CHEMMEDCHEM

CHEMISTRY ENABLING DRUG DISCOVERY

Accepted Article

Title: Novel BQCA and TBPB derived M1 Receptor Hybrid-Ligands: Orthosteric Carbachol Differentially Regulates Partial Agonism

Authors: Simon Schramm, Luca Agnetta, Marcel Bermudez, Hubert Gerwe, Timo Littmann, Matthias Irmen, Janine Holze, Gerhard Wolber, Christian Tränkle, and Michael Decker

To be cited as: *ChemMedChem* 10.1002/cmdc.201900283

Link to VoR: http://dx.doi.org/10.1002/cmdc.201900283

A Journal of

ChemPubSoc Europe

WILEY-VCH

www.chemmedchem.org

WILEY-VCH

COMMUNICATION

Novel BQCA and TBPB derived M_1 Receptor Hybrid-Ligands: Orthosteric Carbachol Differentially Regulates Partial Agonism

Dr. Simon Schramm,[a] Luca Agnetta[a], Dr. Marcel Bermudez[b], Hubert Gerwe[a], Matthias Irmen[c], Dr. Janine Holze[c], Dr. Timo Littmann[d], Prof. Gerhard Wolber[b], Dr. Christian Tränkle[c] and Prof. Michael Decker[a]

[a] Pharmazeutische und Medizinische Chemie
Institut für Pharmazie und Lebensmittelchemie, Julius-Maximilians-Universität Würzburg
Am Hubland, D-97074 Würzburg, Germany
E-mail: michael.decker@uni-wuerzburg.de
[b] Institute of Pharmacy, Freie Universität Berlin, Königin-Luise-Straße 2 + 4, 14195 Berlin, Germany.
[c] Pharmacology and Toxicology, Institute of Pharmacy, University of Bonn, Gerhard-Domagk-Straße 3, 53121 Bonn, Germany.
[d] Institute of Pharmacy, University of Regensburg, 93053 Regensburg, Germany.

To this work have equally contributed S. Schramm (synthesis) and L. Agnetta (pharmacology).

Supporting information for this article is given via a link at the end of the document.

Abstract: Recently, investigations of the complex mechanisms of allostery have led to a deeper understanding of G protein-coupled receptor (GPCR) activation and signaling processes. In this context, muscarinic acetylcholine receptors (mAChRs) are highly relevant according to their exemplary role for the study of allosteric modulation. In this work, we compare and discuss two sets of putatively dualsteric ligands, which were designed to connect carbachol to different types of allosteric ligands. We chose derivatives of TBPB [1-(1'-(2-tolyl)-1,4'-bipiperidin-4-yl)-1H-benzo[*d*]imidazol-2(3*H*)-one] as M_1-selective putatively bitopic, i.e. orthosteric/allosteric agonist, i.e. TBPBds, and a benzyl quinolone carboxylic acid derivative (BQCAd) as an M_1- positive allosteric modulator, varying the distance between the allosteric and orthosteric building blocks. Luciferase protein complementation assays demonstrated that linker length must be carefully chosen to yield agonist or antagonist behavior, respectively. These findings may help to design biased signaling and/or different extents of efficacy.

Muscarinic acetylcholine receptors (mAChRs or M receptors) are involved in the regulation of a variety of physiological functions depending on their localization in both the central and peripheral nervous system.[1] Among them are actions of the central nervous system like cognitive, sensory, motor, behavioral and autonomic processes.[2] In the pathophysiology of schizophrenia, depression, Parkinson's disease and Alzheimer's disease (AD), changes in mAChR activities and levels have been described.[3] In AD, postsynaptic AChRs remain mostly intact during presynaptic cholinergic hypofunction. Hence, depletion of the endogenous neurotransmitter acetylcholine (ACh) may be circumvented by targeting the mAChRs directly.[4]

The M_1 receptor is expressed in brain regions responsible for functions like cognition, learning and memory, which are impaired in AD.[5]It was shown that activation of this subtype has a positive influence on the aggregation of Aβ and neurofibrillary tangles, the key pathophysiological hallmarks of AD.[6-9] By stimulation of the receptor, PKC is activated, and the non-amyloidogenic pathway is favored. An increase in α secretase production suppresses the formation of toxic Aβ plaques. The activation of PKC also regulates the GSK-3β cascade, which leads to stabilization of microtubules and therefore mitigating τ-pathology. Moreover, activation of the M_1 receptor leads to positive influences on decreased cerebral blood flow, caspase activation, DNA damage, oxidative stress and mitochondrial impairment in cells.[10-11] The positive effects that the activation of M_1 can produce were also shown in transgenic mice.[12]

Complex downstream signaling and their interactions complicate elucidation of the physiological roles of individual mAChR subtypes. Furthermore, a lack of subtype selective ligands hampers pharmacological examinations. Subtype-selective targeting is essential for investigations of receptor pharmacology and AD pathology alike.

This is, however, rather difficult, as the orthosteric, i.e. neurotransmitter binding sites of the five M-receptor subtypes show a high degree of homology. This results in major challenges when developing ligands selective for a specific receptor subtype. In contrast, allosteric binding sites at the extracellular vestibule of the receptor are less conserved throughout all five subtypes and show greater topological differences while being conformationally linked to the orthosteric site. Allosteric sites are thus easier to address than the orthosteric binding pockets with regard to selectivity and have been used for the development of selective ligands.[13] Allosteric ligands are divided into allosteric agonists, able to cause a signal by themselves, and allosteric modulators, changing the affinity and/or function and/or signaling preference[14] of an orthosteric ligand.[15-18] Allosteric modulators are, however, dependent on an orthosteric ligand, e.g. an agonist, to transduce signals.

The combination of orthosteric and allosteric moieties connected by a linker moiety has been established as a dualsteric/bitopic ligand approach. Dualsteric/bitopic ligands are designed to selectively target a receptor subtype, by concomitantly addressing both the orthosteric and the allosteric binding site.[19] This approach has the advantage that it is not reliant on the endogenous neurotransmitter and that biased signaling by activation of a specific downstream signal is possible via a single molecule.[16-18]

The design of "dualsteric" ligands can be challenging as they need to meet clear prerequisites to bind simultaneously to the orthosteric and allosteric binding site. For this purpose, the connection points have to be chosen carefully in order to avoid alteration of essential functional groups responsible for receptor binding and function. In addition, the linker length has to be taken into consideration. There is evidence that only an optimal spacer length is able to allow an active hybrid binding, while shorter or longer spacers result in inactive allosteric binding of dualsterically conceptualized hybrid ligands.[20] In earlier studies, a variety of

dualsteric M_1 ligands have been developed, including a photoswitchable BQCA-iperoxo hybrid.[21-24]
Furthermore, also the design of the connecting linker represents challenge also, not only for dualsteric GPCR ligands, but any kind of bivalent ligand. For this reason, random walk as well as computational approaches have been used to guide linker design.[25]

Since the orthosteric and the allosteric binding pocket of the M_1 receptor are separated by the so-called tyrosine lid formed by three tyrosine residues (Figure 1), dualsteric ligands have to bridge these two sites by a linker. We have chosen three different linker lengths for this study. First, a C_3-alkyl chain linker, because it represents the shortest possible linker length to bypass the tyrosine lid. Second an C_8-alkyl chain, which represents the longest plausible linker length according to our model and previously reported dualsteric ligands for muscarinic receptors.[20, 26-27] Between these two extremes, the C_5-alkyl chain represents the optimal linker length for the M_1 receptor.

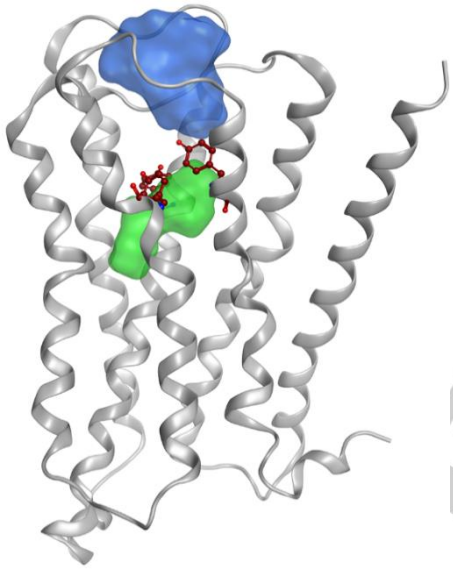


Figure 1. Active M_1 receptor model with the orthosteric and the allosteric binding site shown as green and blue surface, respectively. These two binding sites are separated by tyrosine residues which are forming a lid-like structure (red).

We now have developed two sets of putatively dualsteric ligands as tools for the investigation of M_1 receptor pharmacology. For both sets, carbachol (CCh), a derivative of the endogenous neurotransmitter ACh, was chosen as orthosteric agonist. In contrast to ACh, CCh does not show off-target effects like inhibition of acetylcholinesterase (AChE) and has a reduced rate of hydrolysis.[28]
The allosteric moieties chosen are a truncated 1-(1'-(2-tolyl)-1,4'-bipiperidin-4-yl)-1*H*-benzo[*d*]imidazol-2(3*H*)-one (TBPB), and derivatives of benzyl quinolone carboxylic acid (BQCA). TBPB is an agonist at the M_1 receptor developed at Vanderbilt University, the binding and action of which may occur in a bitopic fashion.[29-31] Remarkably, TBPB showed a shift towards the non-amyloidogenic pathway and reduced aggregation of Aβ *in vitro*.[29]
In previous work, TBPB had been connected to AF292, a selective M_1 agonist.[32-33] These putatively dualsteric compounds, however, did not show the agonism intended since AF292 acted as a weak partial agonist only. Therefore, in the current work AF292 was replaced by carbachol. **Figure 2** shows the design of target structures **1** combining a truncated TBPB with carbachol to form hybrid ligands. The benzylated second piperidine moiety had been shown to be dispensable for allosteric agonism.[20, 34]

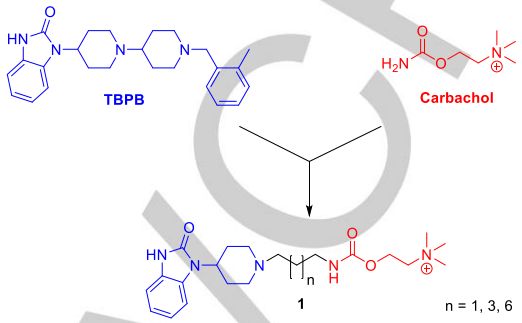


Figure 2. Design of target structures **1** combining the agonist TBPB and the orthosteric carbachol by alkylene linkers of variable lengths.

BQCA (cf. Fig. 3) is described as a positive allosteric modulator and allosteric agonist.[35-36] This compound was shown to be able to reduce the necessary amount of ACh to activate the receptor 129-fold and showed no enhancing activity at other mAChR subtypes.[36-37] We could delineate recently, that the connection of a spacer to a BQCAd canceled the allosteric agonistic action.[20] Structure-activity relationships of BQCA derivatives were conducted thoroughly.[34, 38] We decided to use a derivative as the second allosteric moiety in the current study carrying a fluorine at position 8 of the A-ring and substitute the benzyl ring (B-ring) by naphthalene for an increased M_1 affinity of the resulting ligands,[39] similar to our previously published compounds.[20] **Figure 3** shows the target structures **2** combining carbachol with the BQCA-derived moiety.

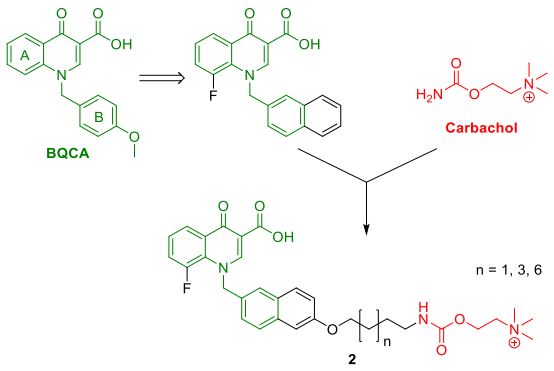
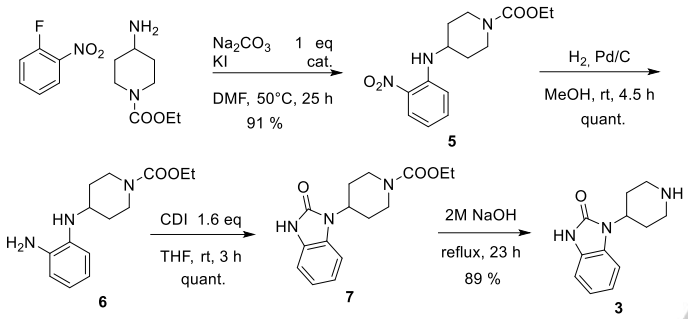


Figure 3. Design of target structures **2** combining derivatives of the allosteric modulator BQCA (BQCAds) and carbachol by alkylene linkers of variable lengths.

Synthesis

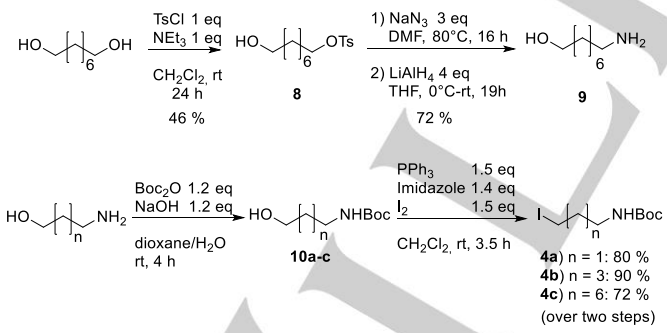
The dualsteric ligands were accessed by connecting the Boc-protected linkers to the allosteric moieties before introducing the carbachol precursor.

TBPB building block **3** was synthesized following literature protocols (**Scheme 1**).[40-42] Nitroaniline **5** was synthesized by reaction of commercially available 1-fluoro-2-nitrobenzene with a carboxyl protected 4-aminopiperidine. The nitro group was reduced by hydrogenation over palladium on activated charcoal. The diamine **6** was reacted with CDI to form the benzimidazolone **7**. Hydrolysis of the ester protection group led to TBPB-building block **3**.



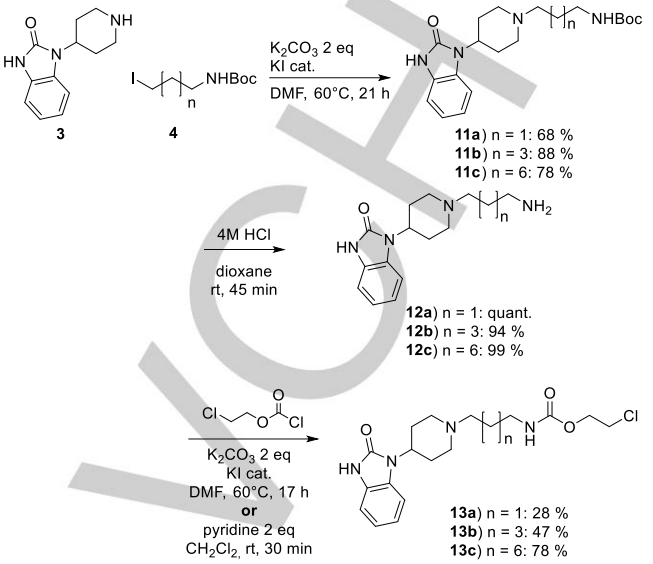
Scheme 1. Synthesis of TBPB-building block **3**.

The C_8-linker **4c** was synthesized from 1,8-octandiol. 1,8-octandiol was mono-tosylated to give compound **8**, which was then substituted with sodium azide in dimethylformamide. Reduction with lithium aluminum hydride yielded 8-aminooctanol **9**. 3-Aminopropanol, 5-aminopentanol and 8-aminooctanol **9** were Boc-protected to give compounds **10a-c**. An Appel reaction to introduce iodine as a leaving group yielded linkers **4a-c** (**Scheme 2**).



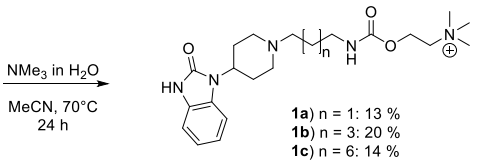
Scheme 2. Synthesis of linkers **4a-c**.

Building blocks **3** and **4a-c** were connected in a substitution reaction to give compounds **11a-c**. Removal of the Boc-protection group was achieved under acidic conditions. 4M HCl in dioxane as solvent gave the free amines **12a-c**. Reaction with 2-chloroethyl chloroformate under basic conditions yielded the carbamates **13a-c**. Compounds **13a** and **13b** were synthesized in dimethylformamide with potassium carbonate as base, but only in low yields. The procedure was changed for compound **13c**, using pyridine as a base in dry dichloromethane. This reaction was finished within 30 min and showed a highly improved yield (**Scheme 3**).



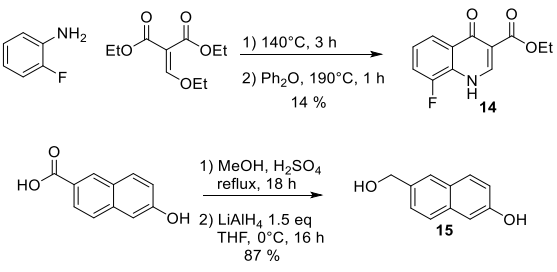
Scheme 3. Synthesis of carbamates **13a-c**.

The final reaction step was the substitution reaction of chloro-compounds **13** with trimethylamine for the formation of the carbachol moiety and target compounds **1a-c** (**Scheme 4**). Due to the large number of byproducts and difficult purification, compounds **1a-c** were obtained in yields of 13% to 20% only (**Scheme 4**).



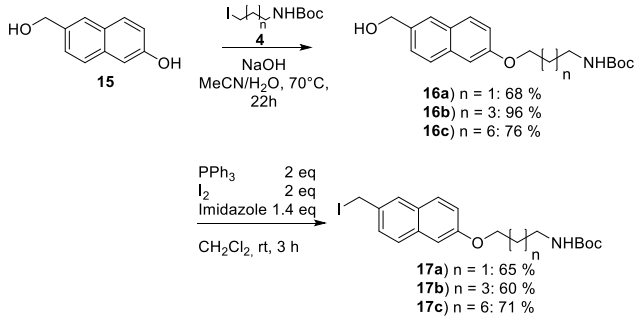
Scheme 4. Last step of the synthesis of dualsteric compounds **1a-c**.

For the BQCA-carbachol hybrids **2**, building block **14** was synthesized by a one-pot Gould-Jacobs synthesis.[38-39] 6-Hydroxy-2-naphthoic acid was esterified with methanol and subsequently reduced to 6-(hydroxymethyl)naphthalen-2-ol **15** (**Scheme 5**).



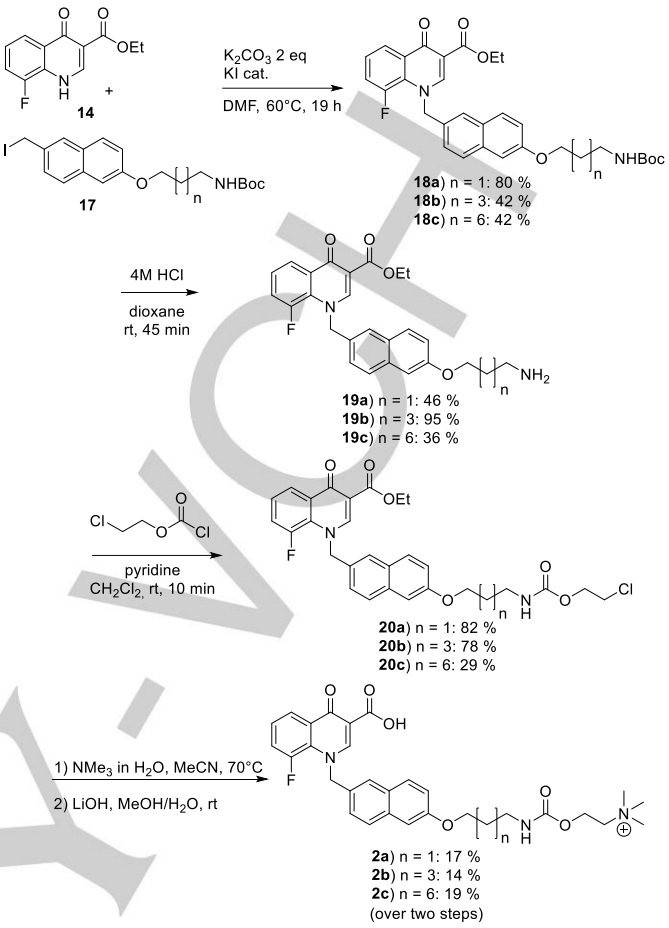
Scheme 5. Synthesis of building blocks **14** and **15**.

Next, the linkers **4a-c** were connected to the naphthyl alcohol **15** in a substitution reaction to yield alcohols **16a-c**. An Appel reaction to introduce iodine as a leaving group gave compounds **17a-c** (**Scheme 6**). The iodine compounds **17a-c**, however, proved to be quite unstable and had to be used for the next reaction step immediately after preparation.



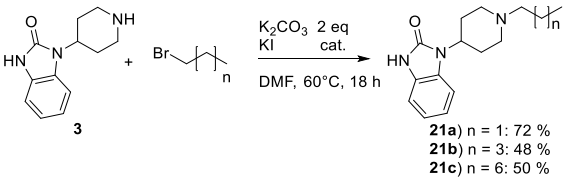
Scheme 6. Synthesis of naphthyl linkers **17**.

Iodine compounds **17a-c** were connected to the quinolone core **14** in dimethylformamide under basic conditions (**Scheme 7**). Boc deprotection of **18** gave the free amines **19a-c**. Carbamate formation was achieved as described above yielding precursors **20a-c**. The last steps were the substitution with trimethylamine and subsequent saponification of the ester using lithium hydroxide. Reaction progression of this step has to be monitored by LCMS. Both the esters and the target compounds **2** showed the same retention on TLC as well as on HPLC. Disappearance of the ester's *m/z* ratio indicated full conversion. The target dualsteric compounds **2** were obtained in yields of 14% to 19% (**Scheme 7**).



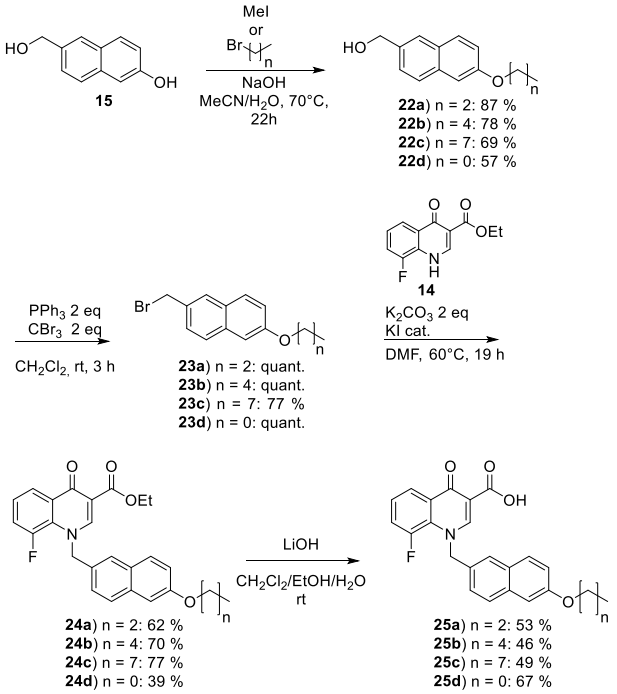
Scheme 7. Synthesis of dualsteric compounds **2a-c**.

For comparison and to investigate binding modes of the designed compounds, reference compounds are required. Thus, the individual allosteric moieties connected to the alkyl linkers were synthesized. Some of these compounds (**21c**, **25c**, **25d**) were previously published.[20] The TBPB reference compounds **21a-c** were synthesized by reaction of the piperidine moiety **3** with the respective halogenated alkane (**Scheme 8**).



Scheme 8. Synthesis of TBPB reference compounds **21a-c**.

BQCA reference compounds were synthesized analogously to the quinolone compounds described above. Alcohol **15** was reacted with the respective halogenated alkane to give ethers **22a-d**. In an Appel reaction, the alcohols **22a-d** were converted into the respective bromines **23a-d**. The bromo-compounds **23a-d** proved more stable than the respective iodo-compounds **17** (*cf*. above). The bromine was then substituted by quinolone **14** yielding esters **24a-d**. By hydrolysis, BQCA reference compounds **25a-d** were obtained (**Scheme 9**).



Scheme 9. Synthesis of BQCA reference compounds **25a-d**.

Biological evaluation

The putative dualsteric compounds **1a-c** and **2a-c**, the corresponding reference compounds **21a-c** and **25a-d** as well as the CCh-alkyl reference compounds **28a-c** were evaluated in a novel luciferase protein complementation assay (**Table 1**).[43] The assay was conducted to evaluate receptor response through the $G_{\alpha q}$ protein specific phospholipase C pathway. For each compound, a nine-point concentration-response-curve was recorded and expressed in percent of the maximal cellular response of CCh.

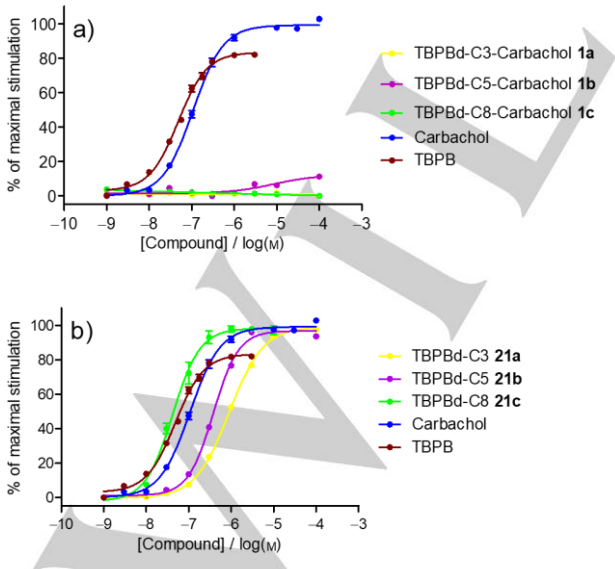


Figure 4. Concentration-response-curves of (a) dualsteric compounds **1a-c** and (b) reference compounds **21a-c** measured in the luciferase complementation *in vitro* assay. Data points represent means ± SEM from three independent experiments, conducted in triplicate.

Figure 4 shows the dose-response curves of the putative dualsteric TBPBd-carbachol compounds **1a-c** and the alkyl reference compounds **21a-c** expressed as a percentage of the maximal cellular response to CCh. No receptor response was caused by the carbachol containing compounds **1a** and **1c.** In contrast, compound **1b** showed weak partial agonism, i.e. its $\%E_{max}$ = 12 ± 2 (cf. **Table 1**) being significantly different from zero (one sample t-test, P>0.05) while the reference compounds **21a, b, c** show full agonism and, depending on their chain lengths, different potencies, with the C8 compound **21c** significantly showing the highest potency of all (One-Way ANOVA with Newman-Keuls post test, P<0.05). Note that the spacers C3, C5 and C8 in compounds **21a**,**b** and **c** enhanced the efficacy of the TBPB (Fig. 4b) and the orthosteric carbachol moiety nearly abolished it in compounds **1a**,**b** and **c** (Fig. 4a).

Table 1. Measures of potency and efficacy induced by muscarinic agonist and hybrids in live HEK 293t cells.[a]

Cmpd.	Cn	pEC_{50}	$\%E_{max}$	Slope
CCh		6.97 ± 0.03	99 ± 1	1.22 ± 0.09
TBPB		7.32 ± 0.02	83 ± 1	1.29 ± 0.07
BQCA		7.20 ± 0.03	90 ± 1	1.60 ± 0.13
1a		n. d.	n. d.	n. d.
1b	3	5.09 ± 0.24	12 ± 2	0.87 ± 0.31
1c	8	n. d.	n. d.	n. d.
2a	3	5.89 ± 0.01	66 ± 0.5	2.02 ± 0.08
2b	5	6.67 ± 0.02	78 ± 1	1.36 ± 0.07
2c	8	6.62 ± 0.03	28 ± 0.5	1.77 ± 0.19
21a	3	6.05 ± 0.01	99 ± 1	1.08 ± 0.03
21b	5	6.42 ± 0.01	97 ± 1	1.46 ± 0.05
21c	8	7.38 ± 0.04	98 ± 2	1.34 ± 0.15
25a	3	5.82 ± 0.02	35 ± 1	2.69 ± 0.23
25b	5	n. d.	n. d.	n. d.
25c	8	n. d.	n. d.	n. d.
25d	1	6.01 ± 0.02	58 ± 1	1.30 ± 0.08
28a	3	4.65 ± 0.18	44 ± 6	0.95 ± 0.21
28b	5	5.27 ± 0.07	28 ± 1	1.24 ± 0.21
28c	8	6.09 ± 0.08	9.4 ± 0.4	1.41 ± 0.37

[a]pEC_{50}, −log EC_{50} value: (-log)concentration of the indicated compounds inducing a half-maximal effect); $\%E_{max}$, maximum effect as a percentage of E_{CCh} (100 µM); Slope factor obtained by curve fitting to data from individual experiments shown in Figures 4-6 using a four-parameter logistic equation. nm: not measurable Data represent means ± SEM from three independent experiments, conducted in triplicate.

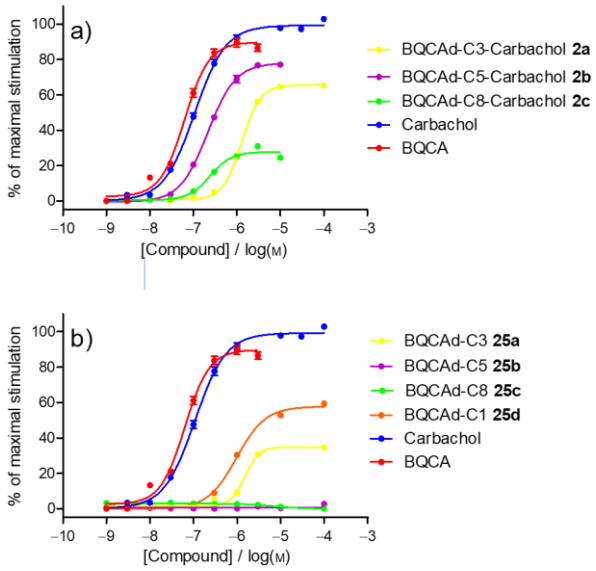


Figure 5. Dose-response curves of (a) dualsteric compounds **2a-c** and (b) reference compounds **25a-d** measured in the Luciferase complementation *in vitro* assay. Data represent means ± SEM from three independent experiments, conducted in triplicate.

Figure 5 shows the dose-response curves of the putative dualsteric BQCAd-carbachol compounds **2a-c** and the corresponding reference compounds **25a-d** in comparison to CCh as the positive control, with its maximum response set to 100%. The reference compounds **25b** and **25c** showed no receptor response as had been expected from a previous study applying deviating assay systems.[20] Interestingly, compounds **25a** and **25d**, however, showed partial agonism suggesting that the minimum spacer length to abolish agonism in the reference compounds is C5 (**25b**) and that C1 (**25d**) and C3 (**25a**) do not suffice. In contrast, all putatively dualsteric compounds **2a-c** showed partial agonism. The C5-linked compound **2b** exhibited the highest efficacy. This finding suggests a putatively dualsteric/bitop ligand binding behavior of **2b** as suggested in Fig. 9 in that its carbachol moiety triggers M_1 receptor activation orthosterically, the efficacy of which decreasing with an increase in spacer-length to C8 as demonstrated by **28c** in Fig. 8. Note that the C5 spacer in compound **2b** abolished efficacy of the allosteric agonist BQCA in compound **25b** (Fig. 5b) and the orthosteric carbachol moiety in **2b** nearly restored it to the level of efficacy of BQCA alone (Fig. 5a). Inactive compounds **1a, 1c, 25b** and **25c** plus the fairly active **1b** were additionally studied which revealed an antagonistic action of **1c** to M_1-receptor activation via the orthosteric carbachol (**Figure 6**). To shed light on the antagonistic properties, the luciferase complementation assay was performed in an antagonist mode, co-incubating the respective test compound with carbachol. BQCAd-reference compounds **25b** and **25c** did not show antagonistic behavior. In contrast, the dualsteric TBPBd compound **1c** revealed clear cut antagonist properties, which were not observed with the shorter ligands **1a** and **b**, indicating that the C8 chain in **1c** is an optimal linker length for compound binding to the receptor without triggering a functional activity. Detailed global data analysis of the functional antagonism between carbachol and **1c** at M_1 receptors was in line with a formally competitive interaction (cf. Fig. 7). Future studies focusing on the binding topography of **1c** will help to elucidate the molecular nature of its antagonistic behavior towards carbachol. Interestingly, the importance for the receptor interaction of the C8 spacer is also confirmed by the corresponding reference compound **21c** which showed the highest potency (**Figure 4**).

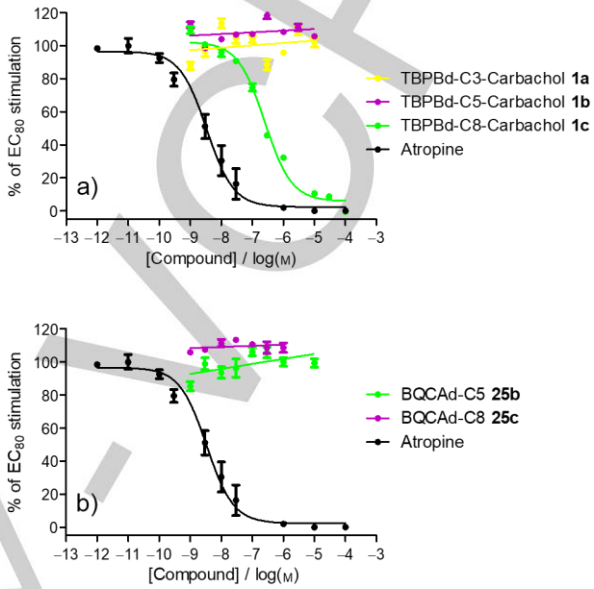


Figure 6. Dose-response curves of (a) putatively dualsteric TBPBd compounds **1a-c** and (b) BQCAd-reference compounds **25b** or **25c** measured in the Luciferase complementation *in vitro* assay in the antagonist mode by co-incubations of EC_{80}-concentration of CCh. Data represent means ± SEM from three independent experiments, conducted in triplicate.

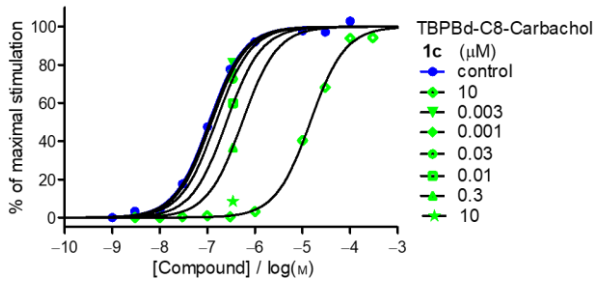


Figure 7. M_1 receptor stimulation by carbachol measured in the Luciferase complementation *in vitro* assay conducted with several concentrations of the dualsteric compound **1c**. Ordinate: response of live HEK296 cells overexpressing the M_1 receptor displayed as a percentage of maximally induced stimulation by CCh. Abscissa: log concentration of CCh. To quantifiy the antagonistic action of **1c**, a control curve with variable concentrations of CCh only (taken from Fig. 4a), a second curve with increasing concentrations of **1c** at one fixed concentration of CCh (taken from Fig. 6a), that induced 80-90% of the maximal receptor response and thirdly, a second full CCh curve shifted by a fixed concentration of **1c** were compiled and fitted by global nonlinear regression to a model assuming competition of **1c** with CCh at a single site.[44] Data represent means ± SEM from three independent experiments, conducted in triplicate.

Cmpd.	Cn	log K_B
1a	3	n. d.
1b	5	n. d
1c	8	7.12 ± 0.02
25b	5	n. d
25c	8	n. d
Atr	-	9.32 ± 0.12

Table 2. Equilibrium log affinity constants K_B of compound **1c** and atropine (**Atr**) for binding to the M_1-receptor according to the competitive model. Log K_B values were determined with s constrained to unity, if s was not statistically different from s = 1 (F test, P > 0.05), suggesting that **1c** and atropine acted formally competitively with CCh.

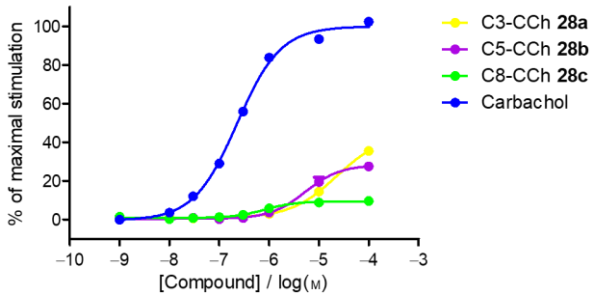


Fig. 8. Dose-response curves of reference compounds **28a-c** and carbachol measured in the Luciferase complementation in vitro assay. Data represent means ± SEM from three independent experiments, conducted in triplicate.

Figure 8 shows the dose-response curves of the CCh-alkyl reference compounds **28a-c** expressed as a percentage of the maximal cellular response to CCh. Compounds **28a-c** showed partial agonism and a decreasing receptor response with increasing chain length of the alkyl rest. Similarly, the potencies of **28a-c** were smaller than that of carbachol (cf. **Table 1**). In contrast to this trend, within this group of carbachol derivatives the potencies increased with chain length, with the C8 compound showing the highest potency (One-Way ANOVA with Newman-Keuls post test, P<0.05). These findings suggest that the CCh-alkyl moieties **28a-c** contribute in part to the agonistic effects of hybrids **1** and **2**, respectively, but do not make up the respective full effects of hybrid efficacy and potency. Specifically, the BQCAd and the TBPBd moieties in hybrids **2a-c** and **1b**, respectively, have, compared to the efficacy of **28a-c,** either *increasing* effects (BQCAd containing hybrids: **Fig. 8**, **Fig. 5**, **Table 1**; E_{max}: **28a**: 44% -> **2a**: 66%, **28b**: 28% -> **2b**: 78%, **28c**: 9,4% -> **2c**: 28%) or *decreasing* effects (TBPBd containing hybrid **1b**: **Fig. 8**, **Fig. 4**, **Table 1**; E_{max}: **28b**: 28% -> **1b**: 12%). Additionally, the BQCAd moieties in the hybrids **2a-c** increased the potency estimate pEC_{50} significantly compared to compounds **28a-c** (**Fig. 8**, **Fig. 5**, **Table 1**; pEC_{50}: **28a**: 4.65 -> **2a**: 5.89, **28b**: 5.27 -> **2b**: 6.67, **28c**: 6.09 -> **2c**: 6.62; One-Way ANOVA with Newman-Keuls post test, P<0.05). The TBPBd moiety decreased the pEC_{50} of **28a** compared to **1b** numerically but not significantly (**Table 1**). Taken together, these results point to the necessity of allosteric hybrid moieties for a full hybrid effect, to a dualsteric/bitopic receptor interaction of the effective hybrids **1** and **2** and show that it is productive to develop ligands that bridge between the M_1 orthosteric and allosteric binding sites.

Molecular Modeling

The dualsteric binding mode of **2b** (BQCAd-C5-Carbachol) was analyzed with regard to receptor-ligand interactions by using three-dimensional pharmacophores (**Figure 9**).[45] The shown binding pose was derived by docking to an active receptor model according to a previously published protocol.[26-27] The ammonium group of the carbachol moiety forms a charge interaction with $D105^{3.32}$ and a cation-π interaction with $Y381^{6.51}$, which represents the key interactions for orthosteric ligands. The carbonyl group of the carbamate serves as hydrogen bond acceptor for the hydroxyl group of $Y408^{7.43}$. This represents a remarkable difference compared to the binding mode of carbachol, in which the carbamate structure shows a different orientation (Supporting information). However, orthosteric key interactions (charge interaction with $D105^{3.32}$ and a cation-π interaction with $Y381^{6.51}$) are present in both binding modes and mainly driving agonist activity. The BQCAd moiety is located in the allosteric vestibule at the extracellular loop region and forms lipophilic contacts with $L174^{EL2}$ and $Y179^{EL2}$. Furthermore, the carboxylic acid forms a charge interaction with $K392^{EL3}$.

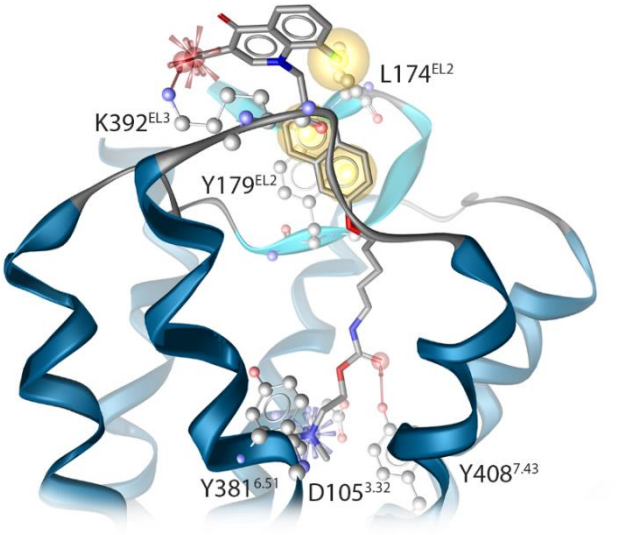


Figure 9. Proposed dualsteric binding mode of **2b** (BQCAd-C5-Carbachol) at the active M_1 receptor model with the carbachol moiety binding at the orthosteric site and the BQCAd building block at the allosteric site. Hydrogen bond acceptors are shown as red arrows, yellow spheres indicate lipophilic contacts, and positive and negative ionizable centers were shown as blue and red stars, respectively.

Conclusion

We have designed and synthesized two sets of novel putative dualsteric hybrid compounds containing the M_1 orthosteric ligand carbachol and either derivatives of BQCA, an allosteric modulator and agonist, or TBPB, a bitopic orthosteric/allosteric agonist. In a first evaluation of their receptor efficacy, agonist activity can be seen for the BQCAd-carbachol-hybrids **2** only, the extent of which is strongly dependent on the spacer length between the moieties.

Different to findings published earlier with other non-agonistically active BQCAd building blocks,[20] in the current study spacer lengths smaller than C5 resulted in partially active BQCAd moieties thus contributing to the orthosteric agonistic effect of the carbachol moiety. Nevertheless, the BQCAd moiety **25b** (which includes a C5 spacer) of the BQCAd-carbachol-hybrid **2b** possessing the highest efficacy (and potency) did not contribute to M_1-receptor activation. This demonstrates that the carbachol moiety pertained part of its agonistic action in this hybrid. Inversely, the orthosteric carbachol massively quenched M_1-receptor activation in the bitopic TBPBd-carbachol-hybrid **1b** to a marginal partial agonism at a high concentration compared to its TBPBd-C5 moiety which behaved as a full agonist. In summary, in the current study, we demonstrate that partial agonism in dualsteric/bitopic compounds can be designed not only by quenching orthosteric receptor activation by an allosteric moiety as in **2b** but also by quenching putative bitopic/dualsteric activation of the receptor protein by an orthosteric moiety such as carbachol in **1b**. These findings practically widen the effect of orthosteric moieties in the concept of putative dualsteric/bitopic ligands. They allow different extents of partial agonism and furthermore enlarge the molecular toolbox of hybrid ligands to investigate mAChR receptor function. More advanced studies with regard to signaling bias and subtype selectivity are ongoing.

Experimental Section

Synthetic procedures and conducted assay procedures are found in the supporting information.

Acknowledgments

Generous support by the Elite Network of Bavaria (ENB) and International Doctoral Program “Receptor Dynamics” for L.A., H. G. and T.L. is gratefully aknowledged.

Keywords: Muscarinic Receptor • GPCR • Dualsteric Ligands • Partial Agonism • Allostery

References

[1] A. C. Kruse; B. K. Kobilka; D. Gautam; P. M. Sexton; A. Christopoulos; J. Wess, *Nat. Rev. Drug Discov.* **2014**, *13*, 549-560.
[2] R. M. Eglen; A. Choppin; N. Watson, *Trends Pharmacol. Sci.* **2001**, *22*, 409-414.
[3] A. Sanabria-Castro; I. Alvarado-Echeverria; C. Monge-Bonilla, *Ann. Neurosci.* **2017**, *24*, 46-54.
[4] R. T. Bartus, *Exp. Neurol.* **2000**, *163*, 495-529.
[5] R. M. Eglen, *Prog. Med. Chem.* **2005**, *43*, 105-136.
[6] A. Fisher, *Neurotherapeutics* **2008**, *5*, 433-442.
[7] A. Fisher, *Neurodegener. Dis.* **2008**, *5*, 237-240.
[8] A. Fisher, *Curr. Alzheimer Res.* **2007**, *4*, 577-580.
[9] A. Fisher, *J. Neurochem.* **2012**, *120*, 22-33.
[10] P. De Sarno; S. A. Shestopal; T. D. King; A. Zmijewska; L. Song; R. S. Jope, *J. Biol. Chem.* **2003**, *278*, 11086-11093.
[11] C. R. Overk; C. C. Felder; Y. Tu; D. A. Schober; K. R. Bales; J. Wuu; E. J. Mufson, *J. Chem. Neuroanat.* **2010**, *40*, 63-70.
[12] E. P. Lebois; J. P. Schroeder; T. J. Esparza; T. M. Bridges; C. W. Lindsley; P. J. Conn; D. L. Brody; J. S. Daniels; A. I. Levey, *ACS Chem. Neurosci.* **2017**, *8*, 1177-1187.
[13] A. Bock; R. Schrage; K. Mohr, *Neuropharmacology* **2018**, *136*, 427-437.
[14] A. E. Davey; K. Leach; C. Valant; A. D. Conigrave; P. M. Sexton; A. Christopoulos, *Endocrinology* **2012**, *153*, 1232-1241.
[15] A. Christopoulos; J. P. Changeux; W. A. Catterall; D. Fabbro; T. P. Burris; J. A. Cidlowski; R. W. Olsen; J. A. Peters; R. R. Neubig; J. P. Pin; P. M. Sexton; T. P. Kenakin; F. J. Ehlert; M. Spedding; C. J. Langmead, *Pharmacol. Rev.* **2014**, *66*, 918-947.
[16] K. Mohr; J. Schmitz; R. Schrage; C. Tränkle; U. Holzgrabe, *Angew. Chem. Int. Ed.* **2013**, *52*, 508-516.
[17] A. Bock; N. Merten; R. Schrage; C. Dallanoce; J. Batz; J. Klöckner; J. Schmitz; C. Matera; K. Simon; A. Kebig; L. Peters; A. Muller; J. Schrobang-Ley; C. Tränkle; C. Hoffmann; M. De Amici; U. Holzgrabe; E. Kostenis; K. Mohr, *Nat. Commun.* **2012**, *3*, 1044.
[18] B. J. Davie; A. Christopoulos; P. J. Scammells, *ACS Chem. Neurosci.* **2013**, *4*, 1026-1048.
[19] J. Antony; K. Kellershohn; M. Mohr-Andrä; A. Kebig; S. Prilla; M. Muth; E. Heller; T. Disingrini; C. Dallanoce; S. Bertoni; J. Schrobang; C. Tränkle; E. Kostenis; A. Christopoulos; H. D. Höltje; E. Barocelli; M. De Amici; U. Holzgrabe; K. Mohr, *FASEB J.* **2009**, *23*, 442-450.
[20] X. Chen; J. Klöckner; J. Holze; C. Zimmermann; W. K. Seemann; R. Schrage; A. Bock; K. Mohr; C. Tränkle; U. Holzgrabe; M. Decker, *J. Med. Chem.* **2015**, *58*, 560-576.
[21] L. Agnetta; M. Bermudez; F. Riefolo; C. Matera; E. Claro; R. Messerer; T. Littmann; G. Wolber; U. Holzgrabe; M. Decker, *J. Med. Chem.* **2019**, *62*, 3009-3020.
[22] L. Agnetta; M. Kauk; M. C. A. Canizal; R. Messerer; U. Holzgrabe; C. Hoffmann; M. Decker, *Angew. Chem. Int. Ed.* **2017**, *56*, 7282-7287.
[23] R. Messerer; C. Dallanoce; C. Matera; S. Wehle; L. Flammini; B. Chirinda; A. Bock; M. Irmen; C. Trankle; E. Barocelli; M. Decker; C. Sotriffer; M. De Amici; U. Holzgrabe, *Med. Chem. Commun.* **2017**, *8*, 1346-1359.
[24] R. Messerer; M. Kauk; D. Volpato; M. C. Alonso Canizal; J. Klockner; U. Zabel; S. Nuber; C. Hoffmann; U. Holzgrabe, *ACS Chem. Biol.* **2017**, *12*, 833-843.
[25] P. I. Kitov; H. Shimizu; S. W. Homans; D. R. Bundle, *J. Am. Chem. Soc*. **2003**, *125*, 3284-3294
[26] M. Bermudez; C. Rakers; G. Wolber, *Mol. Inform.* **2015**, *34*, 526-530.
[27] M. Bermudez; A. Bock; F. Krebs; U. Holzgrabe; K. Mohr; M. J. Lohse; G. Wolber, *ACS Chem. Biol.* **2017**, *12*, 1743-1748.
[28] K. M. Sanders; M. H. Zhu; F. Britton; S. D. Koh; S. M. Ward, *Exp. Physiol.* **2012**, *97*, 200-206.
[29] C. K. Jones; A. E. Brady; A. A. Davis; Z. Xiang; M. Bubser; M. N. Tantawy; A. S. Kane; T. M. Bridges; J. P. Kennedy; S. R. Bradley; T. E. Peterson; M. S. Ansari; R. M. Baldwin; R. M. Kessler; A. Y. Deutch; J. J. Lah; A. I. Levey; C. W. Lindsley; P. J. Conn, *J. Neurosci.* **2008**, *28*, 10422-10433.
[30] S. J. Bradley; C. Molloy; C. Bundgaard; A. J. Mogg; K. J. Thompson; L. Dwomoh; H. E. Sanger; M. D. Crabtree; S. M. Brooke; P. M. Sexton; C. C. Felder; A. Christopoulos; L. M. Broad; A. B. Tobin; C. J. Langmead, *Mol. Pharmacol.* **2018**, *93*, 645-656.
[31] A. Christopoulos, *Mol. Pharmacol.* **2014**, *86*, 463-478.
[32] A. Fisher, *Jpn. J. Pharmacol.* **2000**, *84*, 101-112.
[33] A. Fisher; R. Brandeis; R. H. N. Bar-Ner; M. Kliger-Spatz; N. Natan; H. Sonego; I. Marcovitch; Z. Pittel, *J. Mol. Neurosci.* **2002**, *19*, 145-153.
[34] M. Decker; U. Holzgrabe, *Med. Chem. Commun.* **2012**, *3*, 752-762.
[35] M. Canals; J. R. Lane; A. Wen; P. J. Scammells; P. M. Sexton; A. Christopoulos, *J. Biol. Chem.* **2012**, *287*, 650-659.
[36] J. K. Shirey; A. E. Brady; P. J. Jones; A. A. Davis; T. M. Bridges; J. P. Kennedy; S. B. Jadhav; U. N. Menon; Z. Xiang; M. L. Watson; E. P. Christian; J. J. Doherty; M. C. Quirk; D. H. Snyder; J. J. Lah; A. I. Levey; M. M. Nicolle; C. W. Lindsley; P. J. Conn, *J. Neurosci.* **2009**, *29*, 14271-14286.
[37] L. Ma; M. A. Seager; M. Wittmann; M. Jacobson; D. Bickel; M. Burno; K. Jones; V. K. Graufelds; G. Xu; M. Pearson; A. McCampbell; R. Gaspar; P. Shughrue; A. Danziger; C. Regan; R. Flick; D. Pascarella; S. Garson; S. Doran; C. Kreatsoulas; L. Veng; C. W. Lindsley; W. Shipe; S. Kuduk; C. Sur; G. Kinney; G. R. Seabrook; W. J. Ray, *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 15950-15955.
[38] S. D. Kuduk; D. C. Beshore, *Curr. Top. Med. Chem.* **2014**, *14*, 1738-1754.

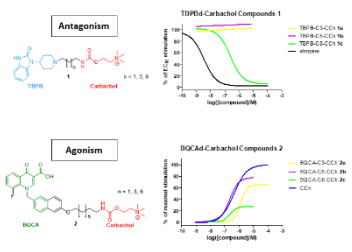
[39] S. D. Kuduk; C. N. Di Marco; V. Cofre; W. J. Ray; L. Ma; M. Wittmann; M. A. Seager; K. A. Koeplinger; C. D. Thompson; G. D. Hartman; M. T. Bilodeau, *Bioorg. Med. Chem. Lett.* **2011**, *21*, 2769-2772.
[40] B. Budzik; V. Garzya; D. Shi; G. Walker; M. Woolley-Roberts; J. Pardoe; A. Lucas; B. Tehan; R. A. Rivero; C. J. Langmead; J. Watson; Z. Wu; I. T. Forbes; J. Jin, *ACS Med. Chem. Lett.* **2010**, *1*, 244-248.
[41] R. Henning; R. Lattrell; H. J. Gerhards; M. Leven, *J. Med. Chem.* **1987**, *30*, 814-819.
[42] N. Xu; C. Yang; X. Gan; S. Wei; Z. Ji, *Int. J. Mol. Sci.* **2013**, *14*, 6790-6804.
[43] T. Littmann; T. Ozawa; C. Hoffmann; A. Buschauer; G. Bernhardt, *Sci. Rep.* **2018**, *8*, 17179.
[44] S. Lazareno; N. J. Birdsall, *Trends Pharmacol. Sci.* **1993**, *14*, 237-239.
[45] T. Seidel; G. Ibis; F. Bendix; G. Wolber, *Drug Discov. Today Technol.* **2010**, *7*, 221-228.

10.1002/cmdc.201900283

WILEY-VCH

COMMUNICATION

Entry for the Table of Contents



Tailor-made: Dualsteric compounds derived from carbachol as orthosteric moiety can be tailored-made to activate or inactivate the muscarinic M_1 acetylcholine receptor to different extents. BQCA-carbachol derivatives and TBPB-carbachol-derived compounds show partial agonism or a formally competitive antagonism with orthosteric carbachol, respectively. The degree of intrinsic receptor response can be controlled in a wide range by carbachol and the length of the linker.