McCombie, S. W.; Lin, S. I.; Tagat, J. R.; Nazareno, D.; Vice, S.; Ford, J.; Asberom, T.; Leone, D.; Kozlowski, J. A.; Zhou, G.; Ruperto, V. B.; Duffy, R. A.; Lachowicz, J. E. Synthesis and structure−activity relationships of M(2)-selective muscarinic receptor ligands in the 1-[4-(4-arylsulfonyl)-phenylmethyl]-4-(4-piperidinyl)-piperazine family. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 795−798.

(41) Watson, N.; Daniels, D. V.; Ford, A. P. D. W.; Eglen, R. M.; Hegde, S. S. Comparative pharmacology of human muscarinic M_3 and M_5 cholino-ceptors expressed in Chinese hamster ovary (CHO) cells. *Br. J. Pharmacol.* **1998**, *123*, U153−U153.

(42) Pulido-Rios, M. T.; Steinfeld, T.; Armstrong, S.; Watson, N.; Choppin, A.; Eglen, R.; Hegde, S. S. In vitro isolated tissue functional muscarinic receptor assays. *Curr. Protoc. Pharmacol.* **2010**, *48*, 4.15.1−4.15.29.

(43) Zhang, W.; Yamada, M.; Gomeza, J.; Basile, A. S.; Wess, J. Multiple muscarinic acetylcholine receptor subtypes modulate striatal dopamine release, as studied with M_1−M_5 muscarinic receptor knock-out mice. *J. Neurosci.* **2002**, *22*, 6347−6352.

(44) Kruse, A. C.; Hu, J.; Pan, A. C.; Arlow, D. H.; Rosenbaum, D. M.; Rosemond, E.; Green, H. F.; Liu, T.; Chae, P. S.; Dror, R. O.; Shaw, D. E.; Weis, W. I.; Wess, J.; Kobilka, B. K. Structure and dynamics of the M3 muscarinic acetylcholine receptor. *Nature* **2012**, *482*, 552−556.

(45) Cheng, Y.; Prusoff, W. H. Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50% inhibition (I_{50}) of an enzymatic reaction. *Biochem. Pharmacol.* **1973**, *22*, 3099−3108.

(46) Dwoskin, L. P.; Zahniser, N. R. Robust modulation of [^{3}H]dopamine release from rat striatal slices by D-2 dopamine receptors. *J. Pharmacol. Exp. Ther.* **1986**, *239*, 442−453.

(47) Zumstein, A.; Karduck, W.; Starke, K. Pathways of dopamine metabolism in the rabbit caudate nucleus in vitro. *Naunyn. Schmiedeberg's Arch. Pharmacol.* **1981**, *316*, 205−217.

(48) Huang, X.; Zheng, G.; Zhan, C. G. Microscopic binding of M_5 muscarinic acetylcholine receptor with antagonists by homology modeling, molecular docking, and molecular dynamics simulation. *J. Phys. Chem. B* **2012**, *116*, 532−541.

(49) Morris, G. M. G., D. S.; Halliday, R. S.; Huey, R.; Hart, W. E.; Belew, R. K.; Olson, A. J. Automated docking using a lamarckian genetic algorithm and empirical binding free energy function. *J. Comput. Chem.* **1998**, *19*, 1639−1662.