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Photopharmacology

# Optical Control of Acetylcholinesterase with a Tacrine Switch**

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***Abstract:*** *Photochromic ligands have been used to control a variety of biological functions, especially in neural systems. Recently, much effort has been invested in the photocontrol of ion channels and G-protein coupled receptors found in the synapse. Herein, we describe the expansion of our photopharmacological approach toward the remote control of an enzyme. Building on hallmark studies dating from the late 1960s, we evaluated photochromic inhibitors of one of the most important enzymes in synaptic transmission, acetylcholinesterase (AChE). Using structure-based design, we synthesized several azobenzene analogues of the well-known AChE inhibitor tacrine (THA) and determined their effects on enzymatic activity. One of our compounds, AzoTHA, is a reversible photochromic blocker of AChE in vitro and ex vivo with high affinity and fast kinetics. As such, AzoTHA can be used to control synaptic transmission on the neuromuscular endplate based on the light-dependent clearance of a neurotransmitter.*

Synaptic communication is largely based on small diffusible molecules that translate electrical signals into chemical ones.[1] Once released from synaptic vesicles, these neurotransmitters cross the synaptic cleft to stimulate receptors, that is, ligand-gated ion channels and G-protein coupled receptors, on the postsynaptic side. A third essential component of synaptic transmission consists of transporters or enzymes that remove or inactivate the neurotransmitter, respectively, to prevent tonic stimulation and allow for the meaningful integration of signals.

Among a diverse set of neurotransmitters that mediate chemical communication in humans, acetylcholine (ACh) is especially important. When released, it stimulates nicotinic and muscarinic acetylcholine receptors to modulate cellular excitability. Fast inactivation is achieved by acetylcholinesterase (AChE), which hydrolyzes ACh to acetate and choline with diffusion-controlled kinetics (Figure 1a).[2] A large number of AChE inhibitors are known, which range from drugs, such as tacrine (THA), to research tools, such as decamethonium, and nerve gases, such as sarin (Figure 1b). Many of these have been co-crystallized with AChE, providing detailed insights into the mode of action of this enzyme and its inhibitors.[3] Some of these are clinically relevant, because they raise ACh levels, which has beneficial effects to patients suffering, for instance, from Alzheimer's disease, myasthenia gravis, and glaucoma.[4]

In recent years, the control of neural systems with light has become a major scientific frontier. Light is noninvasive and can be applied with very high temporal and spatial control. It can interact with genetically encoded photoreceptors ("optogenetics")[5] or with photoswitchable drugs that target native receptors and change their efficacy upon photoisomerization (Figure 1c). We call the latter approach "photopharmacology".[6] It has been applied to the optical control of ion channels[7] and GPCRs[8] and has been used to restore visual response in blind mice[9] and control nociception mediated by DRG neurons.[10]

Enzymes such as AChE, which have fast kinetics and a large influence on the activity of neural networks, are also a prime target for photopharmacology. Indeed, AChE has been investigated in this respect early on. Pioneering studies from the late 1960s and early 1970s by Erlanger and colleagues show that photoregulation of AChE could be achieved with azobenzene-based quaternary ammonium ions that mimic ACh, such as *p*-phenylazophenyl-trimethylammonium and *N*-*p*-phenylazophenylcarbamylcholine (Figure 1d).[11] Photocontrol of an AChE isolated from the scorpion *Heterometus fulvipes* was achieved by exposure to sunlight.[11b] Activity was measured by a reaction between nonhydrolyzed ACh and hydroxylamine, followed by photometric quantification of an iron complex,[12] thus providing a proof-of-concept for a photoswitchable enzyme inhibitor.

Since Erlanger's pioneering studies, the ability to heterologously express enzymes, reliably assay them, study them with X-ray crystallography, and rationally design ligands has much improved. In addition, the precise delivery of light of a given wavelength and intensity has become more practical, recently driven by the rapid development of optogenetics. Given our longstanding interest in the optical control of neural systems, we therefore decided to reinvestigate photochromic blockers of mammalian AChE, that is, human AChE, and to explore new chemotypes that could prove to be useful in neuroscience.

In order to quickly assay human AChE activity in the presence of a photoswitchable inhibitor, we modified a commercially available kit, which is used to measure the AChE

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Supporting information for this article (experimental details including all synthesis, chemical characterizations, and assay conditions) is available on the WWW under http://dx.doi.org/10.1002/anie.201403666.

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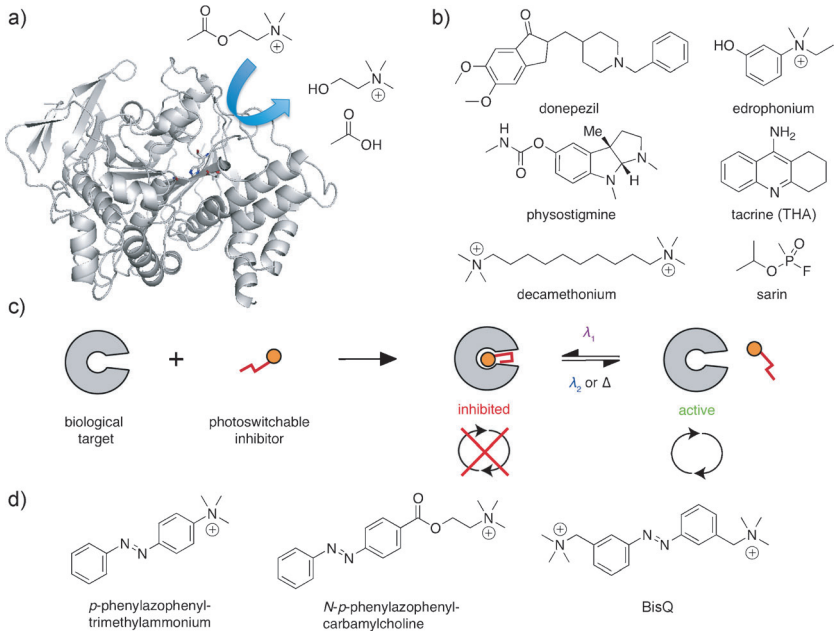


***Figure 1.*** AChE function and inhibition. a) AChE (pdb: 1ACJ)[13] catalyzes the hydrolysis of acetylcholine (ACh) to give acetate and choline. b) Noncovalent and covalent inhibitors of AChE activity. c) Photopharmacological control of an enzyme with a photochromic inhibitor. d) Photochromic molecules used to control the cholinergic system.

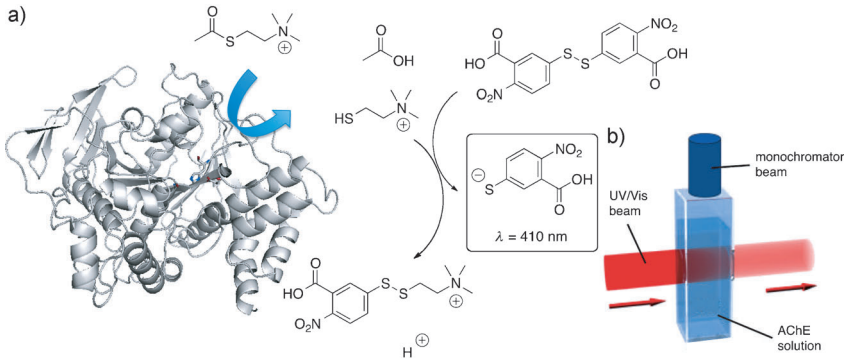


***Figure 2.*** Colorimetric assay for measuring the AChE activity in the presence of a photoswitchable blocker. a) Hydrolysis of acetylthiocholine gives thiocholine, which reacts with Ellman's reagent to give a colored thiolate ($\lambda_{max}$ = 410 nm). b) Schematic representation of the experimental configuration.

content in a variety of biological samples.[14] It utilizes the intrinsic ability of AChE to cleave acetylthiocholine, which subsequently reacts with Ellman's reagent to liberate a colored thiolate (Figure 2a). Using a UV/Vis spectrometer, we were able to photometrically follow the appearance of the thiolate at $\lambda = 410$ nm, while alternately irradiating with an orthogonal light beam of different wavelengths, to reversibly trigger photoswitching (Figure 2b).

Our initial efforts were directed at the doubly charged ACh mimic BisQ (Figure 1d), which was developed by Erlanger and Lester as a photoswitchable agonist for the nicotinic acetylcholine receptor,[15] but had not been described as photoswitchable inhibitor of AChE. Given its resemblance with decamethonium, we wondered whether it could also act as a photochromic inhibitor of the enzyme as well. We found that BisQ is indeed a blocker of human AChE in the dark ($K_i = 3.13$ μM, Table 1), but does not significantly change its activity upon photoisomerization. This result and an inspection of the X-ray structure of decamethonium bound to AChE (pdb: 1ACL)[13] imply that BisQ does not simultaneously occupy both cationic binding sites when blocking the enzyme. Thus we hypothesized that both isomeric forms of BisQ exhibit comparable blocking characteristics.

A systematic exploration of AChE X-ray structures showed that the inhibitors tacrine (THA; pdb: 1ACJ)[13] and 9-benzyl THA (pdb: 1DX4)[16] occupy the catalytic site of the enzyme, while being partially solvent-exposed. The knowledge that substitution in the 9-position of THA is tolerated enabled the design of several azobenzene derivatives that we call AzoTHAs (Scheme 1a). Our small series includes a minimally substituted azobenzene (AzoTHA), an ester derivative thereof (AzoTHA-2), and a triazole (AzoTHA-3), which was prepared by "click chemistry" to pave the way for rapid diversification. The synthesis of AzoTHA started with the condensation of anthranilic acid (**1**) and cyclohexanone (**2**) in refluxing phosphoryl chloride to give 9-chloro-THA (**3**).[17] Substitution of the chloride with 4-(2-aminoethyl)aniline (**4**) enabled by catalytic amounts of sodium iodide in refluxing phenol gave phenethylamine derivative **5**. Finally, the azobenzene moiety was installed through Mills reaction using nitrosobenzene (**6**; Scheme 1b). As a "regular" azobenzene, AzoTHA could be switched to its *cis* form with UV light ($\lambda = 350$ nm) and back to its *trans* form using blue light ($\lambda = 440$ nm; Scheme 1b,c). The small change in the π–π* band upon irradiation can be partly attributed to the underlying absorption of the quinoline chromophore of THA (black line, Scheme 1c). Details of the switching kinetics of AzoTHA and synthesis of AzoTHA-2 and AzoTHA-3 can be found in the Supporting Information.

AzoTHA in its dark-adapted form inhibits AChE with a $K_i$ of approximately 100 nM (Table 1), while AzoTHA-2 and

***Table 1:*** Inhibitory constants ($K_i$) of AChE blockers ($N = 3$, mean ± standard deviation).

| | THA | AzoTHA | decamethonium | BisQ |
|---|---|---|---|---|
| $K_i$ [nM] | 6.40 ± 3.20 | 95.35 ± 12.39 | 457.8 ± 52.0 | 3129.3 ± 1181.4 |


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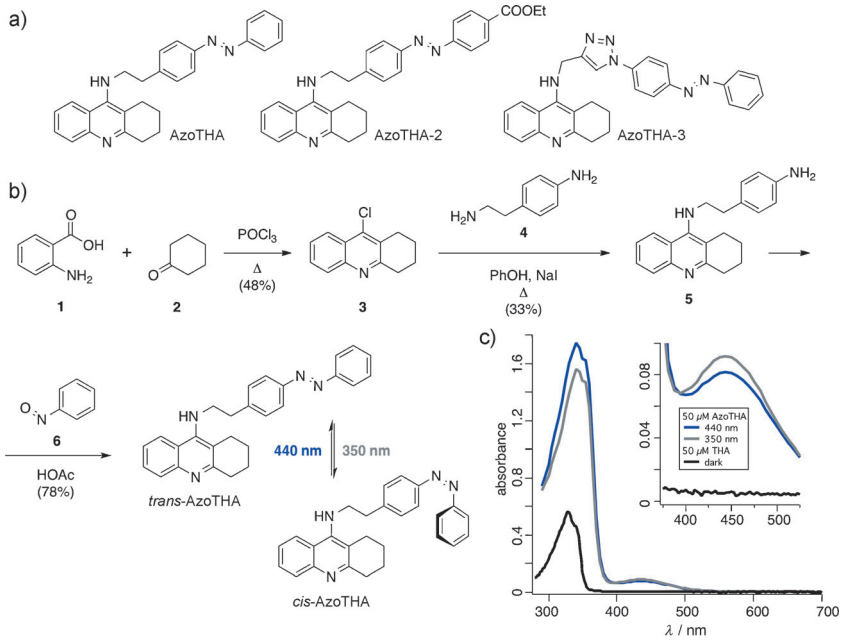


***Scheme 1.*** Design, synthesis, and spectroscopy of AzoTHAs. a) A small compound library of tacrine-derived photoswitches. b) Three-step synthesis gives the photochromic AChE blocker AzoTHA, which exhibits reversible *cis–trans* isomerization using UV light ($\lambda = 350$ nm) and blue light ($\lambda = 440$ nm). c) UV/Vis spectra of the *cis* (gray) and *trans* isomer (blue) of AzoTHA (inset: expansion of the n–π* band) and THA (black).

AzoTHA-3 showed significantly lower affinities (see the Supporting Information). As such, *trans*-AzoTHA is about ten times less active than THA, but more active than decamethonium and BisQ.

Having determined the $K_i$ of AzoTHA, we assessed the photo-dependent activity of our enzyme inhibitor at this concentration. In photopharmacology, it is generally advisable to employ concentrations close to the $K_i$ (or $EC_{50}$) of the dark-adapted state, as the largest dynamic change can be expected in this region. When we incubated 0.05 U mL$^{-1}$ of AChE with 1.0 μM of AzoTHA, we were able to induce photodependent activity (Figure 3a). By contrast, AzoTHA-2 and AzoTHA-3 exhibited no and low photodependent activity. Our investigations therefore focused on AzoTHA (Figure 3). Activity was reduced to 17% compared with unblocked AChE when the sample was irradiated with blue light ($\lambda = 440$ nm) and was almost abolished (4% activity) when the light was switched to UV ($\lambda = 350$ nm). Thus, AzoTHA is more active as an inhibitor in its *cis* state and becomes less active in the dark-adapted *trans* state. This effect could be repeated over many cycles with no apparent loss of activity (Figure 3b,c). Fitting the off- and on-sets-slopes of photoswitching exponentially, the $\tau_{on/off}$ values remain stable (Figure 3d) over the cycles and their average can be determined as $\tau_{off} = (4.74 \pm 1.81)$ s and $\tau_{on} = (4.17 \pm 1.09)$ s.

To establish that AzoTHA can be used to control physiological responses with light, we next turned to mouse trachea. The smooth muscle of this airway constricts in response to ACh, which can be recorded by force transducers, an assay known as tracheal tensometry.[18] Electrical field stimulation (EFS) leads to the release of ACh at the neuromuscular endplate, in an experimental setting similar to the classical Loewi experiment.[19] When the electrical stimulation is shut off, ACh release from nerve fibers ceases and the relaxation kinetics of the trachea reflect AChE activity as the enzyme is degrading ACh. The high affinity AChE blocker physostigmine was used as reference in our experiments. At 10 μM, physostigmine induced a constriction of the preparation even without further EFS (Figure 4a), which is attributed to a direct activation of acetylcholine receptors in addition to AChE inhibition.[20] In contrast, 50 μM AzoTHA in the dark did not evoke an increase in the basal tone (Figure 4a), providing a pharmacologically cleaner tool to study cholinergic systems. When AzoTHA treated (50 μM) tracheas were stimulated by EFS (100 Hz, 10 V, 2 ms) different relaxation kinetics were observed under irradiation with UV and blue light (Figure 4b). The observed kinetics ($\tau_{off}$) are in agreement with our previously performed in vitro experiments: In the presence of *cis*-AzoTHA (UV irradiation), AChE is inhibited to a larger extent and ACh clearance kinetics are significantly slower ($\tau_{off,cis} = (15.25 \pm 1.48)$ s) with

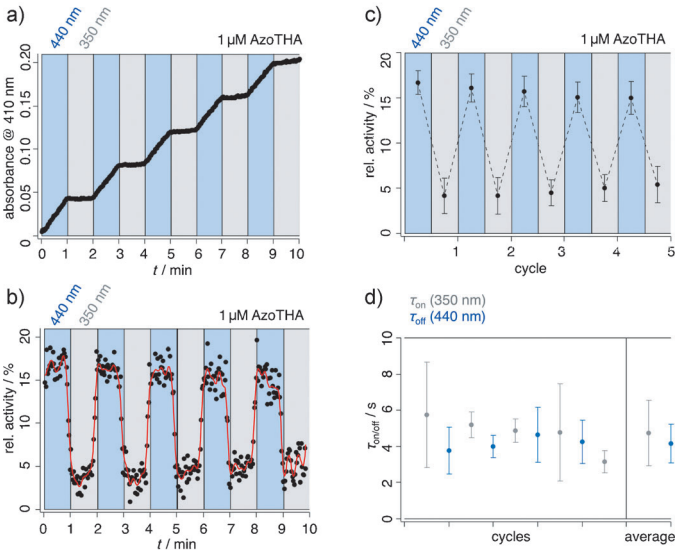


***Figure 3.*** In vitro photocontrol of AChE with AzoTHA. a) Hydrolysis of acetylthiocholine in the presence of AChE (0.05 U mL$^{-1}$), AzoTHA (1 μM) and irradiated with either blue light ($\lambda = 440$ nm, blue bars) or UV light ($\lambda = 350$ nm, grey bars). b) Reversible photocontrol of AChE (0.05 U mL$^{-1}$) with 1 μM AzoTHA controlled by blue light ($\lambda = 440$ nm, blue bars) and UV light ($\lambda = 350$ nm, grey bars). Black dots show data points derived from the initially recorded slope (see a) of catalytic activity, red line shows binomial fit. c) Average light-dependent activity calculated from b over 5 cycles. d) $\tau_{off}$ (gray) and $\tau_{on}$ (blue) values for the repeated deactivation and activation over several cycles (left side) and their averaged values (right side). Error bars show standard deviation; $N = 3$ for all experiments.


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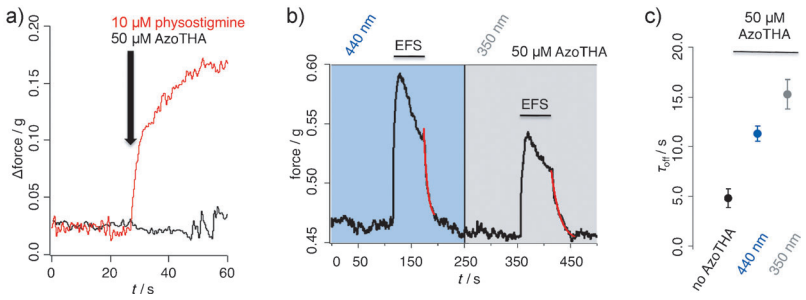


***Figure 4.*** Ex vivo photocontrol of AChE with AzoTHA. a) Basal tonus is not affected by AzoTHA (50 μM, black) but with physostigmine (10 μM, red) in mouse trachea preparations. b) Relaxation kinetics are slower under irradiation with UV light (350 nm) than with blue light (440 nm) in mouse trachea preparations (50 μM AzoTHA, EFS, 100 Hz, 10 V, 2 ms). c) $\tau_{off}$ values fitted from (b) with no blocker (black) and with AzoTHA (50 μM) under blue ($\lambda = 440$ nm, blue) and UV light ($\lambda = 350$ nm, gray); ($N = 3$, mean ± standard deviation, t-test: $p = 0.02$).

respect to *trans*-AzoTHA (blue light, $\tau_{off,trans} = (11.29 \pm 0.77)$ s). In the absence of an inhibitor, ACh is cleared even faster ($\tau_{off} = (4.81 \pm 0.95)$ s; Figure 4c). Our results demonstrate an increase in inhibition of 61% under UV light irradiation when background is subtracted. It should be noted that a decrease in amplitude was also observed in control experiments, when EFS was performed without any AChE blockers. Nevertheless, kinetics under UV and blue light followed the same trend, regardless in which order they were applied and EFS was conducted (see the Supporting Information).

In summary, we have rationally designed and synthesized several novel photochromic inhibitors of AChE, one of the most important enzymes involved in synaptic transmission. Starting with a commercially available AChE assay kit, we developed a generally applicable method to monitor photochromic AChE inhibitors. Using this in vitro assay, we found that one of our molecules, AzoTHA, can reversibly switch enzymatic activity in a fast manner using light. We also determined that BisQ, a known photochromic agonists of acetylcholine receptors, has no photodependent effect on AChE activity. Further biological evaluation showed that AzoTHA does have a light-dependent effect on AChE kinetics in mouse trachea preparations with improved characteristics compared to physostigmine. In accordance with in vitro data, AChE activity can be reduced to a larger extent with UV light than with blue light.

While our paper was in revision, a publication from Decker and colleagues appeared that demonstrates control of AChE activity with a dithienylethene photoswitch, allowing the optical control of β-amyloid aggregation.[21] However, because of the long irradiation times necessary to achieve photoswitching and the different biological target, this tool is not comparable with AzoTHA, which works on a second timescale and operates on the neuromuscular endplate. Our study remains the first example of manipulating neural communication by optical control of an enzyme that clears a neurotransmitter from the active zone.

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