

Synthesis and Pharmacological Characterization of Novel Tetrahydropyridine-Based Muscarinic M₅ Receptor Antagonists to Investigate the Difference in the SARs of Muscarinic Receptor Subtypes Utilizing Induced Fit Docking Studies

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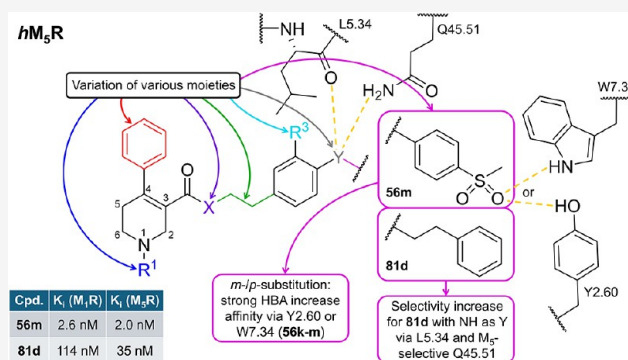
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Supporting Information

ABSTRACT: Many studies and experiments suggest the muscarinic M₅ receptor (M₅R) as a potential target for the clinical treatment of drug abuse, schizophrenia, or Alzheimer's disease. In the present study, a library containing 39 novel hM₅R antagonists based on an arecaidine (tetrahydropyridine) core was synthesized, pharmacologically characterized in radioligand competition binding or mini-G protein assays and analyzed in molecular docking studies to elucidate their binding modes and in vitro characteristics. Compound **56m** showed low single-digit nanomolar affinity on hM₅R combined with a 17-fold selectivity over hM₂R, while compound **81d** displayed good affinity on hM₅R with a 3- to 12-fold selectivity over the other subtypes. Induced fit docking studies suggest the conserved Y2.60 and hM₅R-unique Q45.51 as the responsible amino acids regarding affinity and selectivity.

KEYWORDS: muscarinic receptors, MSR, induced fit docking, tetrahydropyridine, SAR



INTRODUCTION

Drug discovery has taken a lot of inspiration from nature; many approved drugs are directly found in nature or derived from compounds present in plants, animals, or fungi.^{1–4} Examples are scopolamine and atropine, tropane alkaloids, found in *atropa bella-donna*, which all are still used in modern medicine.^{5,6} Among the ligands for the muscarinic receptors, a wide range of natural compounds and their derivatives are known. Tropane alkaloids like scopolamine and atropine served as lead compounds for the launched drugs tiotropium and ipratropium bromide. Both antagonize muscarinic receptor activity and are essential for the treatment of chronic obstructive pulmonary disease (COPD) and asthma.^{7,8} Among the agonists for the muscarinic receptor family, the fungus toxin muscarine found in *amanita muscaria* and arecoline found in *areca catechu* are the most prominent ones.^{2,3,9–15} Xanomeline, which has a similar core structure as arecoline, is an unselective muscarinic receptor agonist and approved to treat schizophrenia in combination with the tropane alkaloid tropism chloride (Figure 1A).^{16–19} Examples of launched muscarinic receptor antagonists are pirenzepine, darifenacin, and umecclidinium bromide, which are used in the treatment of ulcers, overactive bladder, and COPD, respectively.^{7,20,21}

The muscarinic receptor family M_xR consists of five subtypes 1–5 and is a widely known and popular target class for drugs due

to their ubiquitous expression throughout the body and their various physiological effects.^{22–27} Muscarinic receptors belong to the class A G protein-coupled receptors (GPCRs).²⁸ M₁R, M₃R, and M₅R are mainly coupled to the G_q protein, which stimulates the PLC-β pathway upon receptor activation and downstream increases intracellular calcium levels in the cytoplasm.^{22–25,29–31} In contrast to that, M₂R and M₄R mostly couple with the G_i protein, which leads upon stimulation to deactivation of adenylyl cyclase and therefore decrease the activity of cAMP-dependent protein kinase.^{23–25,30,31} Out of all five subtypes, M₅R is the least studied one with no approved drug on the market yet.^{25,27} Additionally, its crystal structure was resolved quite late in 2019, while the structures of the other subtypes were resolved 2016 or earlier.^{32–35} Histologically, M₅R is mainly found within the central nervous system, with the substantia nigra pars compacta and ventral tegmental area having the highest prevalence. This makes it the only muscarinic receptor subtype detectable in the midbrain, where it modulates

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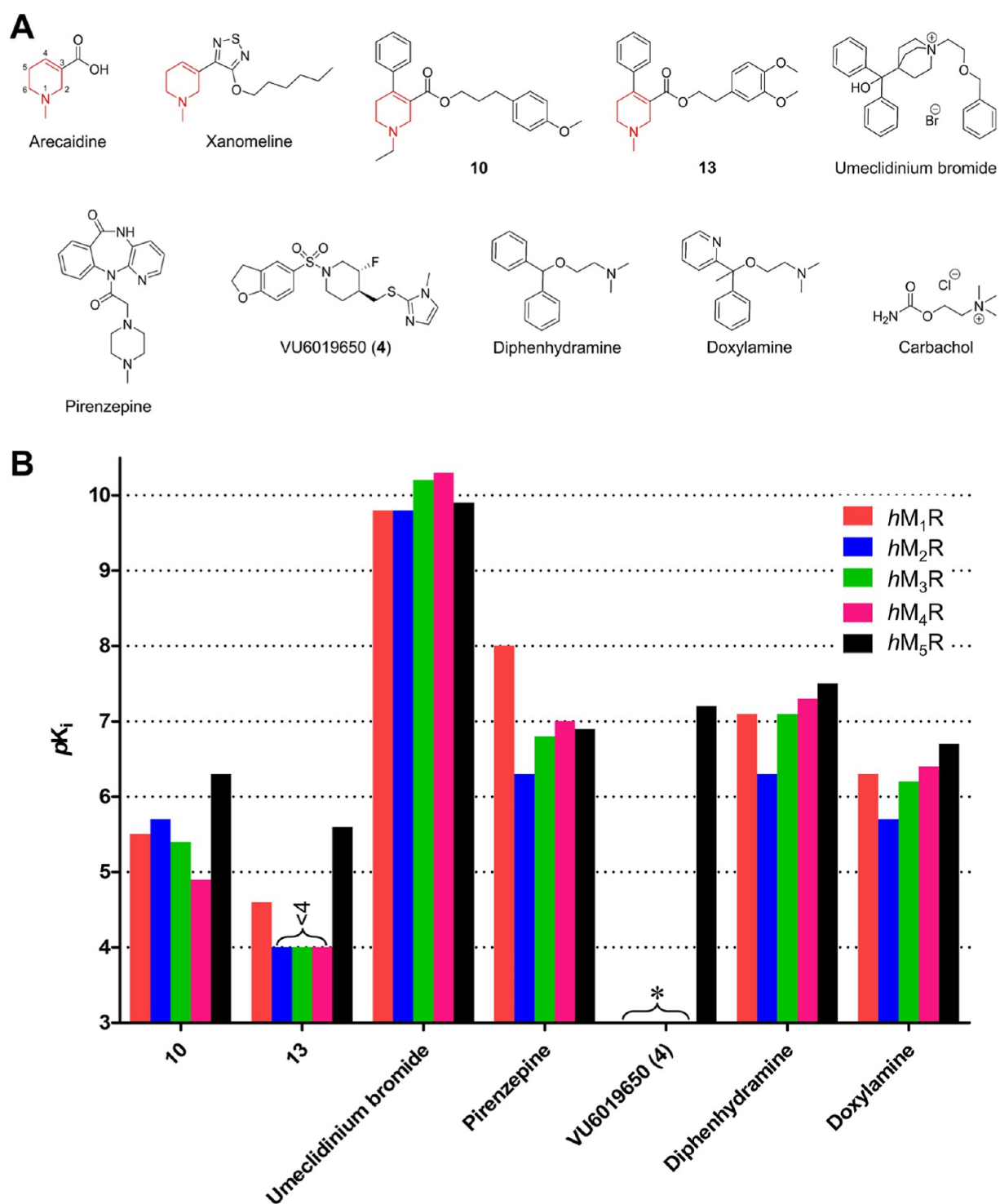


Figure 1. A: Structures of arecaidine, xanomeline, 10, 13, umecleidinium bromide, pirenzepine, VU6019650 (4), diphenhydramine, doxylamine, and carbachol. B: Reported radioligand binding affinities of the antagonists 10, 13, umecleidinium bromide, pirenzepine, VU6019650 (4), diphenhydramine, and doxylamine displayed in grouped columns. *No radioligand binding affinity values have been published in the literature for cpd. 4 on $hM_{1-4}R$. Published functional IC_{50} values: hM_1R = 4384 nM and hM_5R = 36 nM.⁷¹

the dopamine signaling of dopaminergic neurons.^{24,29,30,36–41} In various studies with KO-mice or previously reported modulators of the receptor, M_3R influences cerebral blood flow, mice memory, and the dopamine-dependent reward cycle produced by addictive substances.^{38,40,42–48} Based on these findings, M_3R holds potential for an application in the treatment of drug addictions, schizophrenia, or possibly Alzheimer's dis-

ease.^{3,10,26,30,40,49–55} Worldwide, roughly 64 million people were diagnosed with a substance use disorder and about 55 million people were living with dementia in 2022; both numbers are predicted to rise in the future.^{56–58} During the treatment of substance use disorders, patients often struggle with intense withdrawal symptoms, which lead to increased relapses and treatment discontinuation.⁵⁸ Providing a tool or rather a drug to

A	TM2												TM3										TM4	ECL2			TM5												
Pocket	-	-	-	-	-	-	-	a	-	-	a	a	-	-	o	o	-	-	o	o	-	-	o	-	o	-	a	a	-	-	-	-	a	-					
BW	2.52	2.53	2.54	2.55	2.56	2.57	2.58	2.59	2.60	2.61	2.62	2.63	3.29	3.30	3.31	3.32	3.33	3.34	3.35	3.36	3.37	3.38	3.39	3.40	4.56	4.57	45.50	45.51	45.52	45.53	45.54	5.34	5.35	5.36	5.37	5.38	5.39	5.40	5.41
hM ₁ R	I	I	G	T	S	M	N	L	Y	T	T	Y	L	A	L	D	Y	V	A	S	N	A	S	V	L	W	C	Y	I	Q	F	L	S	Q	P	I	I	T	F
hM ₂ R	I	I	G	V	S	M	N	L	Y	T	L	Y	L	A	L	D	Y	V	V	S	N	A	S	V	L	W	C	Y	I	Q	F	F	S	N	A	A	V	T	F
hM ₃ R	I	I	G	V	S	M	N	L	F	T	T	Y	L	A	I	D	Y	V	A	S	N	A	S	V	L	W	C	F	I	Q	F	L	S	E	P	T	I	T	F
hM ₄ R	I	I	G	A	S	M	N	L	Y	T	V	Y	L	A	L	D	Y	V	V	S	N	A	S	V	L	W	C	F	I	Q	F	L	S	N	P	A	V	T	F
hM ₅ R	I	I	G	I	S	M	N	L	Y	T	T	Y	L	A	L	D	Y	V	A	S	N	A	S	V	L	W	C	Q	I	Q	F	L	S	E	P	T	I	T	F
	TM5						TM6								ECL3				TM7																				
Pocket	-	oa	oa	-	-	o	-	-	o	-	-	o	o	-	-	oa	-	-	a	-	-	a	-	-	a	-	-	a	a	-	-	o	-	-	o	o	-	-	-
BW	5.42	5.43	5.44	5.45	5.46	5.461	5.47	6.47	6.48	6.49	6.50	6.51	6.52	6.53	6.54	6.55	6.56	6.57	6.58	6.59	67.48	67.49	67.50	67.51	7.31	7.32	7.33	7.34	7.35	7.36	7.37	7.38	7.39	7.40	7.41	7.42	7.43	7.45	7.46
hM ₁ R	G	T	A	M	A	A	F	T	W	T	P	Y	N	I	M	V	L	V	S	T	K	D	C	V	E	T	L	W	E	L	G	Y	W	L	C	Y	V	N	S
hM ₂ R	G	T	A	I	A	A	F	T	W	A	P	Y	N	V	M	V	L	V	N	T	A	P	C	I	N	T	V	W	T	I	G	Y	W	L	C	Y	I	N	S
hM ₃ R	G	T	A	I	A	A	F	T	W	T	P	Y	N	I	M	V	L	V	N	T	D	S	C	I	K	T	F	W	N	L	G	Y	W	L	C	Y	I	N	S
hM ₄ R	G	T	A	I	A	A	F	T	W	T	P	Y	N	V	M	V	L	V	N	T	Q	S	C	I	D	T	V	W	S	I	G	Y	W	L	C	Y	V	N	S
hM ₅ R	G	T	A	I	A	A	F	T	W	T	P	Y	N	I	M	V	L	V	S	T	D	K	C	V	V	T	L	W	H	L	G	Y	W	L	C	Y	V	N	S

B

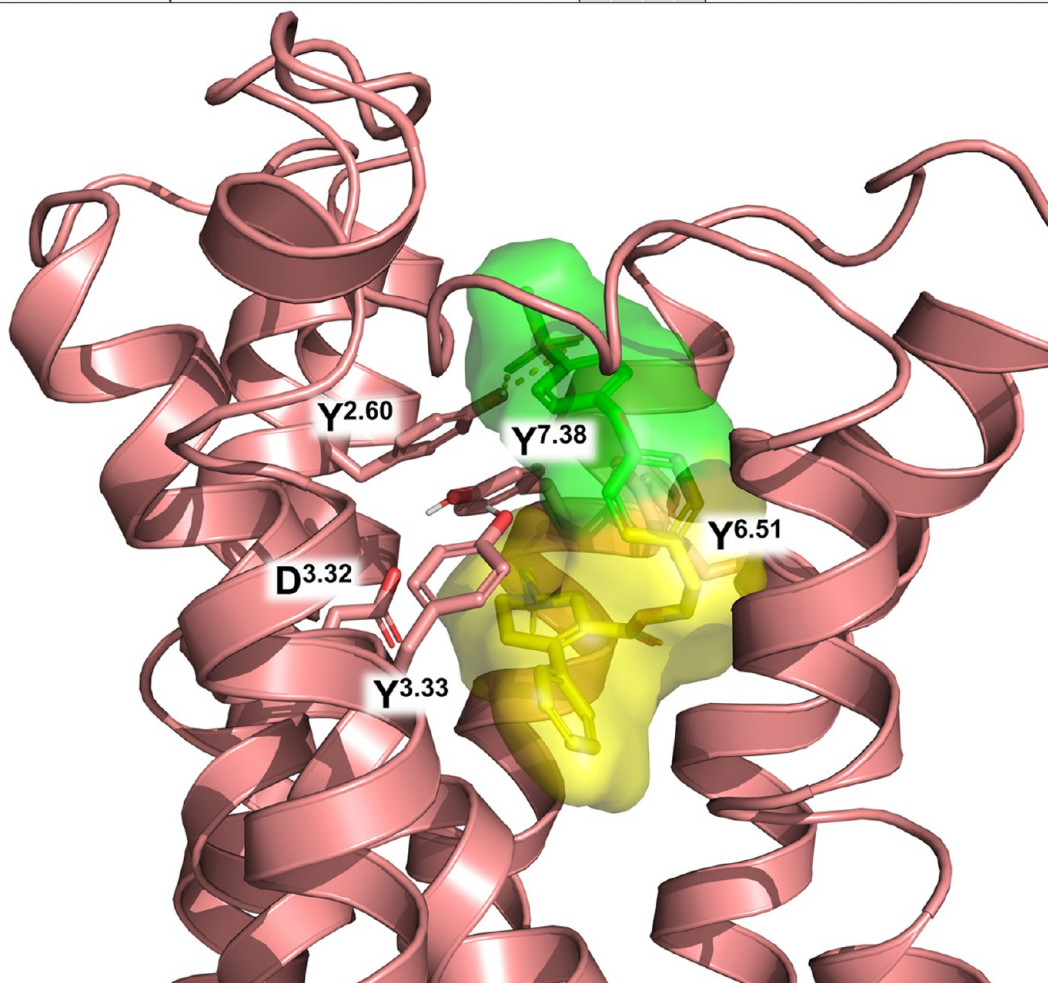
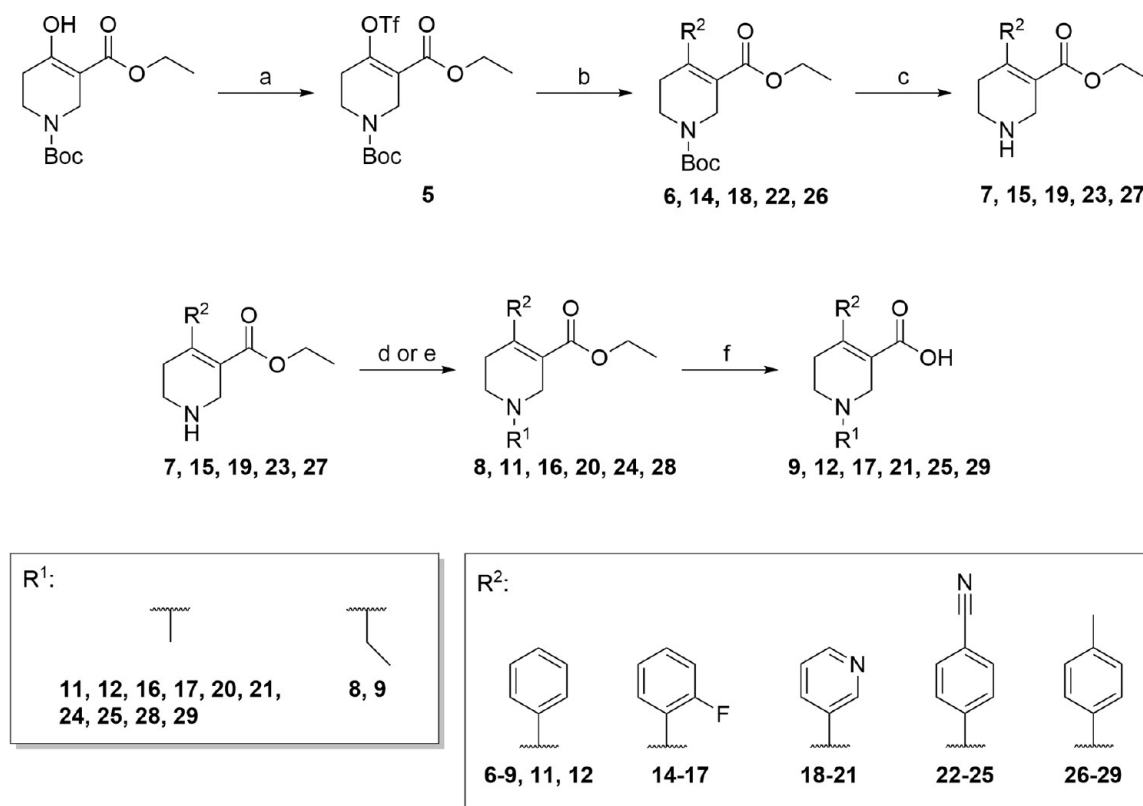


Figure 2. A: Amino acid alignment with generic Ballesteros–Weinstein numbering for potential orthosteric (o, yellow) and allosteric (a, green) binding sites of all five M_xR with all differences in amino acids highlighted in red.⁶³ The allocation of the amino acids to the respective pockets was based on exemplary induced fit docking poses. B: Exemplary docking pose of **56m** on hM_5R with crucial amino acids displayed and the orthosteric binding site highlighted in yellow and the allosteric binding site in green. The amino acids were numbered according to the generic Ballesteros–Weinstein nomenclature obtained from the GPCR database (www.gpcrdb.org), see Figure S137 in the Supporting Information.^{63,95}

Scheme 1. Synthesis of the Modified Arecaidine Core Structure^a

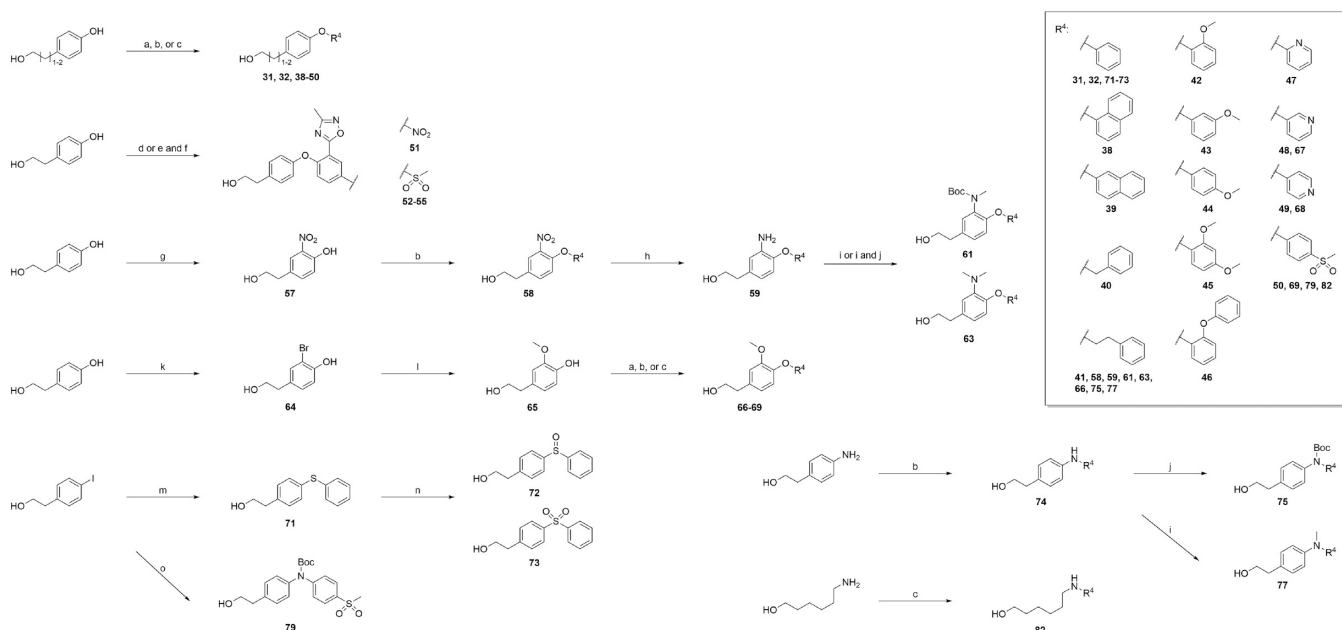
^aReagents and conditions: a) NaH, PhNTf₂, THF, 0 °C to rt, 24 h; b) R²-B(OH)₂, Pd(PPh₃)₄, 2 M aq. Na₂CO₃, THF, 65 °C, 2–4 h; c) TFA, DCM, 0 °C to rt, 4–18 h; d) Paraformaldehyde, NaCNBH₃, EtOH, rt, 18 h; e) Ethyl iodide, dry K₂CO₃, EtOH, 0 °C to rt, 26 h; f) 10% aq. KOH, EtOH, rt, 18 h; For more details and yields see the [Supporting Information](#).

lessen such a burden on the patient would be clinically valuable and improve the efficacy of the treatment greatly. For the treatment of Alzheimer's disease, several drugs, such as acetylcholinesterase inhibitors (rivastigmine, galantamine, and donepezil) and a NMDA receptor antagonist like memantine, have been established in clinical use.^{59,60} However, the clinical benefit of these drugs is rather small and only delays disease progression.⁶¹

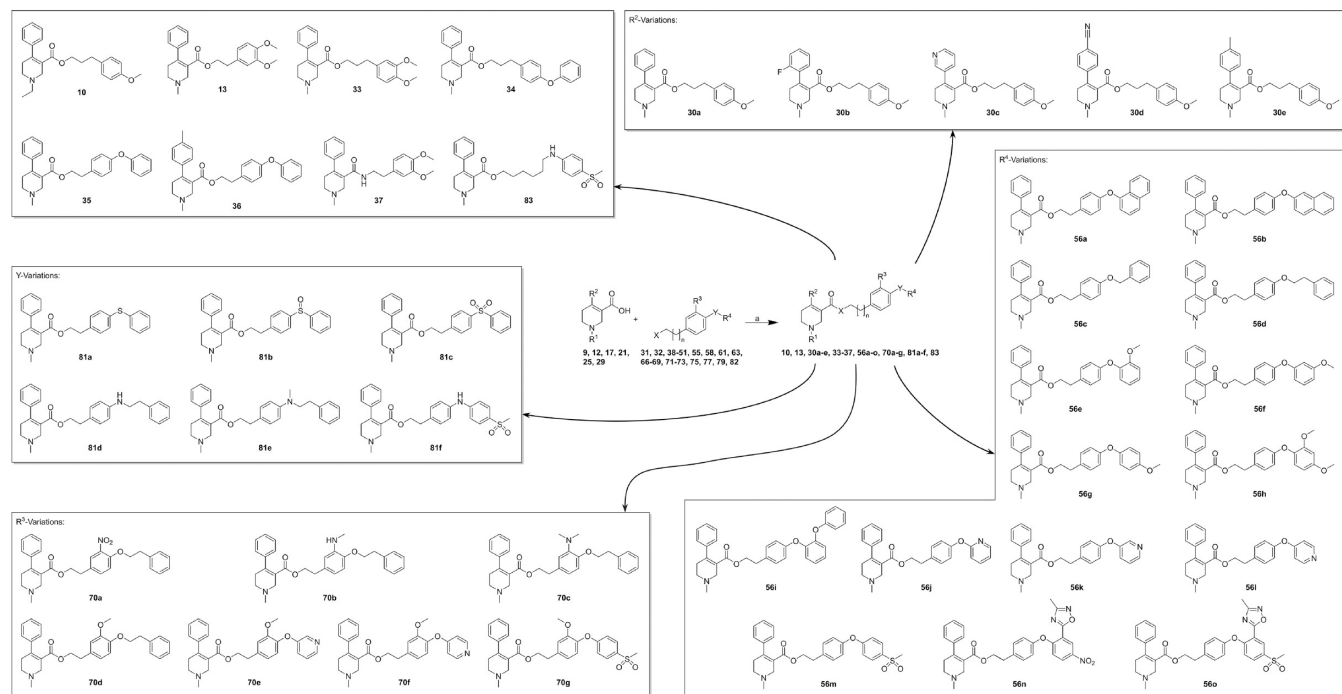
For the clinical evaluation of M₅R as a therapeutic target for the treatment of drug addiction or Alzheimer's disease, a truly selective and affine M₅R antagonist is highly required as a tool compound. The muscarinic receptor family is known to be a challenging target family in terms of selectivity among its subtypes.^{27,53,62} Most commercially available drugs targeting M_xR are not truly selective and bear a concomitant affinity toward at least one other subtype (Figure 1A,B). This is due to their high overall homology of about 50% depending on the subtype and up to 100% for the orthosteric binding site (Figure 2A).^{63–65} Especially the orthosteric binding sites barely differ in relevant amino acids, therefore making addressing the allosteric binding sites even more attractive (Figure 2A).^{54,66} These differences in amino acids have been utilized to develop positive and negative allosteric modulators for M₅R and subsequently lead to the development of potential antagonists like compound VU6019650 (4, Figure 1A).^{46,50,54,67–73} To examine and elucidate the physiological effect of M₅R, an orthosteric antagonist would be more suited, because it decreases receptor activation without depending on the endogenous ligand acetylcholine (ACh). However, due to the difficulty presented

by the high orthosteric homology, developing a bitopic ligand to address allosteric sites for selectivity and orthosteric sites for affinity became a promising approach (Figure 2B).^{49,74–77} A known unselective agonist for the muscarinic receptors is xanomeline, which contains a *N*-methylated 1,2,5,6-tetrahydropyridine ring like the naturally occurring M_xR agonist arecaidine (Figure 1A).^{3,9,11–13,16,17} The introduction of a bulky moiety at the 4-position of this tetrahydropyridine core turns the agonist into an antagonist on M_xR.⁷⁸

Reported M₅R antagonists 10 and 13 by Zheng et al., based on the substituted tetrahydropyridine and naturally occurring tyrosol, showed promising results in terms of selectivity and physiological affinity (Figure 1A,B).^{78,79} Starting from compounds 10 and 13, various moieties were altered and pharmacologically characterized regarding their influence on affinity, selectivity, and activity at M₁R and M₅R using radioligand competition binding and mini-G protein assays. To obtain a full selectivity profile, the most promising compounds were screened on all M_xR subtypes for affinity and on other exemplary aminergic receptors for activity. Additionally, induced fit docking studies were chosen to elucidate the binding mode of the compounds and identify crucial amino acids, relevant for binding and selectivity at hM₁R and hM₅R.^{80–90} With the data obtained from all those experiments, the structure activity and affinity relationships (SARs) were discussed to obtain a larger understanding of the binding mode and its influences on affinity and selectivity and how the tetrahydropyridine-based compounds could be used or further optimized in the future.

Scheme 2. Synthesis Pathways of the Modified Tyrosol Side Chains^a

^aReagents and conditions: a) I-Ar, CuI, picolinic acid, K₃PO₄, DMSO, argon, 80–90 °C, 18 h; b) Br-R⁴, dry K₂CO₃, MeCN, reflux, 18–72 h; c) F-R⁴, dry K₂CO₃, DMF, 120 °C, 18 h; d) (i) F-R⁴, dry K₂CO₃, DMF, 80 °C, 18 h; (ii) acetamidine x HCl, HATU, DIPEA, DMF, rt, 8 h; (iii) H₂NOH x HCl, AcOH, DMF, 80–90 °C, 18 h; e) F-R⁴, dry K₂CO₃, DMF, 80 °C, 18 h; f) (i) acetamidine x HCl, HATU, DIPEA, DMF, rt, 8 h; (ii) H₂NOH x HCl, AcOH, DMF, 80–90 °C, 18 h; g) AcOH, HNO₃, 0 °C to rt, 1 h; h) Fe, 50% aq. AcOH, EA, 50 °C, 1–2 h; (i) Paraformaldehyde, NaCNBH₃, AcOH, EtOH, rt, 18 h; j) Boc₂O, THF, rt, 18 h; k) Br₂, NaHCO₃, CHCl₃, 0 °C, 3–4 h; l) CuBr, DMF, 25% NaOMe in MeOH, 120 °C, 18–72 h; m) HS-Ph, CuI, picolinic acid, K₃PO₄, DMSO, argon, 80–90 °C, 18 h; n) NFSI, H₂O, rt, 18 h; o) BocNH-R⁴, CuI, picolinic acid, K₃PO₄, DMSO, argon, 90 °C, 21 h. For more details and yields see the [Supporting Information](#).

Scheme 3. Synthesis of the Target Compounds 10, 13, 30a–e, 33–37, 56a–o, 70a–g, 81a–f, and 83^a

^aReagents and conditions: a) respective alcohol or amine side chain, EDC, DMAP, DMF, DCM, rt, 18 h. For more details and yields, see the [Supporting Information](#).

Table 1. Binding Data of the Compounds 10, 13, 30a–e, 33–37, 56a–o, 70a–g, 81a–f, and 83 on Human Muscarinic M₁ and M₅ Receptors^a

Cpd.	n	R ¹	R ²	R ³	R ⁴	X	Y	K _i [nM] ± SEM [N]		<i>h</i> M ₅ R selectivity K _i (<i>h</i> M ₁ R)/ K _i (<i>h</i> M ₅ R)
								<i>h</i> M ₁ R	<i>h</i> M ₅ R	
10*	2	Et		H	Me	O	O	75 ± 14 [4]	446 ± 38 [3]	0.2
13*	1	Me		OMe	Me	O	O	4709 ± 778 [4]	3505 ± 1135 [4]	1.3
30a	2	Me		H	Me	O	O	70 ± 5 [3]	294 ± 105 [3]	0.2
30b	2	Me		H	Me	O	O	3497 ± 895 [3]	5179 ± 231 [3]	0.7
30c	2	Me		H	Me	O	O	3106 ± 365 [4]	5337 ± 175 [3]	0.6
30d	2	Me		H	Me	O	O	1360 ± 245 [3]	4130 ± 281 [3]	0.3
30e	2	Me		H	Me	O	O	1278 ± 117 [3]	4874 ± 1234 [3]	0.3
33	2	Me		OMe	Me	O	O	5793 ± 1749 [3]	> 10,000 [3]	< 0.6
34	2	Me		H		O	O	273 ± 48 [3]	1182 ± 189 [3]	0.2
35	1	Me		H		O	O	30 ± 2 [3]	36 ± 6 [3]	0.8
36	1	Me		H		O	O	228 ± 26 [4]	922 ± 169 [3]	0.3
37	1	Me		OMe	Me	NH	O	> 10,000 [3]	> 10,000 [2]	-
56a	1	Me		H		O	O	435 ± 29 [3]	681 ± 197 [3]	0.6
56b	1	Me		H		O	O	48 ± 8 [3]	96 ± 33 [3]	0.5
56c	1	Me		H		O	O	125 ± 14 [3]	189 ± 51 [3]	0.7
56d	1	Me		H		O	O	43 ± 6 [3]	32 ± 6 [4]	1.4
56e	1	Me		H		O	O	168 ± 15 [3]	184 ± 19 [3]	0.9
56f	1	Me		H		O	O	33 ± 7 [3]	165 ± 60 [3]	0.2
56g	1	Me		H		O	O	30 ± 7 [4]	62 ± 15 [4]	0.5
56h	1	Me		H		O	O	255 ± 60 [4]	120 ± 16 [4]	2.1
56i	1	Me		H		O	O	321 ± 62 [3]	537 ± 86 [4]	0.6

Table 1. continued

Cpd.	n	R ¹	R ²	R ³	R ⁴	X	Y	K _i [nM] ± SEM [N]		<i>h</i> M ₅ R selectivity K _i (<i>h</i> M ₁ R)/ K _i (<i>h</i> M ₅ R)
								<i>h</i> M ₁ R	<i>h</i> M ₅ R	
56j	1	Me		H		O	O	8.1 ± 0.9 [5]	15 ± 3 [3]	0.5
56k	1	Me		H		O	O	4.2 ± 0.4 [5]	8.6 ± 2.1 [3]	0.5
56l	1	Me		H		O	O	3.3 ± 0.4 [3]	4.3 ± 0.4 [3]	0.8
56m	1	Me		H		O	O	2.6 ± 0.3 [5]	2.0 ± 0.2 [4]	1.3
56n	1	Me		H		O	O	236 ± 25 [4]	319 ± 50 [3]	0.7
56o	1	Me		H		O	O	155 ± 12 [3]	63 ± 7 [4]	2.5
70a	1	Me		NO ₂		O	O	415 ± 88 [4]	413 ± 58 [3]	1.0
70b	1	Me		NHMe		O	O	578 ± 38 [4]	477 ± 52 [4]	1.2
70c	1	Me		NMe ₂		O	O	599 ± 44 [3]	267 ± 15 [3]	2.2
70d	1	Me		OMe		O	O	452 ± 70 [4]	259 ± 29 [3]	1.7
70e	1	Me		OMe		O	O	250 ± 11 [3]	141 ± 13 [4]	1.8
70f	1	Me		OMe		O	O	152 ± 17 [4]	124 ± 13 [3]	1.2
70g	1	Me		OMe		O	O	110 ± 21 [4]	54 ± 9 [3]	2.0
81a^a	1	Me		H		O	S	30 ± 2 [3]	27 ± 6 [4]	1.1
81b	1	Me		H		O	SO	77 ± 5 [3]	170 ± 16 [3]	0.5
81c	1	Me		H		O	SO ₂	181 ± 36 [3]	232 ± 48 [3]	0.8
81d	1	Me		H		O	NH	114 ± 5 [4]	35 ± 9 [3]	3.3
81e	1	Me		H		O	NMe	266 ± 22 [3]	145 ± 25 [3]	1.8
81f	1	Me		H		O	NH	14 ± 1 [3]	10 ± 3 [3]	1.4
83								173 ± 19 [3]	142 ± 24 [4]	1.2

^aRadioligand competition binding assay with CHO cells expressing *h*M₁R or *h*M₅R, values represent mean binding affinity ± SEM from *N* independent experiments, each performed as triplicates. ^{*}Previously published compound synthesized as described in the literature. Published K_i values for **10** (*h*M₁R: 3290 nM; *h*M₅R: 470 nM) and for **13** (*h*M₁R: 25,300 nM; *h*M₅R: 2240 nM).⁷⁹ ^bCompound **81a** was only 94% pure as determined by HPLC.

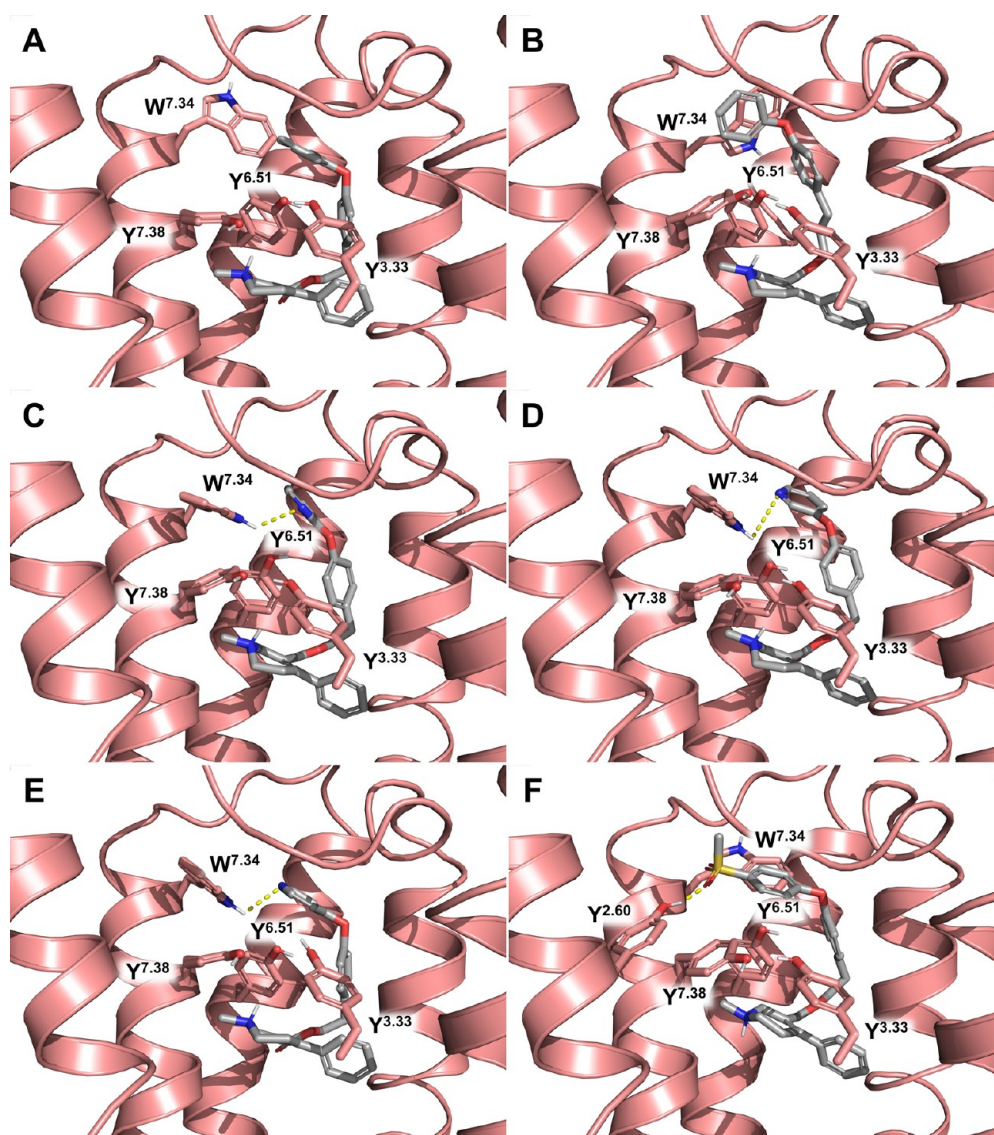


Figure 3. Induced fit docking poses of *hM₅R* (PDB: 6OL9) with **35** (A), **34** (B), **56j** (C), **56k** (D), **56l** (E), or **56m** (F) in the binding pocket. Important amino acids interacting with each compound are highlighted, and relevant hydrogen bond interactions are displayed as yellow dashed lines. The cartoon representation of TM3 was hidden to allow a better view on the interactions.

RESULTS AND DISCUSSION

Chemistry

All compounds were synthesized analogous to known protocols reported in the literature or described in the [Supporting Information](#).⁷⁹ The arecaidine derivatives **9**, **12**, **17**, **21**, **25**, and **29** were prepared using a modified synthetic procedure adapted from the literature ([Scheme 1](#)).⁷⁹ Starting from tyrosol or other commercially available building blocks, the side chains **31**, **32**, **38–51**, **55**, **58**, **61**, **63**, **66–69**, **71–73**, **75**, **77**, **79**, and **82** were prepared ([Scheme 2](#)). These side chains were then coupled with the carboxylic acid **9**, **12**, **17**, **21**, **25**, and **29** using EDC and DMAP to afford the final compounds **10**, **13**, **30a–e**, **33–37**, **56a–o**, **70a–g**, **81a–f**, and **83** ([Scheme 3](#)).

Pharmacology and Docking

***hM₅R* Affinity and Receptor Subtype Selectivity.** The *K_i* values of all target compounds (**4**, **10**, **13**, **30a–e**, **33–37**, **56a–o**, **70a–g**, **81a–f**, and **83**) were determined in competition binding studies on CHO cells expressing *hM₅R* or other subtypes (*hM_{1–4}R*) using [³H]-*N*-methylscopolamine ([³H]-

NMS) as a radioligand. Due to the high structure similarity between *hM₁R* and *hM₅R* with 55% overall sequence homology and 89% for the ortho- and allosteric binding pocket, *hM₁R* was used as a primary marker for subtype selectivity of all ligands.⁶⁴ Subsequently, the influence of various moieties and substituents on binding affinity and selectivity of the compounds was investigated. The first step in altering the arecaidine core was replacing the ethyl substituent (*R*¹) on the tertiary amine present in reported compound **10** by a smaller methyl group (**30a**). This modification resulted in a slight gain in affinity at *hM₅R*, while the affinity for *hM₁R* was not affected ([Table 1](#)). The methyl group was kept constant at the amino function. It is important to note that the obtained data for reported compounds **10** and **13** in our assay match well with the published affinities on *hM₅R* but not on *hM₁R*, indicating them as nonselective compounds in the assay setup of the present study ([Table 1](#)). The series of compounds with variations of the aromatic moiety at *R*² (**30b–30e**) displayed no improvements in affinity or selectivity. Exchanging the methyl group of the ether

function (R^4) of compound **30a** by a phenyl group (**34**) resulted in a 4-fold decrease in affinity in the case of both receptors.

Next, the influence of the alkyl linker length between the ester group and the phenyl ring on the M_1R and M_5R affinity was investigated. The propyl linker present in **34** was shortened to an ethyl spacer in **35**. This modification resulted in 9- and 33-fold increases in affinity for M_1R and M_5R , respectively, leading to a balanced dual M_1R and M_5R ligand (Table 1). Docking studies suggested that the phenyl moiety at R^4 of compound **35** with the shorter linker might be able to interact more with the tyrosine lid (Y3.33, Y6.51, and Y7.38) and tryptophane W7.34 present in all muscarinic subtypes (Figure 3A,B). A similar but less pronounced trend was observed in the case of compounds **13** (ethyl linker) and **33** (propyl linker). Replacing the ester with an amide group (compound **37**) resulted in a complete loss in binding affinity. The increased rigidity and different hydrogen-bonding profile of an amide moiety do not seem to be compatible with the bulky phenyl tetrahydropyridine core in this case.

Considering these results, the phenyl arecaine derivative **12** was chosen as a core structure for all the following compounds (Scheme 3). To improve the interaction with the tyrosine lid, another aromatic moiety R^4 was introduced to the hydroxy group of the tyrosol moiety (Table 1). Bulky and lipophilic substituents like 1- (**56a**) and 2-naphthyl (**56b**) were less favorable and decreased affinity on both subtypes. When the R^4 substituent was elongated to a benzyl moiety **56c**, binding affinities decreased but then returned to similar values as **35** in the case of the longer phenyl ethyl derivative **56d** (**56d**: $K_i(hM_5R) = 32$ nM, Table 1). Additionally, the phenylethyl derivative showed no preference for M_1R (hM_1R/hM_5R ratio: 1.4). Introducing methoxy substituents on varying positions of the ring always led to a decrease in affinity on M_5R (**56e**: ortho, $K_i(hM_5R) = 184$ nM; **56f**: meta, $K_i(hM_5R) = 165$ nM; and **56g**: para, $K_i(hM_5R) = 62$ nM), with para being the least detrimental position. At M_1R , however, only the ortho-substituted **56e** led to a decrease in affinity, while **56f** and **56g** maintained affinity compared to **35** (**56e**: ortho, $K_i(hM_1R) = 168$ nM; **56f**: meta, $K_i(hM_1R) = 33$ nM; and **56g**: para, $K_i(hM_1R) = 30$ nM). When introducing methoxy groups in the ortho and para position at the phenyl ring at R^4 (**56h**), the loss of affinity was less pronounced compared to the ortho methoxy-substituted **56e**, but M_5R preference was unexpectedly improved (**56h**: $K_i(hM_5R) = 120$ nM, hM_1R/hM_5R ratio: 2.1). Bulkier substituents in the ortho position, like in **56i**, were too large despite their potential for multiple π - π interactions as indicated by the decrease in affinity compared to **35** and **56e**.

In the next step, a pyridine ring was used as R^4 , and while analyzing the influence of the substitution pattern, a similar trend as previously mentioned (**56e-g**), with the meta- and para-substituted derivatives being favored, was noticeable. Providing a suitable hydrogen bond acceptor in the para position (**56l**) increased affinity nearly 10-fold (**56j**: ortho, $K_i(hM_5R) = 15$ nM; **56k**: meta, $K_i(hM_5R) = 8.6$ nM; and **56l**: para, $K_i(hM_5R) = 4.3$ nM). Computational induced fit docking revealed a potential new hydrogen bond interaction of the pyridine nitrogen with W7.34, whose orientation and distance were depending on the substitution pattern of R^4 (Figure 3C-E). This W7.34 is conserved among all subtypes, leading to a similar increase in affinity to a low nanomolar range on hM_1R as well (Table 1). Additionally, due to the large allosteric binding pockets of muscarinic receptors, the distance between the pyridine nitrogen of the docked **56l** and the aromatic bound hydrogen

of W7.34 was not optimal. Thus, a larger more polar substituent was necessary in the para position to form stronger hydrogen bonds with the tryptophane. After examining the docking poses and interactions of various virtual compounds, compound **56m** having a methyl sulfone moiety as a hydrogen bond acceptor at the phenyl ring was synthesized. In the docking experiment, an interaction of the oxygen atoms in the sulfone group with the phenolic hydroxy group of Y2.60 was observed (Figure 3F). The introduced methylsulfone substituent of **56m** improved affinity to a low single-digit nanomolar range on both M_1R and M_5R , confirming the hypothesis of a hydrogen bond interaction at that position (**56m**: $K_i(hM_5R) = 2.0$ nM, $K_i(hM_1R) = 2.6$ nM; Table 1). To increase M_5R preference, as seen in **56g**, an additional ortho substituent was introduced in **56n** and **56o** to potentially address the hM_5R -selective lysine K67.49 with a π -cation interaction as predicted in induced fit docking studies. Both compounds **56n** and **56o** lost affinity greatly, but **56o**, however, displayed improved selectivity toward M_5R (**56n**: hM_1R/hM_5R ratio: 0.7; **56o**: $K_i(hM_5R) = 63$ nM, hM_1R/hM_5R ratio: 2.5; Table 1). Therefore, the new nitro group and its polarized oxygen atoms of **56n** do not seem to form any beneficial interactions with Y2.60 or the receptor overall. Contrary to the nitro-substituted **56n**, the methylsulfone substituent of **56o** seems to allow the oxadiazol moiety in the ortho position to properly address K67.49 and therefore increasing selectivity for **56o** slightly.

Last, the influence of the tyrosol moiety connecting the arecaine-like core and terminal aromatic ring structure was investigated. With tyrosol being a readily available precursor, a substituent in the ortho position (R^3) to the phenolic hydroxy group was introduced first. Starting with the easily accessible nitro-substituted compound and its reduced and methylated amine derivatives, a negative influence on hM_5R affinity was observed for all three compounds (Table 1) compared to **56d**. However, compound **70c** bearing the dimethyl amine showed an improved preference toward hM_5R (hM_1R/hM_5R ratio: 2.2) compared to the other compounds. Introducing hydrogen bond donors in that position is not favorable, but flexible acceptors, like the dimethyl amine, might help to improve selectivity. Compounds **13** and **33** were substituted with a methoxy group at R^3 , which served as a potential hydrogen bond acceptor in that position as well. Therefore, the new ortho-substituted tyrosol moiety was combined with various terminal substituents (R^4) to investigate how their size and polarity influence affinity and selectivity: compound **70d** shows reduced affinity with a minor improvement in selectivity but not as distinct as previously observed with **70c** (Table 1). Combining the methoxy-substituted tyrosol with the previously affine hydrogen bond acceptor containing the aromatic ring from **56k**, **56l**, or **56m** yielded unexpected results: Contrary to the nonmethoxylated counterparts, **70e** demonstrated a similar affinity on hM_5R as its para-substituted analogue **70f** (**70f**: $K_i(hM_5R) = 124$ nM; **70e**: $K_i(hM_5R) = 141$ nM). In terms of hM_1R affinity, **70f** was higher than **70e** (**70f**: $K_i(hM_1R) = 152$ nM, hM_1R/hM_5R ratio: 1.2; **70e**: $K_i(hM_1R) = 250$ nM, hM_1R/hM_5R ratio: 1.8). Based on induced fit docking studies, the additional methoxy group causes the ligand to shift, pushing the pyridine moiety (R^4) away from W7.34 toward the ECL2 amino acids Q45.51 and I45.52. The pyridine nitrogen can form a new interaction with the amide NH of one of the amino acids, but unlike before, the para- or meta-substitution has no direct influence on this interaction. Compound **70g**, which is derived from the previously most affine **56m**, lost about 27-fold affinity but gained some selectivity

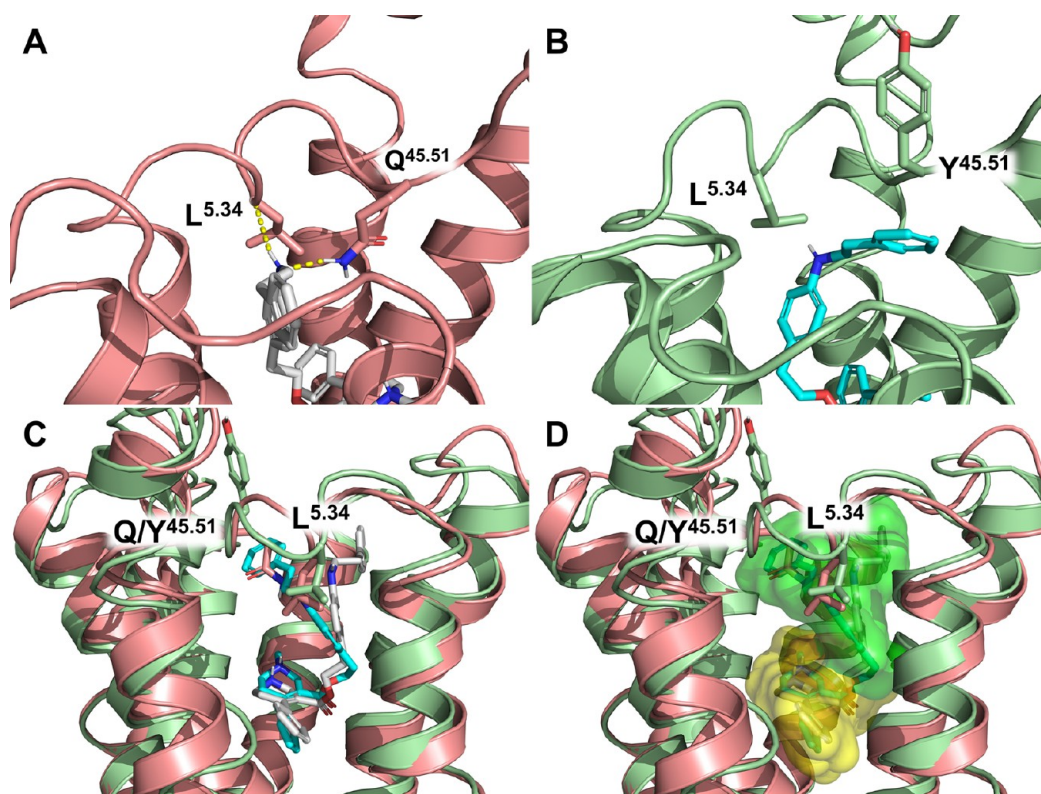


Figure 4. A: Representative induced fit docking pose of the compound **81d** with *hM₃R* (PDB: 6OL9). B: Representative induced fit docking pose of the compound **81d** with *hM₁R* (PDB: 5CXV). C: Overlay of the docking poses at *hM₃R* (ligand: gray, receptor: red) and at *hM₁R* (ligand: cyan, receptor: pale green). D: Overlay with the subtypes *hM₁R* and *hM₃R* as seen in C, but the orthosteric binding site is highlighted in yellow and the allosteric binding site in green. Important amino acids are highlighted, and relevant hydrogen bond interactions are displayed as yellow dashed lines.

compared to its parent compound. The increased spatial demand of the methoxy substituent positively influenced selectivity but considerably decreased the affinity overall, matching the results from **70a–c**, all having substituents with similar sizes.

The next step focused on the oxygen atom (Y) serving as an ether bridge between the tyrosol partial structure and terminal aromatic moiety. Hereby, the influence of other heteroatoms and the effect of a hydrogen bond acceptor–donor swap were investigated to elucidate whether it was possible to address the unique Q45.51 or not: exchanging the ether to a sulfide (**81a**) bridge did not significantly affect selectivity or affinity. However, the sulfoxide and sulfone derivatives lead to a roughly 5-fold decrease in affinity on both subtypes (Table 1). This might be due to their different geometric angles or differences in size, which can also influence how well the phenyl (*R*⁴) substituent interacts with the receptor. Introducing a tertiary methylated amine as bridge reduced affinity on both receptors but increased selectivity slightly. The nonmethylated secondary amine in position Y showed improved selectivity while maintaining affinity (**81d**: $K_i(hM_3R) = 35$ nM, *hM₁R*/*hM₃R* ratio: 3.3). For compound **81f**, this effect was not observed. The exchange of an oxygen to a nitrogen atom only lowered affinity 5-fold without providing any benefit toward selectivity compared to **56m** (Table 1). Unlike **70c** and its substitution on *R*³, position Y favors secondary amines as seen in **81d** and **81f** as hydrogen bond donors with leucine 5.34 over tertiary amines as pure acceptors, respectively. After examining various binding poses of **81d** in the docking studies, it was concluded that the gain in selectivity stems from hydrogen-bonding interactions with Q45.51 and the backbone carbonyl of L5.34. The flexible

amide side chain of Q45.51 helped in stabilizing the binding mode as a hydrogen-bond donor, allowing the secondary amine to directly interact with L5.34 (Figure 4A). At *M₁R*, these hydrogen bonds with Q45.51 or L5.34 were not observed in the docking poses, leading to the assumption that the lack of the stabilizing hydrogen bond of Q45.51 or steric hindrance of Y45.51 (*M₁₋₂R*) or F45.51 (*M₃₋₄R*) resulted in a different orientation compared to Q45.51. Therefore, preventing the formation of the hydrogen bonds as seen at *M₃R* (Figure 4B). The glutamine at *M₃R*, however, is small and flexible enough to accommodate this, resulting in a gain in selectivity toward *M₃R* for **81d** (Figure 4C). Since both amino acids L5.34 and Q/Y45.51 are located in the allosteric binding site, compound **81d** can be considered as a bitopic binder (Figure 4D). To simplify synthesis and access more options for derivatization, the central 4-aminotyrosol moiety was replaced with a 6-aminohexanol linker in compound **83**. Compared to **81f**, this exchange lowered the affinity about 14-fold, showing the necessity of the rigid planar ring and its aromatic interaction with the receptor to maintain affinity. In terms of selectivity, this linker exchange of **83** did not have any significant effect toward either of the two subtypes.

While *hM₁R* was suitable for a general selectivity screening, it is important to investigate ligand affinities on all *M_xR* subtypes to avoid any unwanted binding and potential side effects. Commercially available pirenzepine (Figure 1A) was used as a reference compound in the in-house radioligand competition binding assay, and all obtained affinities and resulting selectivity are displayed in Table 2. The most promising ligands of this series, reference compound pirenzepine, as well as reported compounds **4** and **10**, were screened on all five receptor

Table 2. Binding Data of the Reference Compounds Pirenzepine, **4**, **10**, and **13** and the Compounds **35**, **56c**, **56d**, **56l-o**, **70e**, **70g**, **81d**, **81f**, and **83** on all Five Human Muscarinic M_x Receptor Subtypes^a

cmpd	K_i [nM] $\pm$ SEM (hM_5R selectivity) $K_i(hM_xR)/K_i(hM_5R)$, $x = 1, 2, 3, 4$ [N] {literature K_i [nM]} ^{71,79,93}				
	hM_1R	hM_2R	hM_3R	hM_4R	hM_5R
pirenzepine ^b	9.2 $\pm$ 0.9 (0.1) [3] {10}	197 $\pm$ 14 (2.7) [3] {501}	248 $\pm$ 20 (3.4) [3] {158}	22 $\pm$ 2 (0.3) [3] {100}	72 $\pm$ 7 [3] {126}
4 ^b	2119 $\pm$ 146 (40.0) [3] {-}	439 $\pm$ 44 (8.3) [4] {-}	>1,000 ^c (>19) [3] {-}	1337 $\pm$ 264 (25.2) [4] {-}	53 $\pm$ 12 [3] {68}
10 ^b	75 $\pm$ 14 (0.2) [3] {3290}	432 $\pm$ 54 (1.0) [3] {2030}	611 $\pm$ 197 (1.4) [3] {4070}	298 $\pm$ 16 (0.7) [3] {11,500}	446 $\pm$ 38 [3] {470}
13 ^b	4709 $\pm$ 778 (1.3) [4] {25,300}	- {>100,000}	>10,000 (>3) [3] {>100,000}	- {>100,000}	3505 $\pm$ 1135 [4] {2240}
35	30 $\pm$ 2 (0.8) [3]	-	242 $\pm$ 45 (6.7) [3]	-	36 $\pm$ 6 [3]
56c	125 $\pm$ 14 (0.7) [3]	-	489 $\pm$ 43 (2.6) [3]	-	189 $\pm$ 51 [3]
56d	43 $\pm$ 6 (1.4) [3]	-	102 $\pm$ 4 (3.2) [3]	-	32 $\pm$ 6 [4]
56l	3.3 $\pm$ 0.4 (0.8) [3]	74 $\pm$ 9 (17.4) [3]	6.1 $\pm$ 0.2 (1.4) [3]	9.1 $\pm$ 0.7 (2.1) [4]	4.3 $\pm$ 0.4 [3]
56m	2.6 $\pm$ 0.3 (1.3) [5]	34 $\pm$ 4 (17.4) [4]	7.0 $\pm$ 0.2 (3.6) [3]	3.8 $\pm$ 0.4 (1.9) [3]	2.0 $\pm$ 0.2 [4]
56n	236 $\pm$ 25 (0.7) [4]	134 $\pm$ 12 (0.4) [3]	482 $\pm$ 29 (1.5) [3]	468 $\pm$ 47 (1.5) [4]	318 $\pm$ 50 [3]
56o	155 $\pm$ 23 (2.5) [3]	116 $\pm$ 9 (1.8) [3]	297 $\pm$ 15 (4.7) [3]	243 $\pm$ 37 (3.8) [4]	63 $\pm$ 7 [4]
70e	250 $\pm$ 11 (1.8) [3]	-	1064 $\pm$ 126 (7.6) [3]	-	141 $\pm$ 13 [4]
70g	109 $\pm$ 21 (2.0) [4]	908 $\pm$ 49 (16.9) [3]	272 $\pm$ 47 (5.1) [3]	248 $\pm$ 39 (4.6) [3]	54 $\pm$ 9 [3]
81d	114 $\pm$ 5 (3.3) [4]	262 $\pm$ 52 (7.6) [5]	417 $\pm$ 51 (12.1) [3]	251 $\pm$ 27 (7.3) [3]	35 $\pm$ 9 [3]
81f	14 $\pm$ 1 (1.4) [3]	39 $\pm$ 5 (3.9) [4]	28 $\pm$ 2 (2.8) [3]	33 $\pm$ 7 (3.3) [3]	10 $\pm$ 3 [3]
83	173 $\pm$ 19 (1.2) [3]	235 $\pm$ 31 (1.7) [4]	332 $\pm$ 9 (2.3) [3]	121 $\pm$ 10 (0.9) [4]	142 $\pm$ 24 [4]

^aRadioligand competition binding assay with CHO cells expressing a singular hM_xR , values represent mean binding affinity $\pm$ SEM from N independent experiments, each performed as triplicates. ^bPreviously published compound purchased or synthesized as described in the literature.^{71,79,93} ^cCompound **4** was not able to fully displace the radioligand from the receptor.

subtypes.^{71,79} The affinities of pirenzepine matched the reported values well, except on hM_4R , where it was slightly higher than reported (Table 2). Compound **4** displayed good selectivity over all subtypes ranging from 8-fold in the case of hM_2R up to 40-fold for hM_1R . On hM_3R , however, **4** was not able to fully displace the radioligand, even at the highest tested concentration. The values reported in the literature for the tetrahydropyridine compounds **10** and **13** could not be reproduced in our assay system. A higher affinity was observed for both compounds for nearly all receptor subtypes, except for hM_5R and in the case of **13** also for hM_3R .⁷⁹ Since compound affinity and therefore selectivity depend on the used assay system, such discrepancies are normal, but they usually follow a symmetrical pattern. Among the high-affinity compounds **56l** and **56m** sharing a 17-fold selectivity over hM_2R , **56m** having a slight preference for hM_5R over hM_3R due to the lack of Y2.60 at hM_3R was noteworthy. These results imply that the hydroxy group of Y2.60 is probably involved in the binding of **56m** because of the lower affinity at hM_3R with its F2.60. It is worth mentioning that on hM_2R a phenylalanine is at position 5.34, whereas all other subtypes have a L5.34 (Figure 2A). This might explain the 17-fold selectivity over hM_2R of **56l** and **56m** due to the more voluminous planar side chain of phenylalanine. The methoxy derivate of **56m** (**70g**) had a lower affinity on hM_5R compared to its parent compound with a K_i value of 54 nM but demonstrated slightly better selectivity over the other subtypes, except on hM_2R (**70g**: hM_1R : 2-fold; hM_2R : 17-fold; hM_3R : 5-fold; and hM_4R : 5-fold). Compound **81d**, having the highest preference toward hM_3R over hM_1R among the tested

tetrahydropyridine-based compounds, shows even better selectivity over the other subtypes (**81d**: hM_1R : 3-fold; hM_2R : 8-fold; hM_3R : 12-fold; and hM_4R : 7-fold; Table 2).

Mini-G Protein Recruitment Assay. A mini-G protein recruitment assay was employed to determine whether the compound exerts agonistic or antagonistic effects on the signaling cascade. This assay relies on recruiting NlucN-modified mini-G proteins to an NlucC-modified receptor after ligand binding and consequently measuring the resulting signal changes obtained from the split NanoLuc interaction. This procedure was established in-house.^{96,97} All tested compounds were measured in the antagonist mode of the assay and were found to be antagonists. The IC₅₀ values of the antagonists and reference compounds pirenzepine and atropine were in accordance with the literature, and **4** matched the published data as well (Table 3). Analogous to the radioligand competition binding assay for compound **13**, the determined IC₅₀ values differ greatly from the published data by Zheng et al.⁷⁹ This might be again related to the use of a different assay system, and it is not uncommon to encounter such a discrepancy.^{98,99} The most affine compounds in the radioligand competition binding assay **56m**, **56o**, **70g**, **81d**, and **81f** demonstrated a 20- to 110-fold difference between the two assay types on the same subtype hM_5R (Tables 1 and 3), while most other compounds only had a 2- to 50-fold difference. The compounds with no additional substituents at R³ on the tyrosol moiety showed the highest potency within this series. This is in good agreement with the results of the radioligand binding assay where the unsubstituted and nonselective compounds (**56k**, **56l**, **56m**, and **81f**)

Table 3. Functional Data of the References Pirenzepine and Atropine and the Compounds 56k-o, 70c, 70g, 81d, 81f, and 83 on NlucC-Modified hM_3R ^a

cmpd	IC ₅₀ [nM] ± SEM at hM_3R {literature IC ₅₀ [nM]} ^{71,79,102}	N
pirenzepine ^b	220 ± 24 {224}	4
atropine ^b	2.0 ± 0.01 {2.2}	3
4 ^b	92 ± 7 {36}	4
13 ^b	>10,000 {0.45}	6
56k	359 ± 74	4
56l	216 ± 11	4
56m	212 ± 16	4
56n	1687 ± 173	4
56o	955 ± 130	3
70c	>10,000	6
70g	1059 ± 98	4
81d	3206 ± 352	4
81f	559 ± 19	4
83	986 ± 98	4

^aMini-G protein recruitment assay using HEK293T cells stably coexpressing NlucN-mG_q/M₃R-NlucC fusion proteins. All antagonists were characterized in the presence of 100 μ M carbachol. Data were normalized to L-15 as solvent control (%) and to maximal responses elicited by 100 μ M carbachol (100%). Values represent mean IC₅₀ values ± SEM from *N* independent experiments, each performed as triplicates. ^bPreviously published compound purchased or synthesized as described.^{71,79}

displayed the highest affinities. These four compounds also had some of the largest discrepancies between *K_i* and IC₅₀ values (40–110-fold) among the examined compounds, whereas compounds 56n, 56o, and 83 only had a less than 20-fold difference at highest. Despite the ligands (56k, 56l, and 56m) possessing a single-digit nanomolar affinity, their inhibitory potency is much lower than expected.

Asparagine 6.52 is claimed to be crucial for receptor activation and inactivation. The substituents on the tyrosol moiety R³, Y, and R⁴ may influence the ligands' binding pose and how the ester carbonyl interacts with the amide side chain of N6.52.^{100,101} Implying the 108-fold difference between the activity and affinity of 56m might result from the methyl sulfone moiety interacting with Y2.60, which causes a change in binding and pulling the ester moiety further away from N6.52, whereas for 56k and 56l, which supposedly address W7.34 with their pyridine moiety, this change does not seem to be as strong as with 56m (56k: 42-fold; 56l: 51-fold; and 56m: 108-fold). Interestingly, for 81f, bearing the methyl sulfone at R⁴, the difference is 56-fold and therefore in a similar range as with 56k and 56l. The difference between 56m and 81f is the swap of an oxygen to a nitrogen atom at the Y position. This nitrogen atom, or rather its positively polarized hydrogen, is able to form an interaction through hydrogen bonding with the backbone of L5.34 and thus might be able to stabilize a more favorable binding position for addressing N6.52. Compound 83 also reaffirms this hypothesis, where the rigid tyrosol moiety was

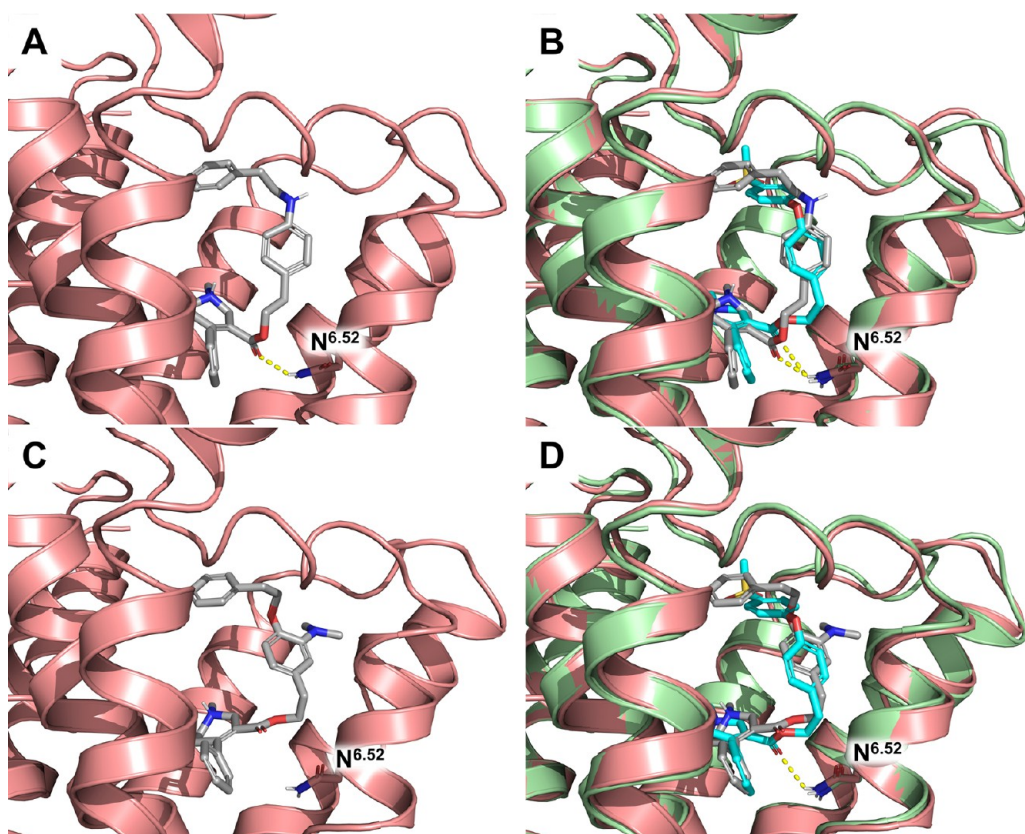


Figure 5. A: Representative induced fit docking pose of the compound 81d with hM_3R . B: Overlay of the docking poses of compounds 81d (ligand: gray, receptor: red) and 56m (ligand: cyan, receptor: pale green) at hM_3R . C: Representative induced fit docking pose of the compound 70c with hM_3R . D: Overlay of the docking poses of compounds 70c (ligand: gray, receptor: red) and 56m (ligand: cyan, receptor: pale green) at hM_3R . Important amino acids are highlighted, and relevant hydrogen bond interactions are displayed as yellow dashed lines (PDB: 5CXV (hM_1R); 6OL9 (hM_3R)).

Table 4. Functional Data of Compounds **4**, **56m**, and **81d** and the References Pirenzepine (hM_1R)^a, Mepyramine (hH_1R)^b, and Haloperidol ($hD_{2long}R$)^c on NlucC-Modified Receptors^a

cmpd	IC ₅₀ [nM] ± SEM (hM_5R selectivity): IC ₅₀ (X)/IC ₅₀ (hM_5R), X = hM_1R , hH_1R , $hD_{2long}R$ [N] {compound K_b , literature K_b [nM]} ^{102,108,109,c}			
	hM_1R	hH_1R	$hD_{2long}R$	hM_5R
pirenzepine ^b	14 ± 2 (0.1) [4] {2.4, 17}	-	-	220 ± 24 [4] {11, 224}
mepyramine ^b	-	16 ± 3 (-) [4] {1.6, 3.3}	-	-
haloperidol ^b	-	-	2.7 ± 0.2 (-) [3] {1.6, 2.1}	-
4 ^b	1796 ± 141 (20) [5] {305, -}	>100,000 (>1000) [4] {>10,000, -}	>100,000 (>1000) [3] {>10,000, -}	92 ± 7 [4] {36, 4.4}
56m	89 ± 20 (0.4) [5] {15, -}	9323 ± 1321 (44) [4] {960, -}	>10,000 (>47) [3] {5799, -}	212 ± 16 [4] {10, -}
81d	982 ± 94 (0.3) [4] {167, -}	8622 ± 1402 (2.7) [4] {888, -}	4851 ± 610 (1.5) [3] {2813, -}	3206 ± 352 [4] {153, -}

^aMini-G protein recruitment assay using HEK293T cells stably coexpressing either NlucN-mG_q/M₁R-NlucC^a, NlucN-mG_q/H₁R-NlucC^b, or NlucN-mG_i/D_{2long}R-NlucC^c fusion proteins. All antagonists were characterized in the presence of either 100 μM carbachol^a, 10 μM histamine^b, or 100 nM dopamine^c. Data were normalized to L-15 as solvent control (%) and to maximal responses elicited by either 100 μM carbachol^a, 10 μM histamine^b, or 100 μM dopamine^c (100%). Values represent mean IC₅₀ values ± SEM from *N* independent experiments, each performed as triplicates. ^bPreviously published compounds were purchased or synthesized as described. ^c K_b was calculated using the Cheng–Prusoff equation with the following pEC₅₀ values: 4.69 (hM_1R , carbachol), 5.94 (hH_1R , histamine), 6.86 ($hD_{2long}R$, dopamine), and 5.30 (hM_5R , carbachol).

exchanged with a flexible hexyl linker while keeping the terminal R⁴ substituent the same. The rigidity of the phenyl ring seemed to be beneficial for affinity, but **83** showed one of the lowest changes in the mini-G protein recruitment assay compared to the radio ligand binding assay (7-fold). Considering these results of **56m** and **81f**, it might be possible that the increased flexibility of the alkyl chain allows **83** to fit more comfortably into the receptor and interact with N6.52 more easily. However, it is important to mention that **81d**, which also has a nitrogen at position Y and can form the mentioned interaction with L5.34, has a 93-fold change for the activity assay compared to the K_i value, meaning the substituent R⁴ plays a major role in how parts of the ligands orientate in the receptor. The ligands **56n** and **56o** with their oxadiazole moiety within R⁴ also show a relatively low discrepancy with a 5- and 15-fold change, respectively. Especially for **56o**, which only differs in the oxadiazol moiety, this change is far lower than the 108-fold difference seen with the parent compound **56m**.

Compounds **70c** and **81d** were among the least active ones in the assay with IC₅₀ values higher than 10,000 nM and 3206 nM, respectively. The docking studies provided no reliable information on why **81d** showed such low activity compared to its affinity (Figure 5A). Only when compared to the binding pose of **56m**, some minor differences are apparent, the ethyl part of the tyrosol moiety is twisted slightly, influencing the angle of interaction with the NH of N6.52 and potentially shifting the conformation of TM6 (Figure 5B). The additional dimethylamine substituent at R³ of **70c** induced a different binding mode, pushing the ligand out of the pocket and preventing the ester from interacting with N6.52 in all examined binding poses (Figure 5C). Especially when compared to an exemplary binding pose of **56m**, this change in orientation of **70c** is even more prominent and might explain the loss in potency despite acceptable affinity (Figure 5D).

Especially among aminergic receptors like muscarine, dopamine, or histamine receptors, specificity is challenging to achieve, as seen with drugs like xanomeline, doxylamine, and diphenhydramine (Figure 1).⁸⁷ Xanomeline, for example, shows high affinity to muscarinic receptors, but also to dopamine D₂ receptor, and diphenhydramine—a potent histamine H₁ receptor antagonist—also binds with reasonable affinity to muscarinic receptors.^{17,94,103,104} Comparing structural homology can be a rational strategy to decide which potential targets are getting screened for compound selectivity and specificity. In the case of M₃R, the subtypes H₁R and D₂R show the highest

overall similarity with 35% and 32% and for the orthosteric binding site 56% and 19% within their respective families.^{105–107}

Therefore, the ligands **4**, **56m**, and **81d** were tested alongside reference compounds on H₁R, D₂R, and M₁R to examine their specificity and selectivity in a functional assay. All three references, pirenzepine, mepyramine, and haloperidol, matched their reported activity data within reason (Table 4). Reported compound **4** displayed weak inhibitory activity at hM_1R with an IC₅₀ value of 1796 nM, which means it has a 20-fold selectivity toward hM_5R over hM_1R (IC₅₀ (hM_5R): 92 nM; Tables 3 and 4). Because no inhibition was measured on hH_1R and $hD_{2long}R$, compound **4** is specific for the muscarinic family compared to hH_1R and $hD_{2long}R$.

Antagonist **56m**, which was the most affine and potent compound within the tetrahydropyridine series, showed higher activity on hM_1R with an IC₅₀ value of 89 nM, compared to 212 nM on hM_5R , making it a nonselective antagonist in the functional assay (Table 4). However, compared to hH_1R and $hD_{2long}R$, **56m** demonstrated a 44-fold or higher specificity toward hM_5R with IC₅₀ values of about 10,000 nM in both cases. The tetrahydropyridine **81d** being the “most” selective compound in the radioligand competition binding assay so far (K_i (hM_1R) = 114 nM, K_i (hM_5R) = 35 nM; Table 2) displayed unexpected results in the mini-G protein recruitment assay. Antagonist **81d** was more potent on hM_1R (IC₅₀ value: 982 nM) compared to hM_5R (IC₅₀ value: 3206 nM), showing a 3.3-fold preference for hM_1R under these conditions. The previously mentioned spatial interaction between the R⁴ substituent of **81d** and L5.34 or Q/Y45.51 was beneficial for the selectivity in the radioligand assay. However, this interaction changed how the compound interacted with the receptor and especially TM6 leading to this discrepancy of selectivity in the functional mini-G protein recruitment assay. Compound **81d** also showed reasonable activity on hH_1R (IC₅₀ value: 8622 nM) and $hD_{2long}R$ (IC₅₀ value: 4851 nM), implying it also has no relevant specificity toward hM_5R (IC₅₀ value: 3206 nM) either (Tables 3 and 4).

While affinity and activity are essential in compound design, in vitro and in vivo stability are important contributors as well. Despite the remarkable benchtop stability of the acidic compound solutions with a 6% or less decrease over 18 months, it is important to consider that in neutral and especially basic solutions, stability will be different, and the lability of the ester might become a concern again (Figures S129–S136). Because ester structures are known to be prone to degradation in human

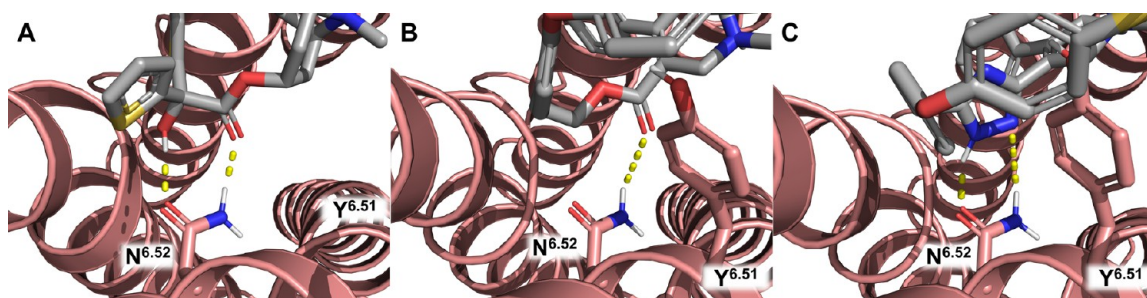


Figure 6. A: Protein complex crystal structure of *hM₁R* with bound antagonist tiotropium (protein complex crystal structure code: 5CXV) and the bidental interaction with N6.52 highlighted. B: Induced fit docking pose of *hM₃R* with **56m** in the binding pocket with the NH—O interaction between **56m** and N6.52 highlighted using yellow dashes. C: Induced fit docking pose of *hM₃R* with an exemplary virtual compound (1-methyl-5-(5-(4-(4-(methylsulfonyl)phenoxy)benzyl)-1H-1,2,4-triazol-3-yl)-4-phenyl-1,2,3,6-tetrahydropyridine) containing a triazole moiety in the position of the ester and the bidental interaction with N6.52 highlighted.

plasma, the compounds **56m** and **81d** were subjected to incubation with human plasma in PBS. Compound **56m** demonstrated surprising stability with roughly 65% of the compound remaining after 24 h and about 39% after 48 h (Table S1).^{110–113} In case of compound **81d**, only 33% and 23% of the parent compound remained after 24 and 48 h, respectively (Table S1). For both compounds, the cleavage of the ester bond was the major cause for the decrease of compound concentration as detected by mass spectrometry.

Discussion. Mutational analysis of the binding sites provides valuable information to determine which amino acids are crucial or relevant for ligand binding. However, because *hM₃R* was first crystallized in 2019 and not a focus of the muscarinic receptor research, the data pool for such mutations is very limited and does not provide a comprehensive overview yet.^{32,100,114} One important finding of the mutagenesis studies at *hM₃R* was that asparagine 6.52 is crucial for ligand binding of carbachol and atropine as well as receptor activation, constitutive activity, and maximum response, mirroring the findings on the other subtypes.^{100,101} Especially, when the hydrogen bonding profile of the amide side chain was altered, affinity and in some cases activity were massively decreased.^{100,101} The ester moiety of the synthesized ligands addresses this asparagine with one hydrogen bond mediated by the carbonyl oxygen (Figure 6A,B). It should be possible to mimic the bidental binding of tiotropium to N6.52 and gain affinity and possibly efficacy, when this ester structure is replaced. The α -hydroxy ester of tiotropium provides a hydrogen bond acceptor/donor pair in proximity to each other which can address the amide side chain of N6.52 in a bivalent manner (Figure 6A). The arecaidine ester structure is inverted compared to tiotropium, so it should be possible to replace it with a different moiety, which can act as a hydrogen bond acceptor/donor pair as seen in tiotropium. A schematic and structural comparison of the binding modes of tiotropium and **56m** can be found in the Supporting Information (Figure S138). The ester moiety can be replaced by a triazole ring, which is a functional bioisostere to an amide group, and offers both hydrogen bond acceptor/donor capabilities (Figure 6C).

Due to the high homology between *hM₁R* and *hM₃R*, it is possible to examine the *hM₁R* subtype data and transfer certain results to *hM₃R*.¹¹⁴ Mutating any of the highly conserved tyrosines (3.33, 6.51, and 7.38) of the previously mentioned “tyrosine lid” drastically lowers affinity on all *M_xR* subtypes for most ligands used.¹⁰⁰ The π -cation interactions formed by these tyrosines and the typical cationic amine structure shared by most ligands and acetylcholine are essential for binding to the receptor. Additionally, their phenolic group can also contribute

hydrogen bond interactions to increase binding affinity with ligands. This leads to the conclusion that these amino acids are either relevant for ligand binding or the overall structural receptor conformation. In the docking studies, compound **56m** interacted with Y2.60 and formed a hydrogen bond, increasing its affinity (Figure 3F). However, due to Y2.60 being present in both *M₁R* and *M₃R*, it was not suited as a target to improve selectivity (Figure 2A). The literature data is not conclusive on the importance of the allosteric Y2.60: some ligands like NMS benefit from an alanine mutation, while other ligands like ACh, carbachol, or xanomeline loose affinity upon mutation.¹⁰⁰ A mutation of Y2.60 seems to have a more negative influence on the affinity of agonists, than on antagonists.¹⁰⁰ The alanine mutant Y2.60A exerts a bigger impact than the phenylalanine one, suggesting the hydroxy group of Y2.60 has no relevant influence on binding for most ligands while the aromatic phenyl moiety holds a crucial role. The likewise conserved Y2.63 in the allosteric pocket shows similar results upon mutation as Y2.60 due to their spatial proximity.

The previously mentioned Q45.51 seems to have influence on the interaction pattern with the receptor by its smaller steric hindrance compared to the tyrosine or phenylalanine residues present in the other subtypes and its ability to form hydrogen bond interactions as examined with compound **81d** (Figure 4A). Interestingly, its neighbor I45.52 influences binding of allosteric modulators positively when mutated to alanine, whereas orthosteric agonists like acetylcholine, carbachol, and xanomeline lost affinity. This aligns with the effect proposed for Q45.51, where the steric properties of the side chain influence the binding of the allosteric part of **81d** directly or indirectly via influencing the conformation of the loop. When W7.34 was mutated, it only influenced allosteric modulators negatively, and orthosteric binders were indifferent to the mutation.

The docking studies of **56j**, **56k**, and **56l** and the indicated hydrogen bond interaction with W7.34 reaffirm the hypothesis of these compounds being bitopic binders with the terminal aromatic moiety R⁴ taking part in allosteric interactions (Figure 3). It is very important to consider that these studies sometimes include different types of assays to determine binding affinity. As seen with compounds **10** and **13**, the affinities depend heavily on the assay used. Especially, the affinity on *hM₁R* and thus their selectivity varied from the reported values (Table 2). However, this might be due to the fundamentally different assay systems and conditions used to determine affinity (in-house: radioligand competition binding assay with whole CHO cells, literature: radioligand competition binding assay with membranes obtained from CHO cells). For published compound **4**, the

obtained value matches the reported value within an acceptable range. Due to steric clashes with the receptor, the used arecaidine core does not allow many variations, and the SARs are very limited for the explored positions. However, introducing an aromatic ring substituted with a strong hydrogen bond acceptor to the tyrosol moiety allowed **56m** to form a hydrogen bond with the hydroxy group of Y2.60 and increase affinity to low single-digit nanomolar values. This tyrosine is highly conserved among all subtypes except for hM_3R causing compound **56m** to be nonselective. Interestingly, the affinity on hM_2R is far lower than on hM_3R , despite having the conserved Y2.60. This suggests that the affinity of compound **56m** does not entirely depend on Y2.60 but can also result from other interactions, like the previously mentioned hydrogen bond of **56j**, **56k**, and **56l** with W7.34. In docking studies on hM_3R , a new interaction of the methylsulfone with the highly conserved Y2.63 was observed, mimicking the interaction with Y2.60 and explaining the maintained affinity on the receptor, despite the Y2.60F difference (Figure 7).

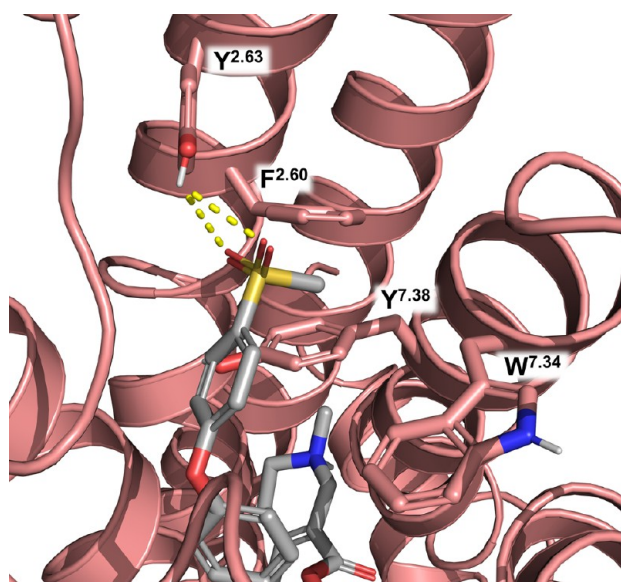


Figure 7. Induced fit docking pose of hM_3R with **56m** in the binding pocket with the hydrogen bond interaction between **56m** and Y2.63 highlighted using yellow dashes (PDB: 4U15).

An ortho substitution of the tyrosol with a methoxy or dimethyl amino group seems beneficial for selectivity but lowers affinity to M_5R as seen with compounds **70c–g**. Based on **56h** with its ortho–para-substituted R^4 moiety and the hydrogen bond interaction of **56m** with Y2.60, the oxadiazole moiety of compounds **56n** and **56o** was designed to address the unique lysine K67.49 in ECL2. However, compound **56o** turned out to be unselective and only displayed a preference for M_5R . Therefore, it is questionable if the goal of addressing this unique lysine was achieved. In this context, it is important to note that M_1R possesses a lysine in position 67.48, which could also form a similar interaction as K67.49 of M_5R and therefore mimic the interaction depending on the flexibility of the extracellular loop and its side chains. This implies that the gain of selectivity of ligand **56o** might result from a different interaction with the receptor, like steric hindrance or a different binding mode and not from the intended π -cation interaction. While discussing induced fit docking studies in context with functional

assays, it is important to note that during the docking protocols, the helices are allowed to move and adjust to the ligand binding. However, this is usually limited to a certain area around the ligand, neglecting any major shifts happening on the intracellular parts of the receptor, where the G proteins bind. The activity and G protein activation, however, is a more complex mechanism mediated by an in- or outward shift of the intracellular side of TM6, caused by ligand binding and the resulted interactions with the receptor helices, allowing the G protein to bind to the activated receptor.^{115–118} More complex and computationally demanding full body simulations of the receptor and ligand would be more suited to predict activity changes.^{119–121}

The determined hM_5R affinity of literature ligand **4** (VU6019650) of 68 nM is in good agreement with the published one (**4**, $K_i(hM_5R) = 53$ nM, Table 2).⁷¹ In the literature, no other radioligand competition binding assay affinities have been reported for the other subtypes. Alongside a good affinity on hM_5R , **4** showed an impressive selectivity over all other muscarinic subtypes, ranging from an 8- to 40-fold difference in K_i value. While compound **56m** was about 25-fold more affine on hM_5R than **4** (**56m**, $K_i(hM_5R) = 2.0$ nM) and displayed a 17-fold selectivity over hM_2R , it lacked selectivity on the other subtypes. Compound **81d** was slightly more affine on hM_5R than **4** (**81d**, $K_i(hM_5R) = 35$ nM) and had a broader selectivity profile compared to **56m**, ranging from the lowest selectivity of 3.3-fold on hM_1R up to a 12-fold selectivity on hM_3R (Table 2). While this was a good balance of selectivity and affinity, **81d** was not able to reach the same selectivity levels as those of **4**. The insights discussed and presented in this work might be able to improve the benefits of **56m** and **81d** further and turn them into usable tool compounds for various purposes, while considering the docking results on the selected M_xR s of the present study.

SUMMARY AND CONCLUSIONS

A library containing 38 novel arecaidine ester derivatives bearing a phenyl ring at the 4-position of the tetrahydropyridine ring (R^2) was synthesized as M_5R antagonists, and useful insights on relevant ligand–receptor interactions with M_1R and M_5R were obtained. Altering the aromatic substituent at R^2 or the oxygen atom X of the ester function resulted in a decreased affinity for both receptors. Shortening the substituent on the arecaidine nitrogen atom (R^1) to a methyl group proved to be beneficial for the affinity toward both receptors. Introducing a substituent at R^3 decreased affinity but had a positive influence on selectivity. Within the tyrosol ester series, the introduction of pyridinyl or substituted phenyl rings on the phenolic position of the tyrosol motif ($Y = O$, R^4) was well tolerated at both M_5R and M_1R . Compound **56m** bearing a para methylsulfonyl group at the phenyl ring at R^4 represents the most balanced dual M_1R/M_5R receptor antagonist of this series ($K_i(hM_1R) = 2.6$ nM; $K_i(hM_5R) = 2.0$ nM; Figure 8). The hydrogen bond acceptor in the para position of the phenyl ring might form a hydrogen bond with Y2.60. Antagonist **56m** shows a 2-, 4-, and 17-fold selectivity for M_5R versus M_4R , M_3R , and M_2R , respectively, and an excellent functional selectivity compared to $hD_{2long}R$ and hH_1R . Swapping the oxygen atom at Y to a group capable of being a hydrogen bond donor (NH) led to the biggest benefit for M_5R selectivity over M_1R , while maintaining affinity for M_5R as seen in phenylethylamine derivative **81d**. M_5R -antagonist **81d** ($K_i(hM_5R) = 35$ nM) has the best selectivity profile of this series versus the other members of the M_xR family (3-, 8-, 12-, and 7-

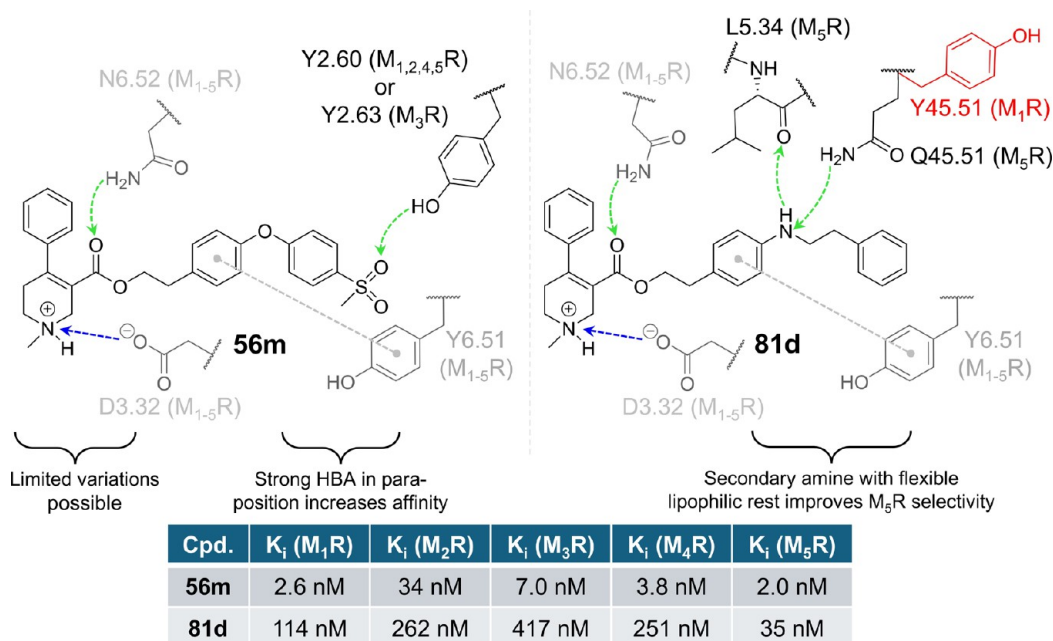


Figure 8. Important interactions of antagonists **56m** and **81d** with surrounding amino acids responsible for either affinity or selectivity increases.

fold selectivity toward M₅R over M₁R, M₂R, M₃R, and M₄R, respectively; **Figure 8**).

Since **81d** is the compound with the best M₃R selectivity, it should be beneficial to introduce a hydrogen bond acceptor in a suitable position at the phenylethyl moiety, as seen in **56m**. Additionally, the phenylethyl moiety can be further derivatized and modified to allow more polar interactions with the receptor. Especially, the M₅R-selective Q45.51 and the unspecific L5.34 (ECL2) or its neighbors are promising targets to increase selectivity. Considering the bidental binding of tiotropium with N6.52, it should be possible to exchange the ester functionality in the shown compounds to heterocycles as seen in xanomeline or other literature to increase affinity further (**Figure 8**).^{16,17,78}

Because of their bitopic character, the ligands **56m** and **81d** can serve as templates to develop fluorescence-labeled tool compounds for examining M₁R and M₃R further. Since the tyrosol-like moiety is bound in the allosteric area of the receptor, a linker carrying a suitable fluorescent dye can be easily attached to it. This fluorescent compound can then be used in fluorescence-based assays as seen in the literature.^{122,123} Overall, the induced fit docking studies helped to understand the ligand–receptor interactions and to improve ligand affinity. Utilizing the findings of the present study and the potential changes mentioned, it should be possible to improve the ligands further and develop a usable tool compound to elucidate the role of M₅R in the human body and gain important SAR information.

EXPERIMENTAL SECTION

Chemistry

General Conditions. Unless otherwise stated, chemicals and solvents were from commercial suppliers and were used as received. All the solvents used in analytics were of analytical grade. For column chromatography, silica gel 60 (0.04–0.063 mm, VWR, Leuven, Belgium) was used. Flash chromatography was performed on a puriFlash XS520Plus or XS530 workstation from Advion Interchim (Ithaca, NY, USA) with puriFlash columns (Si15–30, 4–40 g) from Advion Interchim (Ithaca, NY, USA). Reactions were monitored by thin layer chromatography on ALUGRAM Xtra SIL G UV254 aluminum sheets by MACHEREY-NAGEL GmbH (Düren, Germany),

and spots were visualized with UV light at 254 nm or potassium permanganate staining. NMR spectra were recorded on a Bruker Avance 300 (¹H: 300 MHz and ¹³C: 76 MHz), a Bruker Avance III HD 400 (¹H: 400 MHz and ¹³C: 101 MHz), and a Bruker Avance Neo (¹H: 500 MHz and ¹³C: 126 MHz) (Bruker, Karlsruhe, Germany) NMR spectrometer with deuterated solvents from Deutero (Kastellaun, Germany). All chemical shifts are reported in δ -scale as parts per million (ppm, multiplicity, coupling constant (*J*), and number of protons) relative to the solvent residual peaks as the internal standard. The spectra were analyzed by first order, and coupling constants are given in Hertz (Hz). Abbreviations for the multiplicities of the signals are s (singlet), d (doublet), t (triplet), q (quartet), and m (multiplet). HRMS was performed on a Q-TOF 6540 UHD LC/MS system (Agilent Technologies) using an ESI source or on an AccuTOF GCX GC/MS system (Jeol, Peabody, MA, USA) using an EI source. Prior to lyophilization (ScanVac CoolSafe 100–9 Pro freeze-dryer from Labogene (Labogene ApS, Lyngby, Denmark), equipped with a RZ 6 rotary vane vacuum pump (Vacuubrand, Wertheim, Germany)), the majority of MeCN was removed under reduced pressure using a SpeedVacPlus SC110A system from Savant (Thermo Fisher Life Technologies GmbH, Darmstadt, Germany), equipped with a Bachofer cold-trap and Edwards HV Pump (Edwards, Sussex, England). Analytical HPLC experiments were performed on a 1100 HPLC system from Agilent Technologies equipped with an Instant Pilot controller, a G1312A binary pump, a G1329A ALS autosampler, a G1330B ALS thermoregulator, a G1379A vacuum degasser, a G1316A column compartment, and a G1314A variable wavelength detector. The column was a Phenomenex Kinetex XB-C18 column (250 × 4.6 mm, 5 μ m) (Phenomenex, Aschaffenburg, Germany), tempered at 30 °C. As the mobile phase, mixtures of MeCN/aqueous TFA were used. The following linear gradients were applied for all compounds: MeCN/TFA (0.05%) (v/v) 0 min: 10:90, 30 min: 95:5, 40 min: 95:5; flow rate: 0.8 mL/min. The injection volume was 5–50 μ L. Absorbance was detected at 220 nm. The signals obtained during the radioligand competition binding assay were measured using a MicroBeta² 2450 Microplate Counter (PerkinElmer, Waltham, MA, USA).

General Procedures

Procedure A: Suzuki Reaction. The enole triflate **5** (1 equiv) was dissolved in THF and 2 molar Na₂CO₃ solution; then, the respective boronic acid (1.2 equiv) and Pd(PPh₃)₄ were added to the flask. A septum was used to seal the flask, and the solution was purged with argon for 15–30 min. After purging, the reaction was initiated by

placing the flask in a 65 °C water bath while being stirred for 4 h. After completion, the flask was cooled to room temperature and opened. The reaction mixture was poured into H₂O and brine to aid phase separation. The aqueous layer was extracted with EA, and organic layers were collected, combined, dried over MgSO₄, and reduced under vacuum. The crude product was then purified by flash column chromatography to obtain the product. Detailed procedures for compounds 6, 14, 18, 22, and 26 can be found in the [Supporting Information](#).

Procedure B: Ester Synthesis via EDC Hydrochloride and Respective Nucleophiles. The carboxylic acid (Scheme 1, 1 equiv) was suspended in dry DCM, and subsequently, the alcohol (Scheme 2, 1.0–1.5 equiv), DMAP (0.2–0.4 equiv), EDC hydrochloride (1.2–1.5 equiv), and optionally DMF were added. The reaction mixture was left to stir at room temperature overnight. After completion, the suspension was diluted with DCM and washed with saturated NaHCO₃ solution to remove side products. The organic layer was kept, dried over MgSO₄, filtered, and reduced under vacuum. The crude product was purified by flash chromatography twice to obtain the final compounds as free amines. Detailed procedures for compounds 4, 10, 13, 30a–e, 33–37, 56a–o, 70a–g, 81a–f, and 83 can be found in the [Supporting Information](#).

Procedure C: Modified Ullmann Coupling. The phenol or aniline derivative (1.0–1.2 equiv), aryl iodide (1.0–1.2), CuI (0.05 equiv), picolinic acid (0.1 equiv), K₃PO₄ (2 equiv), and DMSO were added to a microwave tube. The tube was sealed and purged with argon for 15–30 min. Then, the vessel was placed in a preheated aluminum block at 80–90 °C and stirred overnight. The next day, the reaction was left to cool to room temperature and opened, and DCM and H₂O were added. The aqueous phase was extracted with DCM, and the organic layers were collected, combined, and reduced under vacuum. The crude product was then purified by flash chromatography to obtain the product. Detailed procedures for compounds 31, 32, 38, 39, 42–49, 67, 68, 71, and 79 can be found in the [Supporting Information](#).

Compound Characterization. Target compounds (4, 10, 13, 30a–e, 33–37, 56a–o, 70a–g, 81a–f, and 83) were characterized by ¹H NMR (for spectra, see the [Supporting Information](#)), ¹³C NMR (for spectra, see the [Supporting Information](#)), HRMS (see the [Supporting Information](#)), and RP-HPLC analysis (see the [Supporting Information](#)). The purities (for chromatograms, see the [Supporting Information](#)) of all hM_xR compounds used for pharmacological investigation were ≥95%, except 81a, which only was 94% pure. For the biological testing, the target compounds (4, 10, 13, 30a–e, 33–37, 56a–o, 70a–g, 81a–f, and 83) were dissolved in DMSO so that the final concentration was 10 mM. [³H]-NMS was purchased from Novandi Chemistry (Södertälje, Sweden). The reference pirenzepine was purchased from Sigma-Aldrich (Merck, Darmstadt, Germany).

Radioligand Competition Binding. Competition binding experiments on the muscarinic acetylcholine M_{1–5} receptors were performed with CHO cells stably expressing a single respective hM_xR ($x = 1–5$) using previously described radioligand competition binding assays.⁹⁶ Compound samples used were freshly taken from frozen aliquots. The binding curves of 4, 56m, 70g, and 81d are available in the SI ([Figure S141](#)).

Mini-G Protein Recruitment Assay. The mini-G protein recruitment assay at hM₅R was performed using HEK293T NlucN-mG_q/M₅R-NlucC cells as previously described in detail similar to the mini-G protein recruitment assays at hM₁R (HEK293T NlucN-mG_q/M₁R-NlucC), at hH₁R (HEK293T NlucN-mG_q/H₁R-NlucC), and at hD_{2long}R (HEK293T NlucN-mG_i/D_{2long}R-NlucC). Coelenterazine was from BioSynth AG (Staad, Switzerland). The reference mepyramine was purchased from Sigma-Aldrich (Merck, Darmstadt, Germany), haloperidol from TCI Europe N.V. (Tokyo Chemical Industry, Tokyo, Japan), pirenzepine, and atropine from Sigma-Aldrich (Merck, Darmstadt, Germany).^{96,97} Compounds samples used were freshly taken from frozen aliquots. The concentration response curves of 4, 13, 56k–o, 70c, 70g, 81d, 81f, and 83 on hM₅R and 4, 56m, and 81d on hM₁R, hH₁R, and hD_{2long}R, alongside the respective reference compounds, are available in the SI ([Figures S142 and S143](#)).

Induced Fit Docking. Models of M_xR ($x = 1,3,5$): For the receptor modeling and induced fit docking studies, the software maestro (Releases: 2018–4 until 2025–1, Schrödinger Inc., New York, NY, USA) was used, and the results were visualized and presented using PyMOL 3.1.3 (Schrödinger Inc., New York, NY, USA). Induced fit docking was chosen because it represents a middle ground between the fast rigid body dockings and more accurate but time-intensive molecular dynamics simulations, and due to the available knowhow and expertise in-house.⁹⁶ The flexibility of the side chains is beneficial for obtaining more reliable results concerning ligand–receptor interactions, as seen in the literature.^{124–127} Maestro provides a validated protocol for induced fit docking studies utilizing single precision (SP) and extra precision (XP) stages of their docking tool GLIDE.^{89,90} At first, various ligand conformations are exhaustively generated for the binding site, which was defined as a 50 Å cube centered around the reference ligand (e.g., tiotropium) in the crystal structures. Then, those resulting ligand poses are fitted into the receptor, and a Monte Carlo search is performed for the torsional angles of the ligand and surrounding amino acids within the specified cube, therefore allowing backbone and side chain flexibility. Since the discussed ligands were antagonists in this study, it was important to use crystal structures for the docking with a bound antagonist and inactive receptor conformation. For hM₁R, the crystal structure with bound tiotropium (protein databank code, www.rcsb.org: 5CXV) was used, and the T4 FLAG peptide, explicit water molecules, and crystallization additives were removed, so only the receptor and tiotropium remained.³⁵ Analogous to this, for rM₃R the T4 lysozyme and for hM₅R the T4 lysozyme and additives were removed, leaving only the receptor and tiotropium without any other molecules (protein databank code, www.rcsb.org: rM₃R: 4U15 and hM₅R: 6OL9).^{32,34} Mutations in the crystal structures were kept and not altered, since they were not part of the binding pockets. All receptors were in their inactive state with the bound reference antagonist tiotropium, with the following resolutions: hM₁R, 5CXV, 2.70 Å; rM₃R, 4U15, 2.80 Å; and hM₅R, 6OL9, 2.54 Å.^{32,34,35} Examples for the influence of crystal structures with different cocrystallized ligands or activation states alongside exemplary RMSDs can be found in the [Supporting Information](#) ([Figure S139](#)). Each receptor was prepared using the internal protein preparation workflow (formerly: wizard) from maestro; if necessary, additional minimization was performed with the Minimize tool. hM₁R and hM₅R each required an additional minimization step. Sequence and viability of the obtained structures were checked with the FASTA sequences, obtained from the protein databank, and maestro's protein reliability report. Aspartate and glutamate were used in their deprotonated forms, while lysine and arginine were protonated during the preparation workflow. All ligands were prepared using the LigPrep function in maestro and then manually checked for viability. All amine moieties of the compounds were used in their quaternary protonated state, if physiologically realistic. The docking poses and interactions between the ligand and receptor, obtained by GLIDE, were analyzed and used to optimize ligand design. The detailed procedures and parameters can be found in the [Supporting Information](#). Redocking of tiotropium into the prepared crystal structure of hM₅R (PDB: 6OL9) resulted in an average RMSD of 0.62 Å for the overall structure and 1.32 Å for ligand-to-ligand across all five obtained poses with the thiophene ring being the only notable difference with its 180° turn ([Table S3](#) and [Figure S140](#)).

Benchtop Stability. After initial concerns about the stability of the compounds in solution, the samples of each compound, used in RP-HPLC analysis for purity, were kept and stored at room temperature on the benchtop. Compounds 35, 56l, 56m, and 81d were then tested again to assess their stability in an acidic aqueous solution. The resulting purities and chromatograms are available in the SI ([Figures S129–S136](#)).

Proteolytic Stability in Human Plasma. Compounds 56m and 81d were investigated in a human blood plasma/PBS 1:2 v/v mixture at 37 °C as described in the literature.^{128,129} Samples were analyzed based on UV detection at 220 nm using the RP-HPLC system with the settings found in *Chemistry: General conditions*. The obtained results for

the measured time frames and recovery rate ratios are summarized in Table S1 and S2 in the Supporting Information.

Data Processing. Compound purities were calculated as the percentage peak area (AU) of the analyzed compound determined by UV detection at 220 nm. Data from radioligand competition binding assays on hM_{1-5} receptors or from mini-G protein on $hM_{1,5}R$, hH_1R , and $hD_{2long}R$ were processed as reported previously.^{96,97} Ligands were compared using their inhibition constant K_i , its negative decimal logarithm pK_i , or IC_{50} values calculated from the assay data according to the Cheng–Prusoff equation.¹³⁰ K_i and IC_{50} values were used directly from GraphPad Prism 5 or 10, and the SEM was calculated based on the individual values obtained from each independent experiment. K_b values were calculated from the respective IC_{50} values using the Cheng–Prusoff equation.^{130,131} Any pK_i values used were either taken from the literature or were obtained by transforming the mean K_i value to pK_i or vice versa ($pK_i = -\log_{10}(K_i)$; $K_i = 10^{-pK_i}$). Any statistical significance tested for the P value was simplified to a summary with the following cutoffs: P value < 0.05: *; P value < 0.005: **; P value < 0.0005: ***.

■ ASSOCIATED CONTENT

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jmedchem.6c00587>.

Experimental synthesis details for all intermediate products; experimental details for all final compounds **4**, **10**, **13**, **30a–e**, **33–37**, **56a–o**, **70a–g**, **81a–f**, and **83**; 1H NMR and ^{13}C spectra for all final compounds **4**, **10**, **13**, **30a–e**, **33–37**, **56a–o**, **70a–g**, **81a–f**, and **83**; RP-HPLC chromatograms purity analysis of final compounds **4**, **10**, **13**, **30a–e**, **33–37**, **56a–o**, **70a–g**, **81a–f**, and **83**; RP-HPLC chromatograms: chemical stability of **35**, **56l**, **56m**, and **81d**; recovery rates and stability results in the human plasma/PBS of **56m** and **81d**; docking parameters used for all final compounds **4**, **10**, **13**, **30a–e**, **33–37**, **56a–o**, **70a–g**, **81a–f**, and **83**; generic Ballesteros–Weinstein numbering scheme for $hM_{1,3,5}R$; schematic structural comparison of the binding modes of tiotropium and compound **56m**; exemplary docking figures and RMSD for the use of crystal structures with different cocrystallized ligands or activation states; calculated ligand-to-ligand and overall RMSD values for the tiotropium redocking on hM_5R , including exemplary docking poses; radioligand competition binding curves of **4**, **56m**, **70g**, and **81d**; and concentration response curves of **4**, **13**, **56k–o**, **70c**, **70g**, **81d**, **81f**, and **83** on hM_5R and **4**, **56m**, and **81d** on hM_1R , hH_1R , and $hD_{2long}R$ (PDF) Molecular formula strings (CSV)

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Author Contributions

S. G. performed the synthesis and analytical characterization of chemical compounds and the investigation of stability in human plasma. S.G. performed the induced fit docking studies and processed the data. A.S. initiated and planned the project. A.S. and P.K. supervised the research. S.G. and A.S. wrote the manuscript. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

δ	chemical shift in ppm
Ac	acetyl
ACh	acetylcholine
Ar	aryl
AU	absorption units
Boc	<i>tert</i> -butoxycarbonyl
c	molar concentration
cAMP	cyclic adenosine monophosphate
CDCl ₃	deuterated chloroform
CHO	Chinese hamster ovary
cmpd	compound
COPD	chronic obstructive pulmonary disease
D	aspartic acid
DAD	diode array detector
DCM	dichloromethane
DIPEA	diisopropylethylamine
DMAP	4-dimethylaminopyridine
DMF	<i>N,N</i> -dimethylformamide
DMSO	dimethyl sulfoxide
DMSO- <i>d</i> ₆	deuterated DMSO
D _x R	dopamine receptor subtype x
EA	ethyl acetate
ECL	extracellular loop
EDC	1-Ethyl-3-(3-dimethylaminopropyl)carbo-diimide
EI	electron ionization
Et	ethyl
EtOH	ethanol
FLAG	polypeptide protein tag with sequence motif DYKDDDD
g	gram(s) or number of times the gravitational force
GPCR	G protein-coupled receptor
h	human

HATU	hexafluorophosphate azabenzotriazole tetra-methyl uronium
HBA	hydrogen bond acceptors
HEK293T	human embryonic kidney 293T cells
H ₁ R	histamine receptor subtype x
I	isoleucine
IC ₅₀	half maximal inhibitory concentration
K	lysine
K _i	dissociation constant obtained from a competi-tion binding experiment
KO	knockout
L	leucine
LC	liquid chromatography
L-15	Leibovitz's L-15 medium
Me	methyl
MeCN	acetonitrile
MeOH	methanol
MF	molecular formula
mG or mini-G	engineered minimal G protein
M ₃ R	muscarinic receptor subtype x
N	asparagine
NFSI	N-fluorobenzenesulfonimide
NMDA	N-methyl-D-aspartic acid
NMS	N-methylscopolamine
Nluc	nanoLuc luciferase
NlucC	C-terminal Nluc fragment
NlucN	N-terminal Nluc fragment
OMe	methoxy/methoxide
PE	petroleum ether
Ph	phenyl
pK _i	negative logarithm of the K _i in M
PLC	phospholipase C
Pr	propyl
Q	glutamine
Q-TOF	quadrupole time-of-flight
r	rat
RP-HPLC	reversed-phase HPLC
SAR	structure activity/affinity relationship
SEM	standard error of the mean
SI	Supporting Information
T	threonine
TEA	triethylamine
Tf	triflate
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TM	transmembrane domain
W	tryptophan
Y	tyrosine.

REFERENCES

- (1) Lurie, Y.; Wasser, S. P.; Taha, M.; Shehade, H.; Nijim, J.; Hoffmann, Y.; Basis, F.; Vardi, M.; Lavon, O.; Suaed, S.; Bisharat, B.; Bentur, Y. Mushroom poisoning from species of genus *Inocybe* (fiber head mushroom): a case series with exact species identification. *Clin. Toxicol.* **2009**, *47* (6), 562–565.
- (2) Waser, P. G. Chemistry and pharmacology of muscarine, muscarone, and some related compounds. *Pharmacol. Rev.* **1961**, *13*, 465–515.
- (3) Du, G.-H. *Natural Small Molecule Drugs from Plants*; Springer Singapore, 2018.10.1007/978-981-10-8022-7.
- (4) Tulp, M.; Bohlin, L. Functional versus chemical diversity: is biodiversity important for drug discovery? *Trends Pharmacol. Sci.* **2002**, *23* (5), 225–231.

- (5) Ashtiania, F. Tropane alkaloids of *Atropa belladonna* L. and *Atropa acuminata* Royle ex Miers plants. *J. Med. Plants Res.* **2011**, *5* (29), 6515–6522.
- (6) World Health Organization. *WHO Model Formulary 2008*, World Health Organization; World Health Organization, 2009.
- (7) World Health Organization. *WHO Model List of Essential Medicines 22nd List (2021)*; WHO Model List of Essential Medicines, 2021; Vol. 22.
- (8) Kato, M.; Komamura, K.; Kitakaze, M. Tiotropium, a Novel Muscarinic M3 Receptor Antagonist, Improved Symptoms of Chronic Obstructive Pulmonary Disease Complicated by Chronic Heart Failure. *Circ. J.* **2006**, *70*, 1658–1660.
- (9) Liu, Y.-J.; Peng, W.; Hu, M.-B.; Xu, M.; Wu, C.-J. The pharmacology, toxicology and potential applications of arecoline: a review. *Pharm. Biol.* **2016**, *54* (11), 2753–2760.
- (10) Li, M.; Pang, X.; Guo, Z.; Yang, Y.; Gu, Z.; Zhang, L. Integrated metabolomics and network pharmacology to reveal the mechanism of areca nut addiction. *Addict. Biol.* **2023**, *28* (12), No. e13352.
- (11) Gao, W.; He, Y.; Zhang, Y.; Sun, M.; Sun, Y. Comprehensive Insights into Arecoline Hydrobromide: Pharmacology, Toxicity, and Pharmacokinetics. *Med. Sci. Monit.* **2024**, *30*, No. e945582.
- (12) Giri, S.; Idle, J. R.; Chen, C.; Zabriskie, T. M.; Krausz, K. W.; Gonzalez, F. J. A metabolomic approach to the metabolism of the areca nut alkaloids arecoline and arecaidine in the mouse. *Chem. Res. Toxicol.* **2006**, *19* (6), 818–827.
- (13) Kilian, J.; Millard, M.; Ozenil, M.; Krause, D.; Ghaderi, K.; Holzer, W.; Urban, E.; Spreitzer, H.; Wadsak, W.; Hacker, M.; Langer, T.; Pichler, V. Synthesis, Biological Evaluation, and Docking Studies of Antagonistic Hydroxylated Arecaidine Esters Targeting mAChRs. *Molecules* **2022**, *27* (10), 3173.
- (14) Gupta, P. C.; Ray, C. S. Epidemiology of Betel Quid Usage. *Ann. Acad. Med.* **2004**, *33* (4 Suppl), 31–36.
- (15) Volgin, A. D.; Bashirzade, A.; Amstislavskaya, T. G.; Yakovlev, O. A.; Demin, K. A.; Ho, Y.-J.; Wang, D.; Shevyryn, V. A.; Yan, D.; Tang, Z.; Wang, J.; Wang, M.; Alpyshov, E. T.; Serikuly, N.; Wappler-Guzzetta, E. A.; Lakstygal, A. M.; Kalueff, A. V. DARK Classics in Chemical Neuroscience: Arecoline. *ACS Chem. Neurosci.* **2019**, *10* (5), 2176–2185.
- (16) Watson, J.; Brough, S.; Coldwell, M. C.; Gager, T.; Ho, M.; Hunter, A. J.; Jerman, J.; Middlemiss, D. N.; Riley, G. J.; Brown, A. M. Functional effects of the muscarinic receptor agonist, xanomeline, at 5-HT1 and 5-HT2 receptors. *Br. J. Pharmacol.* **1998**, *125*, 1413–1420.
- (17) Bender, A. M.; Jones, C. K.; Lindsley, C. W. Classics in Chemical Neuroscience: Xanomeline. *ACS Chem. Neurosci.* **2017**, *8* (3), 435–443.
- (18) Brannan, S. K.; Sawchak, S.; Miller, A. C.; Lieberman, J. A.; Paul, S. M.; Breier, A. Muscarinic Cholinergic Receptor Agonist and Peripheral Antagonist for Schizophrenia. *N. Engl. J. Med.* **2021**, *384* (8), 717–726.
- (19) Sauerberg, P.; Olesen, P. H.; Nielsen, S.; Treppendahl, S.; Sheardown, M. J.; Honore, T.; Mitch, C. H.; Ward, J. S.; Pike, A. J.; Bymaster, F. P.; Sawyer, B. D.; Shannon, H. E. Novel functional M1 selective muscarinic agonists. Synthesis and structure-activity relationships of 3-(1,2,5-thiadiazolyl)-1,2,5,6-tetrahydro-1-methylpyridines. *J. Med. Chem.* **1992**, *35* (12), 2274–2283.
- (20) Carmine, A. A.; Brogden, R. N. Pirenzepine. A review of its pharmacodynamic and pharmacokinetic properties and therapeutic efficacy in peptic ulcer disease and other allied diseases. *Drugs* **1985**, *30* (2), 85–126.
- (21) Croom, K. F.; Keating, G. M. Darifenacin: in the treatment of overactive bladder. *Drugs Aging* **2004**, *21* (13), 885–892.
- (22) Caulfield, M. P.; Birdsall, N. J. International Union of Pharmacology. XVII. Classification of Muscarinic Acetylcholine Receptors. *Pharmacol. Rev.* **1998**, *50* (2), 279–290.
- (23) Eglen, R. M. Muscarinic receptor subtype pharmacology and physiology. *Prog. Med. Chem.* **2005**, *43*, 105–136.
- (24) Caulfield, M. P. Muscarinic receptors - characterization, coupling and function. *Pharmacol. Ther.* **1993**, *58*, 319–379.

- (25) Ishii, M.; Kurachi, Y. Muscarinic Acetylcholine Receptors. *Curr. Pharm. Des.* **2006**, *12* (28), 3573–3581.
- (26) Yohn, S. E.; Weiden, P. J.; Felder, C. C.; Stahl, S. M. Muscarinic acetylcholine receptors for psychotic disorders: bench-side to clinic. *Trends Pharmacol. Sci.* **2022**, *43* (12), 1098–1112.
- (27) Lavrador, M.; Cabral, A. C.; Verissimo, M. T.; Fernandez-Llimos, F.; Figueiredo, I. V.; Castel-Branco, M. M. A Universal Pharmacological-Based List of Drugs with Anticholinergic Activity. *Pharmaceutics* **2023**, *15* (1), 230.
- (28) Alexander, S. P. H.; Christopoulos, A.; Davenport, A. P.; Kelly, E.; Mathie, A.; Peters, J. A.; Veale, E. L.; Armstrong, J. F.; Faccenda, E.; Harding, S. D.; Pawson, A. J.; Sharman, J. L.; Southan, C.; Davies, J. A. THE CONCISE GUIDE TO PHARMACOLOGY 2019/20: G protein-coupled receptors. *Br. J. Pharmacol.* **2019**, *176* (S1), S21–S141.
- (29) Eglén, R. M.; Nahorski, S. R. The muscarinic M5 receptor: a silent or emerging subtype? *Br. J. Pharmacol.* **2000**, *130*, 13–21.
- (30) Langmead, C. J.; Watson, J.; Reavill, C. Muscarinic acetylcholine receptors as CNS drug targets. *Pharmacol. Ther.* **2008**, *117* (2), 232–243.
- (31) Andersson, K.-E. Muscarinic acetylcholine receptors in the urinary tract. *Handb. Exp. Pharmacol.* **2011**, *202* (202), 319–344.
- (32) Vuckovic, Z.; Gentry, P. R.; Berizzi, A. E.; Hirata, K.; Varghese, S.; Thompson, G.; van der Westhuizen, E. T.; Burger, W. A. C.; Rahmani, R.; Valant, C.; Langmead, C. J.; Lindsley, C. W.; Baell, J. B.; Tobin, A. B.; Sexton, P. M.; Christopoulos, A.; Thal, D. M. Crystal structure of the M5 muscarinic acetylcholine receptor. *Proc. Natl. Acad. Sci. U.S.A.* **2019**, *116* (S1), 26001–26007.
- (33) Haga, K.; Kruse, A. C.; Asada, H.; Yurugi-Kobayashi, T.; Shiroishi, M.; Zhang, C.; Weis, W. I.; Okada, T.; Kobilka, B. K.; Haga, T.; Kobayashi, T. Structure of the human M2 muscarinic acetylcholine receptor bound to an antagonist. *Nature* **2012**, *482* (7386), 547–551.
- (34) 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* (7386), 552–556.
- (35) Thal, D. M.; Sun, B.; Feng, D.; Nawaratne, V.; Leach, K.; Felder, C. C.; Bures, M. G.; Evans, D. A.; Weis, W. I.; Bachhawat, P.; Kobilka, T. S.; Sexton, P. M.; Kobilka, B. K.; Christopoulos, A. Crystal structures of the M1 and M4 muscarinic acetylcholine receptors. *Nature* **2016**, *531* (7594), 335–340.
- (36) Vilaro, M. T.; Palacios, J. M.; Mengod, G. Localization of M5 muscarinic receptor mRNA in rat brain examined by in situ hybridization histochemistry. *Neurosci. Lett.* **1990**, *114*, 154–159.
- (37) Levey, A. I. Immunological localization of M1-M5 muscarinic acetylcholine receptors in peripheral tissues and brain. *Life Sci.* **1993**, *52* (5–6), 441–448.
- (38) Foster, D. J.; Gentry, P. R.; Lizardi-Ortiz, J. E.; Bridges, T. M.; Wood, M. R.; Niswender, C. M.; Sulzer, D.; Lindsley, C. W.; Xiang, Z.; Conn, P. J. M5 receptor activation produces opposing physiological outcomes in dopamine neurons depending on the receptor's location. *J. Neurosci.* **2014**, *34* (9), 3253–3262.
- (39) Berizzi, A. E.; Gentry, P. R.; Rueda, P.; Den Hoedt, S.; Sexton, P. M.; Langmead, C. J.; Christopoulos, A. Molecular Mechanisms of Action of M5 Muscarinic Acetylcholine Receptor Allosteric Modulators. *Mol. Pharmacol.* **2016**, *90* (4), 427–436.
- (40) Yamada, M.; Lamping, K. G.; Duttaroy, A.; Zhang, W.; Cui, Y.; 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 M5 muscarinic acetylcholine receptor knockout mice. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98* (24), 14096–14101.
- (41) Razidlo, J. A.; Fausner, S. M. L.; Ingebreton, A. E.; Wang, L. C.; Petersen, C. L.; Mirza, S.; Swank, I. N.; Alvarez, V. A.; Lemos, J. C. Chronic Loss of Muscarinic M5 Receptor Function Manifests Disparate Impairments in Exploratory Behavior in Male and Female Mice despite Common Dopamine Regulation. *J. Neurosci.* **2022**, *42* (36), 6917–6930.
- (42) Wess, J. Muscarinic acetylcholine receptor knockout mice: novel phenotypes and clinical implications. *Annu. Rev. Pharmacol. Toxicol.* **2004**, *44*, 423–450.
- (43) Wess, J.; Eglén, R. M.; Gautam, D. Muscarinic acetylcholine receptors: mutant mice provide new insights for drug development. *Nat. Rev. Drug Discovery* **2007**, *6* (9), 721–733.
- (44) Fink-Jensen, A.; Fedorova, I.; Wörtwein, G.; Woldbye, D. P.; Rasmussen, T.; Thomsen, M.; Bolwig, T. G.; Knitowski, K. M.; McKinzie, D. L.; Yamada, M.; Wess, J.; Basile, A. S. Role for M5 muscarinic acetylcholine receptors in cocaine addiction. *J. Neurosci. Res.* **2003**, *74*, 91–96.
- (45) Basile, A. S.; Federova, I.; Zapata, A.; Liu, X.; Shippenberg, T.; Duttaroy, A.; Yamada, M.; Wess, J. Deletion of the M5 muscarinic acetylcholine receptor attenuates morphine reinforcement and withdrawal but not morphine analgesia. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99* (17), 11452–11457.
- (46) Bender, A. M.; Garrison, A. T.; Lindsley, C. W. The Muscarinic Acetylcholine Receptor M5: Therapeutic Implications and Allosteric Modulation. *ACS Chem. Neurosci.* **2019**, *10* (3), 1025–1034.
- (47) Araya, R.; Noguchi, T.; Yuhki, M.; Kitamura, N.; Higuchi, M.; Saido, T. C.; Seki, K.; Itoharu, S.; Kawano, M.; Tanemura, K.; Takashima, A.; Yamada, K.; Kondoh, Y.; Kanno, I.; Wess, J.; Yamada, M. Loss of M5 muscarinic acetylcholine receptors leads to cerebrovascular and neuronal abnormalities and cognitive deficits in mice. *Neurobiol. Dis.* **2006**, *24* (2), 334–344.
- (48) Teal, L. B.; Bubser, M.; Duncan, E.; Gould, R. W.; Lindsley, C. W.; Jones, C. K. Selective M5 muscarinic acetylcholine receptor negative allosteric modulator VU6008667 blocks acquisition of opioid self-administration. *Neuropharmacology* **2023**, *227*, 109424.
- (49) Kruse, A. C.; Kobilka, B. K.; Gautam, D.; Sexton, P. M.; Christopoulos, A.; Wess, J. Muscarinic acetylcholine receptors: novel opportunities for drug development. *Nat. Rev. Drug Discovery* **2014**, *13* (7), 549–560.
- (50) Berizzi, A. E.; Perry, C. J.; Shackelford, D. M.; Lindsley, C. W.; Jones, C. K.; Chen, N. A.; Sexton, P. M.; Christopoulos, A.; Langmead, C. J.; Lawrence, A. J. Muscarinic M5 receptors modulate ethanol seeking in rats. *Neuropsychopharmacology* **2018**, *43* (7), 1510–1517.
- (51) Gould, R. W.; Gunter, B. W.; Bubser, M.; Matthews, R. T.; Teal, L. B.; Ragland, M. G.; Bridges, T. M.; Garrison, A. T.; Winder, D. G.; Lindsley, C. W.; Jones, C. K. Acute Negative Allosteric Modulation of M5 Muscarinic Acetylcholine Receptors Inhibits Oxycodone Self-Administration and Cue-Induced Reactivity with No Effect on Antinociception. *ACS Chem. Neurosci.* **2019**, *10* (8), 3740–3750.
- (52) Tsimpili, H.; Zoidis, G. A New Era of Muscarinic Acetylcholine Receptor Modulators in Neurological Diseases, Cancer and Drug Abuse. *Pharmaceutics* **2025**, *18* (3), 369.
- (53) Foster, D. J. Muscarinic receptors: from clinic to bench to clinic. *Trends Pharmacol. Sci.* **2022**, *43* (6), 461–463.
- (54) Conn, P. J.; Jones, C. K.; Lindsley, C. W. Subtype-selective allosteric modulators of muscarinic receptors for the treatment of CNS disorders. *Trends Pharmacol. Sci.* **2009**, *30* (3), 148–155.
- (55) Xu, Z.; Adilijiang, A.; Wang, W.; You, P.; Lin, D.; Li, X.; He, J. Arecoline attenuates memory impairment and demyelination in a cuprizone-induced mouse model of schizophrenia. *NeuroReport* **2019**, *30* (2), 134–138.
- (56) United Nations Office on Drugs and Crime. *World Drug Report 2024: Special Points of Interest*; United Nations Office on Drugs and Crime, 2024.
- (57) World Health Organization. *Global Status Report on the Public Health Response to Dementia*, 1st ed.; World Health Organization, 2021.
- (58) World Health Organization 2024. *Global Status Report on Alcohol and Health and Treatment of Substance Use Disorders*; World Health Organization, 2024.
- (59) Deutsche Gesellschaft für Psychiatrie und Psychotherapie, Psychosomatik und Nervenheilkunde e. V. (DGPPN). *S3-Leitlinie Demenzen Living Guideline*; Deutsche Gesellschaft für Neurologie e. V. Guideline for Alzheimer's disease, 2025.

- (60) Birks, J. S.; Harvey, R. J. Donepezil for dementia due to Alzheimer's disease. *Cochrane Database Syst. Rev.* **2018**, 2018 (6), CD001190.
- (61) Fink, H. A.; Linskens, E. J.; MacDonald, R.; Silverman, P. C.; McCarten, J. R.; Talley, K. M. C.; Forte, M. L.; Desai, P. J.; Nelson, V. A.; Miller, M. A.; Hemmy, L. S.; Brasure, M.; Taylor, B. C.; Ng, W.; Ouellette, J. M.; Sheets, K. M.; Wilt, T. J.; Butler, M. Benefits and Harms of Prescription Drugs and Supplements for Treatment of Clinical Alzheimer-Type Dementia. *Ann. Intern. Med.* **2020**, 172 (10), 656–668.
- (62) Myslivecek, J. Multitargeting nature of muscarinic orthosteric agonists and antagonists. *Front. Physiol.* **2022**, 13, 974160.
- (63) GPCRdb. Sequence alignments of all human muscarinic receptors. <https://gpcrdb.org/alignment/targetselection> (accessed Aug 25, 2025).
- (64) GPCRdb. Structure similarity and homology within the muscarine receptor family MxR. <https://gpcrdb.org/similaritysearch/referenceselection> (accessed Sep 01, 2025).
- (65) Goodwin, J. A.; Hulme, E. C.; Langmead, C. J.; Tehan, B. G. Roof and floor of the muscarinic binding pocket: variations in the binding modes of orthosteric ligands. *Mol. Pharmacol.* **2007**, 72 (6), 1484–1496.
- (66) Burger, W. A. C.; Gentry, P. R.; Berizzi, A. E.; Vuckovic, Z.; van der Westhuizen, E. T.; Thompson, G.; Yeasmin, M.; Lindsley, C. W.; Sexton, P. M.; Langmead, C. J.; Tobin, A. B.; Christopoulos, A.; Valant, C.; Thal, D. M. Identification of a Novel Allosteric Site at the M5 Muscarinic Acetylcholine Receptor. *ACS Chem. Neurosci.* **2021**, 12 (16), 3112–3123.
- (67) Gunter, B. W.; Gould, R. W.; Bubser, M.; McGowan, K. M.; Lindsley, C. W.; Jones, C. K. Selective inhibition of M5 muscarinic acetylcholine receptors attenuates cocaine self-administration in rats. *Addict. Biol.* **2018**, 23 (5), 1106–1116.
- (68) Jakubik, J.; El-Fakahany, E. E. Current Advances in Allosteric Modulation of Muscarinic Receptors. *Biomolecules* **2020**, 10 (2), 325.
- (69) Gentry, P. R.; Kokubo, M.; Bridges, T. M.; Cho, H. P.; Smith, E.; Chase, P.; Hodder, P. S.; Utley, T. J.; Rajapakse, A.; Byers, F.; Niswender, C. M.; Morrison, R. D.; Daniels, J. S.; Wood, M. R.; Conn, P. J.; Lindsley, C. W. Discovery, synthesis and characterization of a highly muscarinic acetylcholine receptor (mAChR)-selective M5-orthosteric antagonist, VU0488130 (ML381): a novel molecular probe. *ChemMedChem* **2014**, 9 (8), 1677–1682.
- (70) Li, J.; Orsi, D. L.; Engers, J. L.; Long, M. F.; Capstick, R. A.; Maurer, M. A.; Presley, C. C.; Vinson, P. N.; Rodriguez, A. L.; Han, A.; Cho, H. P.; Chang, S.; Jackson, M.; Bubser, M.; Blobaum, A. L.; Boutaud, O.; Nader, M. A.; Niswender, C. M.; Conn, P. J.; Jones, C. K.; Lindsley, C. W.; Han, C. Development of VU6036864: A Triazolopyridine-Based High-Quality Antagonist Tool Compound of the M5 Muscarinic Acetylcholine Receptor. *J. Med. Chem.* **2024**, 67 (16), 14394–14413.
- (71) Garrison, A. T.; Orsi, D. L.; Capstick, R. A.; Whomble, D.; Li, J.; Carter, T. R.; Felts, A. S.; Vinson, P. N.; Rodriguez, A. L.; Han, A.; Hajari, K.; Cho, H. P.; Teal, L. B.; Ragland, M. G.; Ghamari-Langroudi, M.; Bubser, M.; Chang, S.; Schnetz-Boutaud, N. C.; Boutaud, O.; Blobaum, A. L.; Foster, D. J.; Niswender, C. M.; Conn, P. J.; Lindsley, C. W.; Jones, C. K.; Han, C. Development of VU6019650: A Potent, Highly Selective, and Systemically Active Orthosteric Antagonist of the M5 Muscarinic Acetylcholine Receptor for the Treatment of Opioid Use Disorder. *J. Med. Chem.* **2022**, 65 (8), 6273–6286.
- (72) Orsi, D. L.; Felts, A. S.; Rodriguez, A. L.; Vinson, P. N.; Cho, H. P.; Chang, S.; Blobaum, A. L.; Niswender, C. M.; Conn, P. J.; Jones, C. K.; Lindsley, C. W.; Han, C. Discovery of a potent M5 antagonist with improved clearance profile. Part 2: Pyrrolidine amide-based antagonists. *Bioorg. Med. Chem. Lett.* **2022**, 78, 129021.
- (73) Burger, W. A. C.; Mobbs, J. I.; Rana, B.; Wang, J.; Joshi, K.; Gentry, P. R.; Yeasmin, M.; Venugopal, H.; Bender, A. M.; Lindsley, C. W.; Miao, Y.; Christopoulos, A.; Valant, C.; Thal, D. M. Cryo-EM reveals a new allosteric binding site at the M5 mAChR. *bioRxiv* **2025**, 2025.02.05.636602.
- (74) Valant, C.; Sexton, P. M.; Christopoulos, A. Orthosteric/Allosteric bitopic ligands-going hybrid at GPCRs. *Mol. Interventions* **2009**, 9 (3), 125.
- (75) Lane, J. R.; Sexton, P. M.; Christopoulos, A. Bridging the gap: bitopic ligands of G-protein-coupled receptors. *Trends Pharmacol. Sci.* **2013**, 34 (1), 59–66.
- (76) Pegoli, A.; She, X.; Wifling, D.; Hübner, H.; Bernhardt, G.; Gmeiner, P.; Keller, M. Radiolabeled Dibenzodiazepinone-Type Antagonists Give Evidence of Dualsteric Binding at the M2 Muscarinic Acetylcholine Receptor. *J. Med. Chem.* **2017**, 60 (8), 3314–3334.
- (77) She, X.; Pegoli, A.; Mayr, J.; Hübner, H.; Bernhardt, G.; Gmeiner, P.; Keller, M. Heterodimerization of Dibenzodiazepinone-Type Muscarinic Acetylcholine Receptor Ligands Leads to Increased M2R Affinity and Selectivity. *ACS Omega* **2017**, 2 (10), 6741–6754.
- (78) Augelli-Szafran, C. E.; Blankley, C. J.; Jaen, J. C.; Moreland, D. W.; Nelson, C. B.; Penrose-Yi, J. R.; Schwarz, R. D.; Thomas, A. J. Identification and characterization of M1 selective muscarinic receptor antagonists. *J. Med. Chem.* **1999**, 42 (3), 356–363.
- (79) Zheng, G.; Smith, A. M.; Huang, X.; Subramanian, K. L.; Siripurapu, K. B.; Deaciuc, A.; Zhan, C.-G.; Dwoskin, L. P. Structural modifications to tetrahydropyridine-3-carboxylate esters en route to the discovery of M5-preferring muscarinic receptor orthosteric antagonists. *J. Med. Chem.* **2013**, 56 (4), 1693–1703.
- (80) Scior, T.; Bernard, P.; Medina-Franco, J. L.; Maggiora, G. M. Large compound databases for structure-activity relationships studies in drug discovery. *Mini Rev. Med. Chem.* **2007**, 7 (8), 851–860.
- (81) Scior, T.; Bender, A.; Tresadern, G.; Medina-Franco, J. L.; Martínez-Mayorga, K.; Langer, T.; Cuanalo-Contreras, K.; Agrafiotis, D. K. Recognizing pitfalls in virtual screening: a critical review. *J. Chem. Inf. Model.* **2012**, 52 (4), 867–881.
- (82) Hammer, S. G.; Gobleder, S.; Naporra, F.; Wittmann, H.-J.; Elz, S.; Heinrich, M. R.; Strasser, A. 2,4-Diaminopyrimidines as dual ligands at the histamine H1 and H4 receptor-H1/H4-receptor selectivity. *Bioorg. Med. Chem. Lett.* **2016**, 26 (2), 292–300.
- (83) Geyer, R.; Nordemann, U.; Strasser, A.; Wittmann, H.-J.; Buschauer, A. Conformational Restriction and Enantioseparation Increase Potency and Selectivity of Cyanoguanidine-Type Histamine H4 Receptor Agonists. *J. Med. Chem.* **2016**, 59 (7), 3452–3470.
- (84) Wittmann, H.-J.; Strasser, A. Binding pathway of histamine to the hH4R, observed by unconstrained molecular dynamics. *Bioorg. Med. Chem. Lett.* **2015**, 25 (6), 1259–1268.
- (85) Asiamah, I.; Obiri, S. A.; Tamekloe, W.; Armah, F. A.; Borquaye, L. S. Applications of molecular docking in natural products-based drug discovery. *Sci. Afr.* **2023**, 20, No. e01593.
- (86) Leelananda, S. P.; Lindert, S. Computational methods in drug discovery. *Beilstein J. Org. Chem.* **2016**, 12, 2694–2718.
- (87) Naporra, F.; Gobleder, S.; Wittmann, H.-J.; Spindler, J.; Bodensteiner, M.; Bernhardt, G.; Hübner, H.; Gmeiner, P.; Elz, S.; Strasser, A. Dibenzo[b,f][1,4]oxazepines and dibenzo[b,e]oxepines: Influence of the chlorine substitution pattern on the pharmacology at the H1R, H4R, 5-HT2AR and other selected GPCRs. *Pharmacol. Res.* **2016**, 113 (Pt A), 610–625.
- (88) Yamali, C.; Gul, H. I.; Tugrak Sakarya, M.; Nurpelin Saglik, B.; Ece, A.; Demirel, G.; Nenni, M.; Levent, S.; Cihat Oner, A. Quinazolinone-based benzenesulfonamides with low toxicity and high affinity as monoamine oxidase-A inhibitors: Synthesis, biological evaluation and induced-fit docking studies. *Bioorg. Chem.* **2022**, 124, 105822.
- (89) Friesner, R. A.; Banks, J. L.; Murphy, R. B.; Halgren, T. A.; Klicic, J. J.; Mainz, D. T.; Repasky, M. P.; Knoll, E. H.; Shelley, M.; Perry, J. K.; Shaw, D. E.; Francis, P.; Shenkin, P. S. Glide: a new approach for rapid, accurate docking and scoring. 1. Method and assessment of docking accuracy. *J. Med. Chem.* **2004**, 47 (7), 1739–1749.
- (90) Friesner, R. A.; Murphy, R. B.; Repasky, M. P.; Frye, L. L.; Greenwood, J. R.; Halgren, T. A.; Sanschagrin, P. C.; Mainz, D. T. Extra precision glide: docking and scoring incorporating a model of hydrophobic enclosure for protein-ligand complexes. *J. Med. Chem.* **2006**, 49 (21), 6177–6196.

- (91) Salmon, M.; Luttmann, M. A.; Foley, J. J.; Buckley, P. T.; Schmidt, D. B.; Burman, M.; Webb, E. F.; DeHaas, C. J.; Kotzer, C. J.; Barrett, V. J.; Slack, R. J.; Sarau, H. M.; Palovich, M. R.; Lainé, D. I.; Hay, D. W. P.; Rumsey, W. L. Pharmacological characterization of GSK573719 (umecclidinium): a novel, long-acting, inhaled antagonist of the muscarinic cholinergic receptors for treatment of pulmonary diseases. *J. Pharmacol. Exp. Ther.* **2013**, *345* (2), 260–270.
- (92) Dörje, F.; Wess, J.; Lambrecht, G.; Tacke, R.; Mutschler, E.; Brann, M. R. Antagonist Binding Profiles of Five Cloned Human Muscarinic Receptor Subtypes. *J. Pharmacol. Exp. Ther.* **1990**, *256* (2), 727–733.
- (93) Sellers, D. J.; Chess-Williams, R. Muscarinic agonists and antagonists: effects on the urinary bladder. *Handb. Exp. Pharmacol.* **2012**, *208* (208), 375–400.
- (94) Krystal, A. D.; Richelson, E.; Roth, T. Review of the histamine system and the clinical effects of H1 antagonists: basis for a new model for understanding the effects of insomnia medications. *Sleep Med. Rev.* **2013**, *17* (4), 263–272.
- (95) Ballesteros, J. A.; Weinstein, H. [19] Integrated methods for the construction of three-dimensional models and computational probing of structure-function relations in G protein-coupled receptors. In *Receptor Molecular Biology; Methods in Neurosciences*; Elsevier, 1995; pp 366–428.10.1016/S1043-9471(05)80049-7.
- (96) Weinhart, C. G.; Wifling, D.; Schmidt, M. F.; Neu, E.; Höring, C.; Clark, T.; Gmeiner, P.; Keller, M. Dibenzodiazepinone-type muscarinic receptor antagonists conjugated to basic peptides: Impact of the linker moiety and unnatural amino acids on M2R selectivity. *Eur. J. Med. Chem.* **2021**, *213*, 113159.
- (97) Höring, C.; Seibel, U.; Tropmann, K.; Grätz, L.; Mönnich, D.; Pitzl, S.; Bernhardt, G.; Pockes, S.; Strasser, A. A. Dynamic, Split-Luciferase-Based Mini-G Protein Sensor to Functionally Characterize Ligands at All Four Histamine Receptor Subtypes. *Int. J. Mol. Sci.* **2020**, *21* (22), 8440.
- (98) Appl, H.; Holzammer, T.; Dove, S.; Haen, E.; Straßer, A.; Seifert, R. Interactions of recombinant human histamine H₁R, H₂R, H₃R, and H₄R receptors with 34 antidepressants and antipsychotics. *Naunyn-Schmiedeberg's Arch Pharmacol* **2012**, *385* (2), 145–170.
- (99) Strasser, A.; Wittmann, H.-J.; Kunze, M.; Elz, S.; Seifert, R. Molecular basis for the selective interaction of synthetic agonists with the human histamine H1-receptor compared with the guinea pig H1-receptor. *Mol. Pharmacol.* **2009**, *75* (3), 454–465.
- (100) Vass, M.; Podlowska, S.; de Esch, I. J. P.; Bojarski, A. J.; Leurs, R.; Kooistra, A. J.; de Graaf, C. Aminergic GPCR-Ligand Interactions: A Chemical and Structural Map of Receptor Mutation Data. *J. Med. Chem.* **2019**, *62* (8), 3784–3839.
- (101) Spalding, T. A.; Burstein, E. S.; Henderson, S. C.; Ducote, K. R.; Brann, M. R. Identification of a Ligand-dependent Switch within a Muscarinic Receptor. *J. Biol. Chem.* **1998**, *273* (34), 21563–21568.
- (102) Littmann, T.; Ozawa, T.; Hoffmann, C.; Buschauer, A.; Bernhardt, G. A split luciferase-based probe for quantitative proximal determination of Gαq signalling in live cells. *Sci. Rep.* **2018**, *8* (1), 17179.
- (103) Ghoneim, O. M.; Legere, J. A.; Golbraikh, A.; Tropsha, A.; Booth, R. G. Novel ligands for the human histamine H1 receptor: synthesis, pharmacology, and comparative molecular field analysis studies of 2-dimethylamino-5-(6)-phenyl-1,2,3,4-tetrahydronaphthalenes. *Bioorg. Med. Chem.* **2006**, *14* (19), 6640–6658.
- (104) Bolden, C.; Cusack, B.; Richelson, E. Antagonism by antimuscarinic and neuroleptic compounds at the five cloned human muscarinic cholinergic receptors expressed in Chinese hamster ovary cells. *J. Pharmacol. Exp. Ther.* **1992**, *260* (2), 576–580.
- (105) GPCRdb. Structure similarity and homology between hMSR and all histamine and dopamine receptors. <https://gpcrdb.org/similaritysearch/referenceselection> (accessed Sep 01, 2025).
- (106) GPCRdb. Generic residue number - Histamine H1 receptor. <https://gpcrdb.org/residue/residuetable> (accessed Sep 18, 2025).
- (107) GPCRdb. Generic residue number - Dopamine D2 receptor. <https://gpcrdb.org/residue/residuetable> (accessed Sep 18, 2025).
- (108) Seifert, R.; Wenzel-Seifert, K.; Burckstummer, T.; Pertz, H. H.; Schunack, W.; Dove, S.; Buschauer, A.; Elz, S. Multiple differences in agonist and antagonist pharmacology between human and guinea pig histamine H1-receptor. *J. Pharmacol. Exp. Ther.* **2003**, *305* (3), 1104–1115.
- (109) Forster, L.; Grätz, L.; Mönnich, D.; Bernhardt, G.; Pockes, S. A Split Luciferase Complementation Assay for the Quantification of β -Arrestin2 Recruitment to Dopamine D2-Like Receptors. *Int. J. Mol. Sci.* **2020**, *21* (17), 6103.
- (110) de Souza, H. M. R.; Guedes, J. S.; Freitas, R. H. C. N.; Gelves, L. G. V.; Fokoue, H. H.; Sant'Anna, C. M. R.; Barreiro, E. J.; Lima, L. M. Comparative chemical and biological hydrolytic stability of homologous esters and isosteres. *J. Enzyme Inhib. Med. Chem.* **2022**, *37* (1), 718–727.
- (111) Reissmann, S.; Greiner, G. Hydrolysis of peptide esters by different enzymes. *Int. J. Pept. Protein Res.* **1992**, *40* (2), 110–113.
- (112) Mendes, E.; Furtado, T.; Neres, J.; Iley, J.; Jarvinen, T.; Rautio, J.; Moreira, R. Synthesis, stability and in vitro dermal evaluation of aminocarbonyloxymethyl esters as prodrugs of carboxylic acid agents. *Bioorg. Med. Chem.* **2002**, *10* (3), 809–816.
- (113) Jordan, C. G. How an increase in the carbon chain length of the ester moiety affects the stability of a homologous series of oxprenolol esters in the presence of biological enzymes. *J. Pharmaceut. Sci.* **1998**, *87* (7), 880–885.
- (114) GPCRdb. Ligand site mutations - Mutations. <https://gpcrdb.org/mutations/> (accessed Aug 25, 2025).
- (115) Altenbach, C.; Kusnetzow, A. K.; Ernst, O. P.; Hofmann, K. P.; Hubbell, W. L. High-resolution distance mapping in rhodopsin reveals the pattern of helix movement due to activation. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105* (21), 7439–7444.
- (116) Farrens, D. L.; Altenbach, C.; Yang, K.; Hubbell, W. L.; Khorana, H. G. Requirement of Rigid-Body Motion of Transmembrane Helices for Light Activation of Rhodopsin. *Science* **1996**, *274*, 768–770.
- (117) Deupi, X.; Standfuss, J.; Schertler, G. Conserved activation pathways in G-protein-coupled receptors. *Biochem. Soc. Trans.* **2012**, *40* (2), 383–388.
- (118) Gether, U.; Asmar, F.; Meinild, A. K.; Rasmussen, S. G. F. Structural basis for activation of G-protein-coupled receptors. *Pharmacol. Toxicol.* **2002**, *91* (6), 304–312.
- (119) Dodda, L. S.; Campos, S.; Ciccone, D.; Carreiro, S.; Leit, S.; Brennan, D.; Zephyr, J.; Jacques-O'Hagan, S.; Kumar, S.; Kuo, F.-S.; Shaik, M. M.; Price, D. J.; Loh, C.; Edmondson, S. D.; Tummino, P.; Kaila, N. Knowledge and Structure-Based Drug Design of 15-PGDH Inhibitors. *J. Med. Chem.* **2025**, *68* (17), 18436–18462.
- (120) Leffler, A. E.; Houang, E. M.; Gray, F.; Placzek, A. T.; Ruvinsky, A. M.; Bell, J. A.; Wang, H.; Sun, S.; Svensson, M.; Greenwood, J. R.; Frye, L. L.; Igawa, H.; Atsriku, C.; Levinson, A. M. Exploiting Solvent Exposed Salt-Bridge Interactions for the Discovery of Potent Inhibitors of SOS1 Using Free-Energy Perturbation Simulations. *ACS Med. Chem. Lett.* **2025**, *16* (3), 444–453.
- (121) Okabe, A.; Carney, D. W.; Tawada, M.; Akther, T.; Aida, J.; Takagi, T.; Dougan, D. R.; Leffler, A. E.; Bell, J. A.; Frye, L.; Hickey, E. R.; Komandla, M.; Tao, W.; Selimkhanov, J.; Yonemori, K.; Chang, E.; Saikatendu, K.; Ochida, A. Discovery of Highly Potent Noncovalent Inhibitors of SARS-CoV-2 Main Protease through Computer-Aided Drug Design. *J. Med. Chem.* **2025**, *68* (20), 21330–21345.
- (122) Gruber, C. G.; Pegoli, A.; Müller, C.; Grätz, L.; She, X.; Keller, M. Differently fluorescence-labelled dibenzodiazepinone-type muscarinic acetylcholine receptor ligands with high M2R affinity. *RSC Med. Chem.* **2020**, *11* (7), 823–832.
- (123) Szabó, R.; Szepesi Kovács, D.; Kiss, D. J.; Yazdi, Z. N.; Tóth, A. D.; Brea, J.; Loza, M. I.; Meszéna, D.; Wittner, L.; Ulbert, I.; Volk, B.; Hunyady, L.; Keserü, G. M. Design, Synthesis, and Evaluation of a New Fluorescent Ligand for the M2 Muscarinic Acetylcholine Receptor. *ACS Med. Chem. Lett.* **2025**, *16* (4), 552–559.
- (124) Miller, E. B.; Murphy, R. B.; Sindhikara, D.; Borrelli, K. W.; Grisewood, M. J.; Ranalli, F.; Dixon, S. L.; Jerome, S.; Boyles, N. A.; Day, T.; Ghanakota, P.; Mondal, S.; Rafi, S. B.; Troast, D. M.; Abel, R.; Friesner, R. A. Reliable and Accurate Solution to the Induced Fit

Docking Problem for Protein-Ligand Binding. *J. Chem. Theory Comput.* **2021**, *17* (4), 2630–2639.

(125) Silky, S.; Neerupma, D.; Arun, G. Inhibitory Perspective of New Synthesized Compounds against Angiotensin Receptor: Schrodinger-based Induced-Fit Molecular Docking. *J. Pharm. Res. Int.* **2021**, 79–87.

(126) Velmurugan, D.; Vijayakumar, B.; Parasuraman, S.; Raveendran, R. Identification of natural inhibitors against angiotensin I converting enzyme for cardiac safety using induced fit docking and MM-GBSA studies. *Pharmacogn. Mag.* **2014**, *10* (39), S639–S644.

(127) Zhong, H.; Tran, L. M.; Stang, J. L. Induced-fit docking studies of the active and inactive states of protein tyrosine kinases. *J. Mol. Graphics Modell.* **2009**, *28* (4), 336–346.

(128) Ertl, F. J.; Kopanchuk, S.; Dijon, N. C.; Veikšina, S.; Tahk, M.-J.; Laasfeld, T.; Schettler, F.; Gattor, A. O.; Hübner, H.; Archipowa, N.; Köckenberger, J.; Heinrich, M. R.; Gmeiner, P.; Kutta, R. J.; Holliday, N. D.; Rinken, A.; Keller, M. Dually Labeled Neurotensin NTS1R Ligands for Probing Radiochemical and Fluorescence-Based Binding Assays. *J. Med. Chem.* **2024**, *67* (18), 16664–16691.

(129) Schindler, L.; Bernhardt, G.; Keller, M. Modifications at Arg and Ile Give Neurotensin(8–13) Derivatives with High Stability and Retained NTS1 Receptor Affinity. *ACS Med. Chem. Lett.* **2019**, *10* (6), 960–965.

(130) 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* (23), 3099–3108.

(131) Craig, D. A. The Cheng-Prusoff relationship: something lost in the translation. *Trends Pharmacol. Sci.* **1993**, *14* (3), 89–91.