Photopharmacology

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A Photoswitchable Dualsteric Ligand Controlling Receptor Efficacy

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Abstract: *The investigation of the mode and time course of the activation of G-protein-coupled receptors (GPCRs), in particular muscarinic acetylcholine (mACh or M) receptors, is still in its infancy despite the tremendous therapeutic relevance of M receptors and GPCRs in general. We herein made use of a dualsteric ligand that can concomitantly interact with the orthosteric, that is, the neurotransmitter, binding site and an allosteric one. We synthetically incorporated a photoswitchable (photochromic) azobenzene moiety. We characterized the photophysical properties of this ligand called BQCAAI and investigated its applicability as a pharmacological tool compound with a set of FRET techniques at the M_1 receptor. BQCAAI proved to be an unprecedented molecular tool; it is the first photoswitchable dualsteric ligand, and its activity can be regulated by light. We also applied BQCCAI to investigate the time course of several receptor activation processes.*

The activity of numerous nervous processes in the human body, such as smooth-muscle contraction, the cardiac rate and force, and glandular secretion, is regulated by the peripheral nervous system, and its parasympathetic nerves are regulated by muscarinic acetylcholine receptors (mAChRs) through the metabotropic action of acetylcholine (ACh).[1] Hence, these receptors have been identified and utilized as therapeutic targets for the treatment of a broad spectrum of diseases.[2]

mAChRs belong to the class A family of G-protein-coupled receptors (GPCRs) and comprise five distinct subtypes.[3] M_1 receptors are abundantly expressed in the cortex, hippocampus, and striatum and play a key role in learning and memory processes. Agonists have been suggested for the treatment of cognitive impairment such as that associated with schizophrenia and Alzheimer's disease (AD).[4] Unfortunately, the tremendous efforts to develop therapeutically applicable selective M_1 ligands have not been successful owing to the high sequence homology of the orthosteric binding sites, that is, the binding sites of ACh. Owing to the plethora of very diverse physiological roles associated with the five mAChR subtypes, the lack of selectivity led to numerous side effects for the experimental therapeutics. As numerous details of the molecular basis of receptor function remain to be elucidated, suitable molecular tools are necessary.

The less conserved allosteric binding sites of the M receptors, including M_1, led to intensive efforts to develop selective allosteric modulators, that is, compounds that affect the binding of an orthosteric ligand or the endogenous neurotransmitter ACh either positively, neutrally, or negatively (Figure 1).[5] Such ligands are able to overcome the problem of subtype selectivity for the M_1 subtype by making use of cooperativity in the selective binding and activation of the receptor.[6]

To take this concept one step further, dualsteric (or bitopic) ligands are developed herein that covalently connect a high-affinity/low-selectivity orthosteric moiety to highly selective allosteric building blocks for concomitant interac-

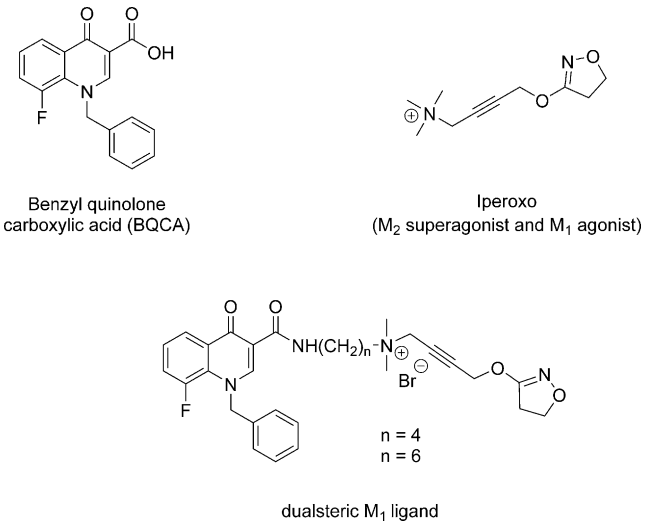

Figure 1. Structures of the non-selective M_1 agonist iperoxo, the positive allosteric modulator (PAM) benzyl quinolone carboxylic acid (BQCA), and a representative dualsteric M_1 ligand consisting of the two building blocks.

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tion with both binding sites. Recently, our groups have reported several sets of dualsteric M_1 and M_2 agonists for which dynamic ligand binding is assumed, leading to partial agonism for the M_1 receptor (Figure 1)[7] as well as for the M_2 receptor.[8]

Based on these findings, dualsteric ligands hold great potential both as future therapeutics and as pharmacological tools owing to their unprecedented selectivity at M receptor subtypes, their specificity for signaling pathways ("biased signaling"), their capability for partial agonism, and their potential for studying the process of receptor activation at the molecular level.[6,9] We have now made use of a photopharmacological approach by incorporating a photoswitchable (or photochromic) azobenzene as a linker into the dualsteric ligands described to specifically design dualsteric ligands for investigating spatiotemporal receptor activation processes with high precision.

The rapidly expanding field of photopharmacology aims to introduce light sensitivity into experimental therapeutics or drugs to control and/or investigate biological processes.[10] In this regard, molecular photoswitches that reversibly change their structure and physicochemical properties upon irradiation with light play an important role. The light-induced isomerization of the azobenzene photoswitch from the *trans* form to the thermodynamically less stable *cis* form is associated with significant changes in geometry and polarity.[11] When an azobenzene unit is incorporated into a bioactive compound, this change can be translated into an alteration in the biological activity towards the respective target.[12]

Following the design strategy for the dualsteric iperoxo/BQCA-type hybrids, we connected the superagonist iperoxo to a positive allosteric modulator with an *N*-benzyl quinolone carboxylic acid type structure. To directly investigate the effect of the spacer on the intrinsic activity of these hybrids at the M_1 receptor, we replaced the aliphatic carbon chain (polymethylene linker) with an azobenzene linker. Introducing the photoswitch into this part of the molecule should significantly change the relative position of the two pharmacophores, namely from a linear to a rectangular arrangement (Figure 2), and thus lead to a different binding mode.

Fluorescence detection and fluorescence resonance energy transfer (FRET) techniques are now well-established in pharmacological research for characterizing various processes,[13] such as receptor activation,[14] G-protein activation,[15] and arrestin signaling,[16] or further downstream detection of calcium, diacylglycerol (DAG), or cyclic AMP.[17] The advantage of such approaches compared to other methods is that they can be performed in living cells under almost physiological conditions.

Herein, we report the synthesis, characterization, and pharmacological testing of a benzyl quinolone carboxylic acid–azobenzene–iperoxo (**BQCAAI**) hybrid compound (Figure 2 and Scheme 1), which represents the first photoswitchable dualsteric ligand described to date. For comparison, we also synthesized a derivative with a benzene-containing alkyne linker (**18**; see the Supporting Information, Scheme S3) and photoiperoxo (Scheme S1), a photochromic iperoxo derivative, in which a hydrogen atom of a methyl

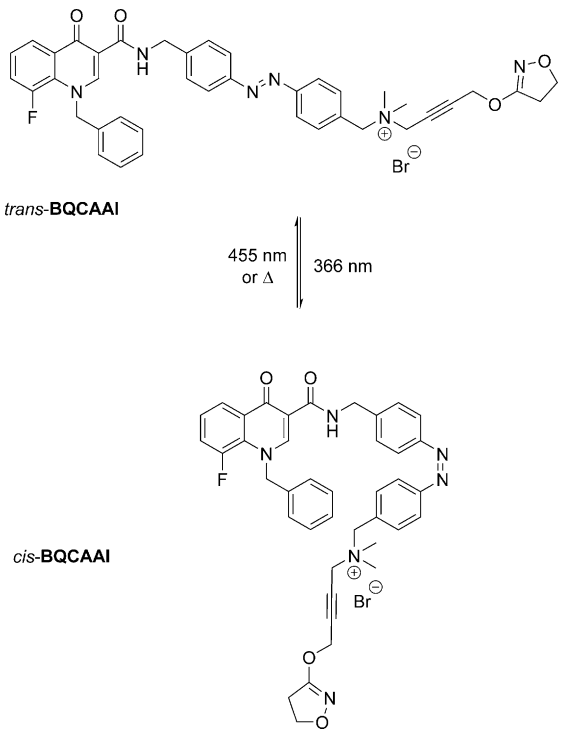
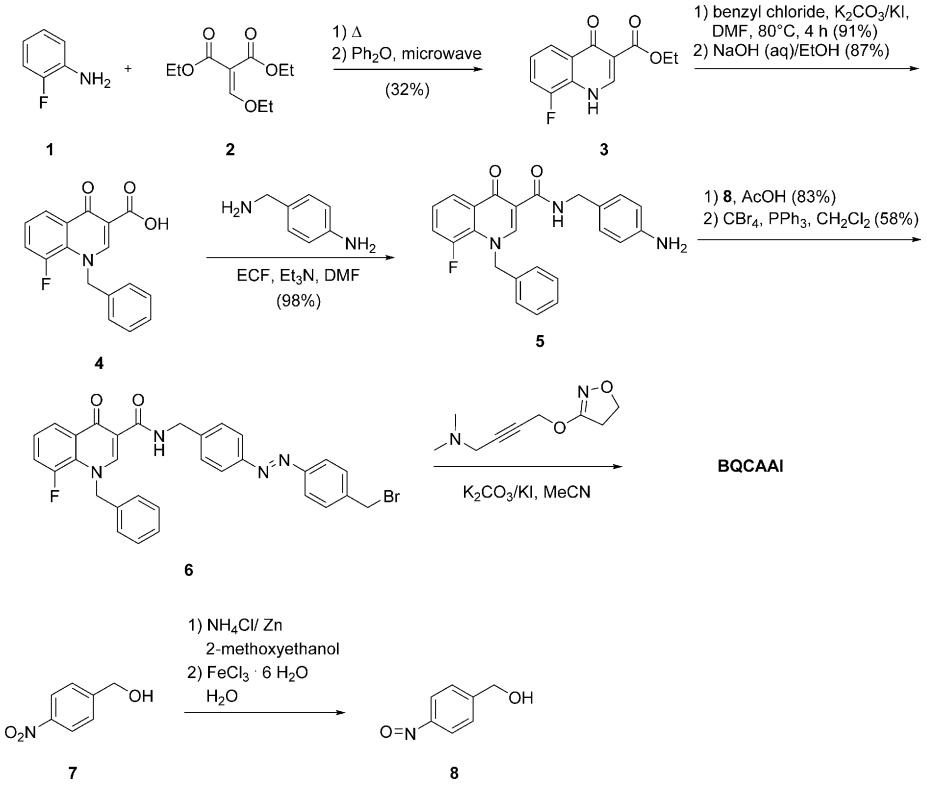


Figure 2. Structure of the photoswitchable dualsteric M_1 ligand **BQCAAI** in the *trans* and *cis* form.

group of the quaternary iperoxo ammonium salt has been replaced by an azobenzene moiety.

The synthesis of **BQCAAI** began with the construction of the quinolone skeleton through a microwave-assisted Gould–Jacobs reaction.[18] Benzylation and subsequent hydrolysis gave acid **4**, which reacted with 4-aminobenzylamine to form amide **5**. To introduce the azobenzene moiety into the target molecule, a Mills reaction was performed. The hydroxy function of the azobenzene compound was replaced by a bromine atom in an Appel reaction to give compound **6**. Finally, iperoxo, which had been synthesized using a convergent synthetic pathway,[19] was connected to the azobenzene group in a microwave-assisted reaction to afford the photoswitchable dualsteric ligand **BQCAAI** (Scheme 1).

As a prerequisite for the light-dependent control of the intrinsic activity (efficacy) at the human M_1 (hM_1) receptor, the photoswitchable compound needs to effectively respond to light. To this end, the structural change between the two photoisomers should be fast and significant, and a high degree of photoconversion (the *trans*/*cis* ratios should differ significantly) is necessary. Furthermore, the stability towards thermal isomerization as well as the intended pharmacological/biological applications have to be taken into account. First, **BQCAAI** was characterized by UV/Vis spectroscopy, which revealed clear photoswitchability (photochromic behavior) and the typical absorption bands of azobenzenes. The absorption maxima at around 325 nm and 430 nm are due to the π–π^* and n–π^* transitions, respectively, which allows for distinct photoswitching between the *trans* and *cis* forms. This process is reversible as switching can be repeated over many cycles without loss of photochromic behavior (Figures 3



Scheme 1. Synthesis of the benzyl quinolone carboxylic acid–azobenzene–iperoxo hybrid **BQCAAI**.

and 4). Second, the photostationary distribution of **BQCAAI** in the dark was determined by HPLC analysis to be 80% in favor of the *trans* form (20% of the *cis* form). Upon irradiation with UV light ($\lambda = 365$ nm), the ratio changed to 52% in favor of the thermodynamically less stable *cis* isomer (Figure 4). Finally, **BQCAAI** shows excellent thermal stability. When the compound is kept in the dark, the photostationary state is stable for several hours after UV light irradiation (Figure S1).

For pharmacological characterization of **BQCAAI** and its individual building blocks, a range of different fluorescence or FRET methods were applied. The orthosteric building block iperoxo, a synthetic agonist for all mAChR subtypes,[20] was used as a reference for all experiments and showed full agonism ($EC_{50} = 0.57$ μM). In contrast, the azobenzene-modified iperoxo derivative (photoiperoxo) **12** was unable to induce conformational changes at the M_1 receptor, both in the *trans* and in the *cis* form (Figure S2a), which is in agreement with previous findings for the M_1 receptor, namely that increasing the linker length of iperoxo derivatives leads to antagonism at this receptor.[21] To evaluate the affinity of compound **12** to the receptor, a competition experiment was performed (Figure S2b). Iperoxo (10 μM solution) induced a 70% receptor response, and compound **12**, when applied alone, did not induce any conformational changes. When iperoxo and compound **12** were applied together, a signal reduction of on average 50% was observed, indicating that both ligands compete for binding at the receptor. From this experimental setting (see Ref. [21a]), we conclude that photoiperoxo must have a distinct affinity for the receptor and can be regarded as an antagonist. This was confirmed by studying the calcium release upon ligand binding (Figure S2c) with a calcium- and DAG-sensitive fluorescent probe. Whereas iperoxo induced a rapid calcium response, no calcium release was observed for compound **12**, even more than 250 s after application (Figure S2d). When compound **12** was first applied for 20 s followed by addition of iperoxo, an immediate calcium response was detectable for iperoxo (Figure S2e), clearly indicating that compound **12** does not exhibit agonism although binding to the receptor has been indirectly shown (Figure S2b).

The same setting was used for investigations with **BQCAAI**, which showed that both isomers interact with the M_1 receptor (Figure 4a). A receptor response of 25% for the *trans* form and a significantly reduced signal of 14% for the *cis* form were detected. Interestingly, the activation is slower than with iperoxo. Comparable results were obtained for derivative **18** (RM405) with a linear alkynylbenzene linker (Figure 4f),[21a] but the process was significantly slower than for related dualsteric ligands bearing a polymethylene linker. This finding suggests that the receptor activation kinetics of a dualsteric ligand and the structure of the linker moiety are closely related.

To gain further insight into the characteristic properties of *trans*- and *cis*-**BQCAAI** with respect to receptor agonism, G-protein activation was investigated using G-protein FRET sensors. As shown in Figure 4b, both isomers were able to induce a G-protein response. With regard to iperoxo, the *trans* isomer induced a more pronounced signal than the *cis* isomer.

However, both of these FRET sensors are excited at 436 nm, a wavelength that induces isomerization of the azobenzene moiety favoring the *trans* isomer. Given the rapid isomerization from the *cis* to the *trans* isomer (Figure 3b), we wanted to investigate if our detection system interferes with the true efficacy of the two ligand configurations owing to the slower kinetics of the G protein response compared to receptor activation. Repeating the experiment with a lower data sampling frequency (1 Hz, taking 1 data point every second, instead of 10 Hz, with taking data points every 100 ms) reduces the light exposure by 90% and should hence influence the *cis*-to-*trans* isomerization (Figure 4c). When the light exposure was reduced, Gq activation by the *trans* isomer was not affected, whereas the signal of the *cis* isomer was significantly reduced by more than 30%.

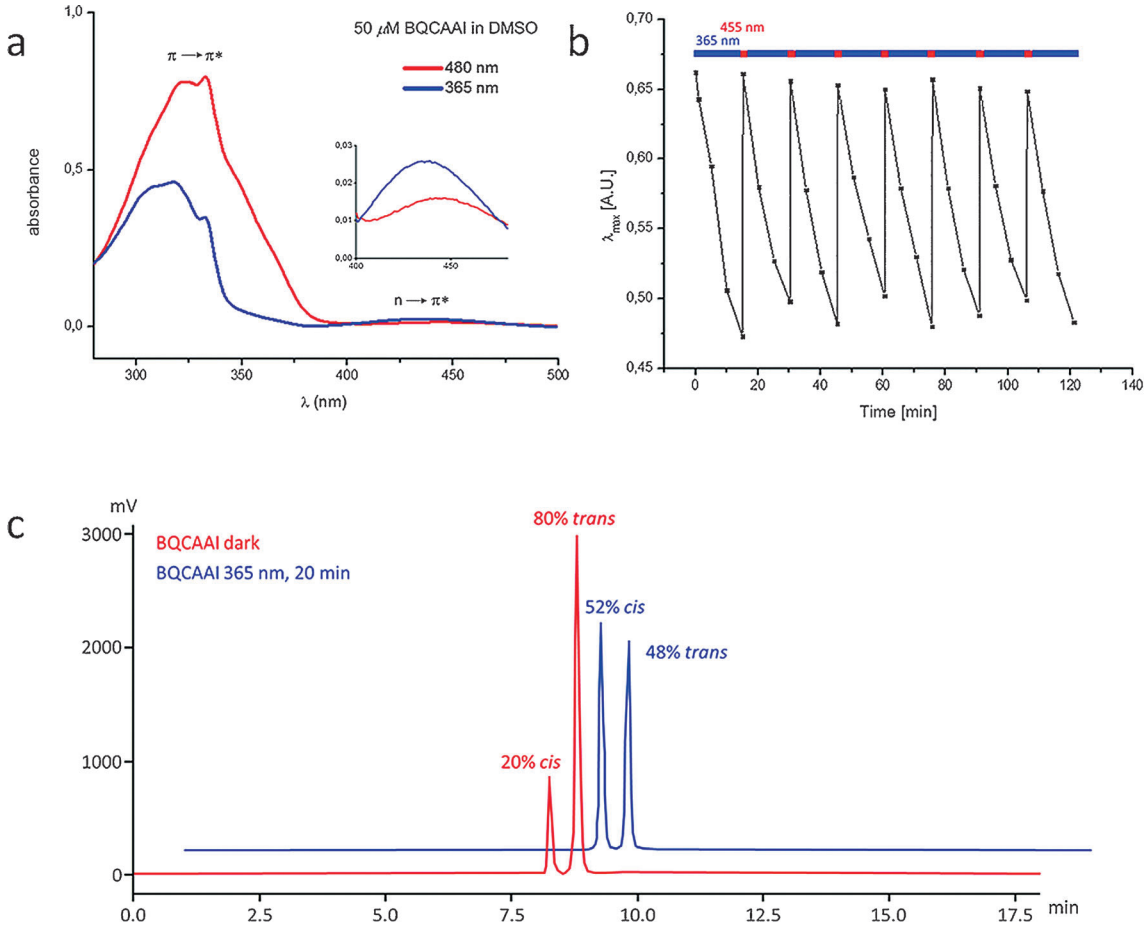


Figure 3. Photochemical characterization. a) Absorption spectra of **BQCAAI**, show distinct photochromic behavior. b) The photoswitching process can be repeated over many cycles without noticeable photofatigue. c) HPLC chromatogram of **BQCAAI** showing the photostationary states in the dark (red) and after irradiation with UV light (blue).

Subsequently, we used a dual Ca^{2+}/DAG fluorescent probe with 488 nm and 562 nm excitation wavelengths,[21a] which can be used simultaneously or separately, with the benefit that one excitation signal lies within the absorption spectrum of **BQCAAI** and the other one does not (Figure 3a). Monitoring calcium only without DAG for **BQCAAI** shows a fast signal for the *trans* isomer (Figure 4d, red trace) whereas the *cis* isomer does not induce a signal over more than 200 s (Figure 4e, red). Exciting both fluorescent probes simultaneously results in the same observation for the *trans* isomer (Figure 4d, green) but in an opposite result for the *cis* isomer (Figure S3), which induces both a calcium and a DAG response, but with a rather long time delay of 45 s. This behavior is likely due to an induced switching process by the light used for detection, which would allow for a certain proportion of the *cis*-**BQCAAI** to switch back to *trans*-**BQCAAI**. This assumption is strengthened by the absence of a calcium signal evoked by *cis*-**BQCAAI** when only the calcium signal is monitored without DAG detection (Figure 4e).

Taken together, we have clear evidence that **BQCAAI** is not only the first reported photoswitchable dualsteric ligand for GPCRs, but also the first "dimmable" one, in the sense that *cis*-**BQCAAI** acts as an antagonist while *trans*-**BQCAAI** is an agonist. We have shown that a direct detection system based on receptor conformational changes has an influence itself because of the current need for an excitation wavelength of 436 nm. The use of a dual Ca^{2+}/DAG sensor helped to overcome this problem. The receptor subtype selectivity of these ligands and the possible application of **BQCAAI** to investigate the receptor activation of other M subtypes are currently unclear and are subject to ongoing research.

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Conflict of interest

The authors declare no conflict of interest.

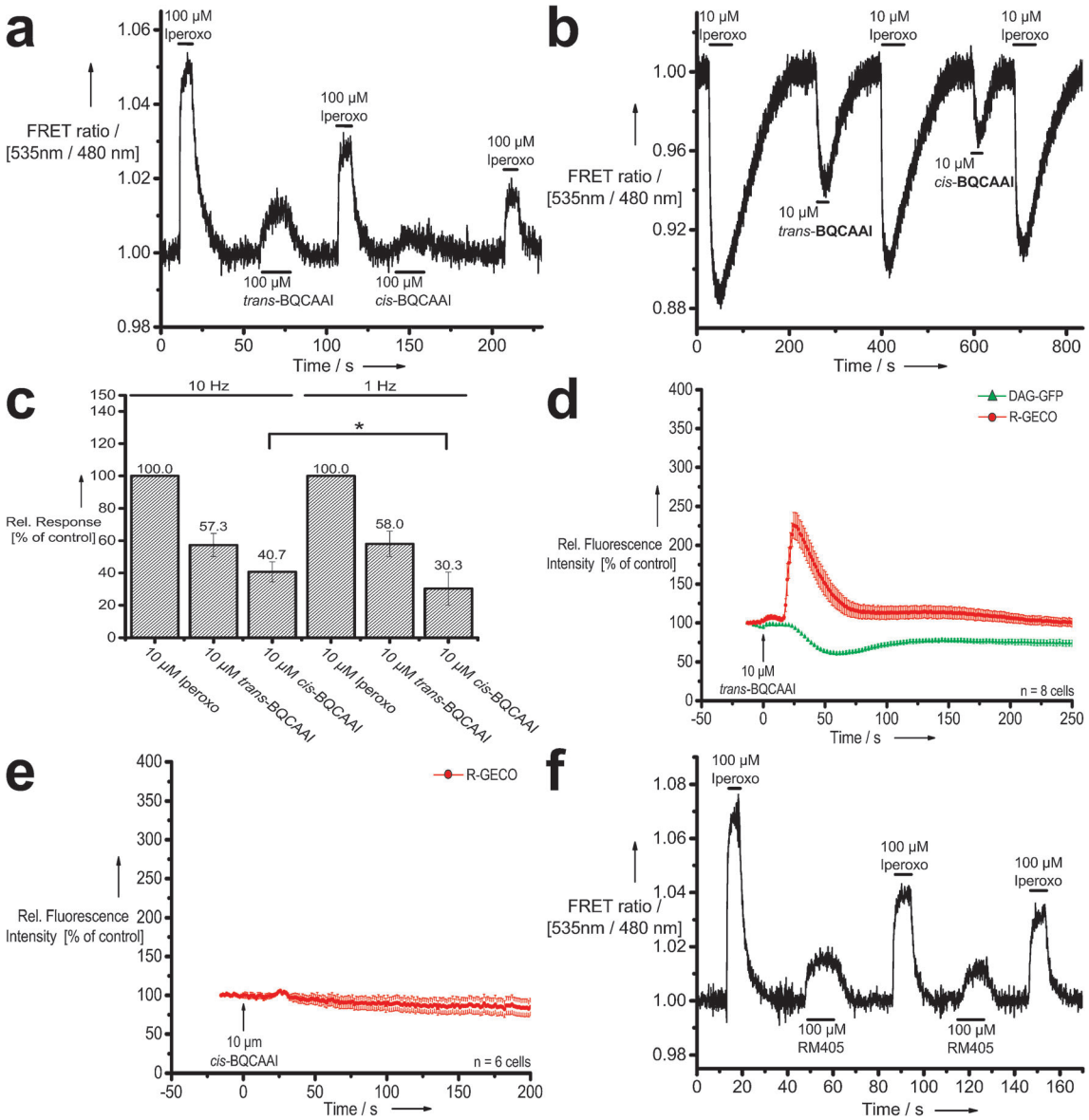

Figure 4. Characterization of the dualsteric **BQCAAI** hybrid compound at the receptor level and its effect on downstream signaling. a) A representative single-cell recording of HEK293 cells stably expressing the M1-IN3-CFP receptor sensor. A solution of 100 µM iperoxo was used as the reference. b) Representative FRET trace of HEK293 cells expressing a Gq sensor. c) Schematic comparison of the Gq activation of both isomers at different irradiation frequencies. d) *trans*-**BQCAAI** induced a detectable Ca^{2+} and DAG response that is significantly lower than the response to iperoxo. e) *cis*-**BQCAAI** was not able to induce a Ca^{2+} response over more than 200 s. f) The *trans*-**BQCAAI** isomer showed reduced activation kinetics at the receptor level compared to a dualsteric ligand with a polymethylene linker. Replacing the polymethylene linker with a benzene-containing linker resulted in comparable activation kinetics.

Keywords: acetylcholine receptors · azobenzenes · dualsteric ligands · G-protein-coupled receptors · photopharmacology

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Communications

Photopharmacology

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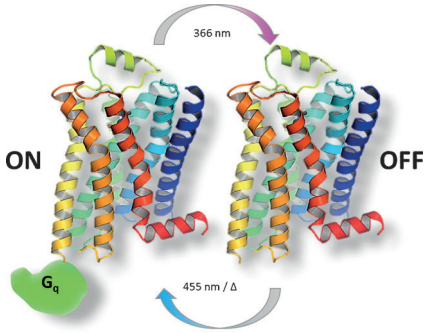
A Photoswitchable Dualsteric Ligand Controlling Receptor Efficacy



A dualsteric acetylcholine receptor ligand that interacts with two binding sites of the receptor and contains a photoswitchable azobenzene unit was synthesized. FRET studies reveal that the activity of the ligand can be reduced with light and that it represents an unprecedented molecular tool to investigate the time course of receptor activation processes.