Design, synthesis, and biological evaluation of orthosteric ligands for the muscarinic acetylcholine receptors

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Background

The trend for developing new bioactive entities for the central nervous system (CNS) has increased significantly in recent years. Despite progress being made, the success of many, at first glance, promising potential drugs or diagnostics is extremely unlikely. An important target is the muscarinic acetylcholine receptor (mAChR) family, which plays a crucial role in neurochemical processes and is involved in a myriad of diseases if perturbated. Consisting of five different subtypes with highly conserved orthosteric binding sites, mAChRs contribute to the complexity of the CNS. The aim of this project was to develop and validate potent and selective orthosteric ligands for mAChR subtypes for potential diagnostic or therapeutic purposes. We used a novel pirenzepine rearrangement product, which was recently discovered by our work group.[1] Previous results showed that the pirenzepine rearrangement product's binding affinity is in the micromolar to high nanomolar range with decent subtype-selectivity, while having a low logP – an ideal starting point for this drug discovery project.

Methods

Throughout our work, the decision-making was guided by *in silico* methods. First, the rearrangement product was docked *via* Autodock Vina into several crystal structures (e.g., 5CXV for M1), followed by an inspection of space-filling properties and ligand-protein interactions. Results were compared to high-affinity co-crystallized ligands. This led to a structural reduction of the rearrangement product. For the following modifications, piperazine bioisosteres were computationally selected *via* a workflow previously used by our group.[2] Briefly, commercially available amines with sterics and electronics similar to piperazine were selected and used for bioisosteric replacement. The virtual derivatives were docked against the orthosteric binding sites of mAChRs crystal structures and scored accordingly. Docking poses lacking an ionic N-ASP interaction were omitted. Furthermore, the scaffold hop was extended by opening the ring system of the phenyl-benzimidazole scaffold and introducing a carbamate moiety. After a final inspection, 15 potential ligands were selected for synthesis and biological evaluation.

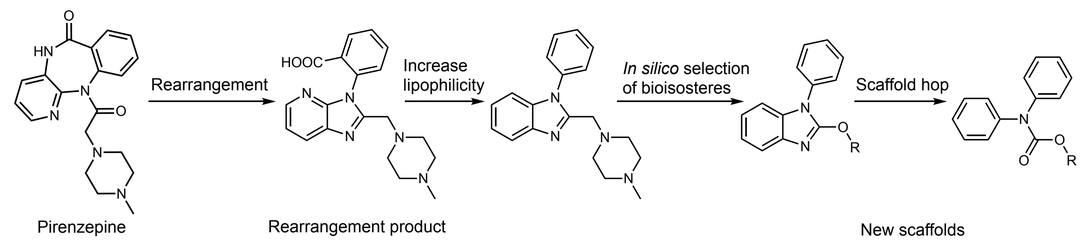
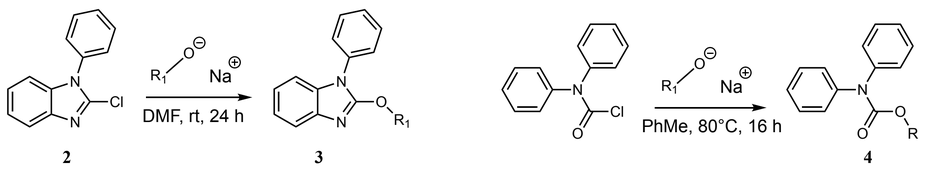


Figure 1: Overview of the workflow towards novel mAChRs orthosteric ligands.

The compounds were synthesized by S_NAr and carbamoylation of the chlorinated precursors and the corresponding alkoxides. The structures were characterized *via* 2D-NMR, HRMS, and purity confirmed with HPLC. Physico-chemical properties such as XlogP were calculated using the CORINA Symphony suit. LogPs at physiological pH were experimentally confirmed by HPLC. The antagonistic agonistic response was evaluated with a Fluo-4 Direct™ Calcium Assay Kit on stably transfected CHO-M1 cells with carbachol and scopolamine as a positive and negative control. Biological affinities towards all five subtypes were assessed *via* a competitive radiolabeling assay.

Table 1: Final steps of the synthesis route and yields.



2 3 4

Compound	Yield (%)	Compound	Yield (%)	Compound	Yield (%)	Compound	Yield (%)
3a	75	**4a**	**70**	**3e**	47	**4f**	**lit. known**
3b	72	**4b**	**76**	**3f**	78	**4f**	**76**
3c	82	**4c**	**63**	**3g**	75	**4g**	**13**
3d	65	**4d**	**74**	**3h**	69	**4h**	**71**

[1] Ozenil, M., Skos, L., Roller, A. et al. Unexpected scaffold rearrangement product of pirenzepine found in commercial samples. Sci Rep 11, 23397 (2021)
[2] Kilian, J.; Ozenil, M.; Millard, M.; Fürtös, D.; Maisetschläger, V.; Holzer, W.; Wadsak, W.; Hacker, M.; Langer, T.; Pichler, V. Design, Synthesis, and Biological Evaluation of 4,4'-Difluorobenzhydrol Carbamates as Selective M1 Antagonists. Pharmaceuticals 2022,

Results and Discussion

Overall, 15 compounds were successfully synthesized in good yields and structurally characterized. Calculated XlogP values of 2.3-3.8 (Table 2) imply a narrow lipophilicity range. Assessment of the experimental lipophilicity under physiological pH showed HPLC-logP values of 1.2-3.2. However, **3e** appears to be an outlier, and its low HPLC-logP of 1.2 may be partly explained by the high pK_a of the quinuclidine feature. Quinuclidine-bearing mAChRs high-affinity ligands such as QNB are known to be able to cross the blood-brain barrier (BBB) but lacking the desired selectivity. Low lipophilicity may also imply reduced unspecific binding, rendering this kind of derivative potentially useful as a diagnostic probe, e.g. as a PET-tracer. Furthermore, the calcium assay confirmed our hypothesis that all compounds are antagonists.

Table 2: Physico-chemical properties of the synthesized compounds.

Cmpd.	HPLC $logP^{pH\ 7.4}$	XlogP	Cmpd.	HPLC $logP^{pH\ 7.4}$	XlogP
3a	2.94 ± 0.03	3.0	**4a**	2.11 ± 0.03	3.2
3b	3.20 ± 0.02	3.0	**4b**	2.69 ± 0.00	3.2
3c	2.81 ± 0.04	2.7	**4c**	1.91 ± 0.01	2.9
3d	2.60 ± 0.07	2.3	**4d**	1.82 ± 0.02	2.5
3e	1.2 ± 0.3	3.1	**4f**	2.69 ± 0.00	3.8
3f	2.73 ± 0.06	3.6	**4g**	1.58 ± 0.05	3.8
3g	3.05 ± 0.01	3.6	**4h**	1.58 ± 0.03	2.3
3h	3.04 ± 0.01	2.6			

Compared to the rearrangement product, the benzimidazole derivatives exhibit significantly improved affinity towards specific subtypes while retaining moderate lipophilicity. The carbamate derivatives' affinities tend to be subpar, with **4g** as the exception but lacking selectivity. The monocyclic tertiary amine set of **3a, 3b,** and **3c** display moderate activity towards the M5 subtype. The small ring size of the methylazidine feature of **3d** results in a negative activity cliff. With an excellent K_i of 14 for the M5 subtype, **3c** shows a promising 4-fold selectivity against $M_{1,3-4}$ and an 18-fold selectivity versus the M2. The low lipophilicity compound **3e** shows a one-digit nanomolar affinity for M_3 and M_5 with low to moderate selectivity against $M_{1-2,4}$.

Table 3: Affinities for the synthesized ligands.

	Affinity: Ki ± SD (nM)						Affinity: Ki ± SD (nM)				
Cmpd.	*h* M1	*h* M2	*h* M3	*h* M4	*h* M5	Cmpd.	*h* M1	*h* M2	*h* M3	*h* M4	*h* M5
3a	20 ± 3	108 ± 16	21 ± 5	32 ± 7	18 ± 6	**4a**	255 ± 7	> 1000	121 ± 40	158 ± 34	255 ± 41
3b	288 ± 47	385 ± 136	236 ± 8	152 ± 28	77 ± 9	**4b**	> 1000	> 1000	> 1000	> 1000	> 1000
3c	51 ± 10	258 ± 71	60 ± 12	65 ± 18	14 ± 7	**4c**	217 ± 22	> 1000	123 ± 19	156 ± 28	105 ± 6
3d	> 1000	> 1000	> 1000	> 1000	> 1000	**4d**	> 1000	> 1000	339 ± 33	244 ± 92	> 1000
3e	16 ± 3	70 ± 28	3.7 ± 1.6	9.4 ± 1.1	2.6 ± 0.7	**4f**	> 1000	> 1000	> 1000	> 1000	> 1000
3f	101 ± 39	442 ± 150	40 ± 11	201 ± 65	> 1000	**4g**	44 ± 2	222 ± 49	108 ± 31	50 ± 20	75 ± 28
3g	9 ± 2	16 ± 3	17 ± 5	11 ± 2	8.1 ± 0.7	**4h**	> 1000	> 1000	> 1000	> 1000	> 1000
3h	285 ± 16	> 1000	350 ± 120	189 ± 57	164 ± 43						

A possible explanation for the low affinity of the carbamates may be found in the higher degree of freedom of the scaffold, which leads in docking experiments to different space-filling properties within the orthosteric binding site.

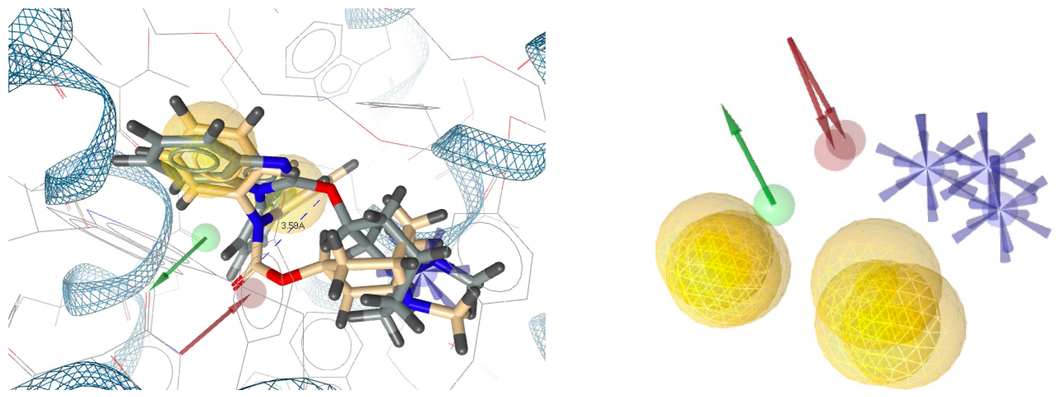


Figure 2: Left: Structure-based view of the orthosteric binding site of the M5 (6OL9) subtype. Highest scored docking poses of **3f** (grey) and **4f** (yellow) are depicted together with the pharmacophore of the co-crystallized ligand tiotropium. **Right**: Overlay of the structure-based generated pharmacophores of docked **3f, 4f** and the the co-crystalized ligand tiotropium

Conclusion

Herein we present the discovery of novel potent ligands for the mAChRs. These ligands' promising biological and physico-chemical properties may lay the groundwork for developing CNS drugs or PET-tracers.