Optical Control of GABA\textsubscript{A} Receptors with a Fulgimide-Based Potentiator**

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Abstract: Optogenetic and photopharmacological tools to manipulate neuronal inhibition have limited efficacy and reversibility. We report the design, synthesis, and biological evaluation of Fulgazepam, a fulgimide derivative of benzodiazepine that behaves as a pure potentiator of ionotropic \(\gamma\)-aminobutyric acid receptors (GABA\textsubscript{A}R\textsubscript{S}) and displays full and reversible photoswitching in vitro and in vivo. The compound enables high-resolution studies of GABAergic neurotransmission, and phototherapies based on localized, acute, and reversible neuroinhibition.

In the quest to understand brain circuits, controlling neuronal activity with light has become an essential tool to manipulate the balance between excitation and inhibition.\cite{1} However, optogenetic tools to inhibit neurons (halorhodopsin pumps, anion-conducting bacterial channelrhodopsins and chloride-conducting ChR2 mutants)\cite{2–4} have very limited conductance and dynamic responses to depolarization. Caged \(\gamma\)-aminobutyric acid (GABA) compounds have been used to inhibit spines and to control seizures, but uncaging is irreversible and their neurotoxicity\cite{5} has been avoided only recently by means of allosteric ligands.\cite{6} A powerful alternative is using reversible medicinal photoswitches\cite{7–10} to harness endogenous anion-conducting receptor-channels like GABA and glycine receptors (GABA\textsubscript{A}R, GlyR), which mediate inhibitory neurotransmission in the mammalian central nervous system.\cite{11} Although some GABA\textsubscript{A}R photoswitches have been reported based on azobenzene,\cite{12–14} this photochromic group displays several shortcomings: it provides incomplete photoconversion due to a substantial overlap of the absorption maxima of cis and trans isomers, and can alter the pharmacophore activity. Indeed, in all azobenzene derivatives of benzodiazepines (allosteric potentiators of GABA\textsubscript{A}R) this characteristic property is abolished, as found in the 7-amino site of nitrazepam, which is reportedly tolerant of other substitutions.\cite{15} In addition, GABA\textsubscript{A}R photoswitches described so far are agonists or antagonists that interfere with endogenous neurotransmission, not pure modulators.\cite{13,16,17}

Here we introduce Fulgazepam (compound 4), a derivative of diazepam based on a different photochromic group (fulgimide), which shows both quantitative reversible switching and GABA\textsubscript{A}R potentiation. It is inactive in its open isomer and potentiates the receptor in its closed isomer without concomitant agonist or antagonist activity, thus overcoming the above mentioned hurdles and displaying an ideal photopharmacological profile. Most importantly, Fulgazepam works both in vitro and in vivo, which indicates that the compound is devoid of toxicity and has favourable molecular properties enabling wide applications.

In contrast to azobenzenes, dithienylethenes, fulgides and their fulgimide-named amide derivatives generally feature high photostationary states (PSS) with both photoisomers being

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thermally stable.\textsuperscript{[7,18]} As dithienylethenes often lack of switching efficiency and stability in polar solvents due to a twisted intramolecular electron charge transfer,\textsuperscript{[20–22]} we chose fulgimides as photochromic scaffold in this study. Both subtypes can be interconverted between their flexible, less-coloured ring-open and their rigid, more coloured ring-closed isomer upon light-induced conrotatory 6π-electrocyclic rearrangement (Figure 1, Scheme 1).\textsuperscript{[18,23]} Although switching from the open to the closed form is usually triggered using UV light, this might be avoided by the isolation and separate application of both isomers. In addition, this ensures the application of quantitative amounts of either the open or the closed form. Thereby, a biological effect can clearly be assigned to one or the other conformation. Synthetic investigations revealed the beneficial effects of an isopropyl group in the alpha bridge position of the fulgide, as the $E$–$Z$ isomerization of the open isomer is suppressed due to steric hindrance and consequently only two distinct isomers are observed (Figure 1A).\textsuperscript{[19]}

One advantage of fulgimides over fulgides is their improved switching in aqueous solutions and high stability. Furthermore, the two-step transformation of fulgides towards fulgimides via nucleophilic ring-opening of the anhydride by a primary amine and subsequent recyclication allows the smooth introduction of amino-functionalized biomolecules.\textsuperscript{[18,19]} However, few biological applications of fulgi(m)ides are reported.\textsuperscript{[24–26]} The transformation of a known ligand into a photosensitive molecule can be designed by either extending the pharmacophore with a photoswitch or via incorporation of the photochromic scaffold as part of the drug’s chemical structure. Once introduced, ideally one isomeric state is biologically active whereas the other loses its required interactions. In the presented work, both approaches were pursued. On the one hand, a furan-fulgide photochromic scaffold was merged with an amino-benzodiazepine under fulgimide formation (Figure 1B, left panel). A difference in activity can be expected from the different flexibility of the isomeric states. On the other hand, a functionalized diazepine was synthesized aiming for a photochromic benzodiazepine core (Figure 1B, right panel). In this case, the difference in activity was expected to be given by the different conjugation of the pharmacophore’s aromatic system upon switching. Unfortunately, the latter modified pharmacophore (compound 7, Figure 2B) was inactive in patch-clamp studies (data not shown) and the synthesis towards the photoswitch was not further pursued.

To obtain a photochromic pharmacophore core, we envisioned a functionalized diazepine derivative (7) providing an acetyl group in position 3 required for Stobbe\textsuperscript{[28–30]} condensation towards fulgide formation and a methyl-group in position 2 beneficial for the fulgide’s switching performance.\textsuperscript{[25]} For diazepine formation, the highly functionalized precursor 7 requires in addition a primary amine in position 5 and a phenone substitution in position 4.\textsuperscript{[27]} Based on the literature known Gewald-reaction\textsuperscript{[26]} and screening of solvents and bases the desired functionalized furan 7 and its diazepine formation towards compound 9 was obtained in good yield in a one-step synthesis starting from commercially available benzoylacetonitrile 5 and 3-chloroacetylaceton 6 (Figure 2B).\textsuperscript{[32,33]} The following ring closure required for diazepine formation of 9 was performed in analogy to literature reports.\textsuperscript{[27]}

Regarding the photochromic properties, the introduction of the bulky isopropyl group on the 1,3,5-hexatriene system of the fulgide avoided the undesired UV light induced $E$–$Z$ isomerization of the open $E$-fulgimide isomer (Figure 1A). Only the $E$ isomer underwent a photocyclization reaction to the thermally stable closed isomer (Figure 3A). The colorless open isomers 3a and 4a were converted to their strongly colored ring closed isomers 3b and 4b upon illumination with UV light of $\lambda = 365$ nm. The absorption maximum of the open...
isomer around 340 nm decreased and a new maximum around 520 nm representing the closed isomer formed (Figure 3B). Both compounds show almost quantitative ring-closing (93% for 3b and 95% for 4b, measured 50 μM in DMSO) and quantitative ring-reopening using green light (λ = 505 nm or 528 nm).

To characterize Fulgazepam in vitro, patch clamp experiments were performed on cells transiently expressing α1β2γ2 subunits of the GABA<sub>R</sub> receptor. This receptor possesses the canonical benzodiazepine allosteric site and its EC<sub>50</sub> for GABA is about 8 μM. The effects of the fulgimide-based benzodiazepine derivatives 3 and 4 on the receptor’s function were studied upon co-application of 0.5 μM GABA, that is, a concentration below the EC<sub>50</sub> (close to EC<sub>3</sub>) that allows to observe allosteric potentiation of GABA<sub>R</sub>-mediated currents.<sup>[34]</sup>

Application of compound 4a (open isomer) (10 μM) caused no significant effect on GABA<sub>R</sub>-mediated currents, while application of 4b (closed isomer), generated by pre-illumination with UV light (365 nm), induced an increase of GABA<sub>R</sub>-mediated current amplitudes (Figure 4A). Thus, the isomers of compound 4 interact differently with GABA<sub>R</sub>Rs, being inactive in the open form and potentiating in the closed form. Analysis of a series of dose-response curves established that the EC<sub>50</sub> for 4b was 13 μM (n = 6; Figure 4B). Figure 4C demonstrates that UV illumination switches the conformation of 10 μM compound 4 (from 4a to 4b) and increases the amplitude of GABA-induced currents by 228 ± 41% (Figure 4D; n = 11). Compound 3a in its open state co-applied with GABA (0.5 μM) induced a strong potentiation of GABA<sub>R</sub>-mediated currents (Figure 3A). This potentiation was not sensitive to illumination by UV light and subsequent isomerization to the closed isomer 3b (Figure S3B) and the kinetics of compound 3b’s development (slow wash-in and slow wash-out) was similar to the one of 4b. Application of 10 μM of 3a increased the current amplitude by 292 ± 65%, while 50 μM of 3a increased the current amplitude by 544 ± 107% (n = 11). The EC<sub>50</sub> of 3a was 12 μM, similar to the one of compound 4b (Figure S3C, n = 11). The degree of GABA<sub>R</sub> potentiation induced by 3a markedly varied for different cells (cf. A and B in Figure S3). A similar behavior was observed for 4b. We suggest that this effect reflects the variability in the EC<sub>50</sub> of GABA in different cells, as it has been shown that allosteric potentiation decreases at high GABA concentrations.<sup>[35]</sup>

The outstanding photopharmacological profile of Fulgazepam (4) as GABA<sub>R</sub> potentiator prompted further assays in vivo. Studies in zebrafish larvae show that the compound alters their behavior depending on isomerization and that this effect can be maintained over time in the absence of illumination. As both compound states 4a and 4b are stable in the dark, larvae behaviors could be studied using pre-illuminated
compounds in the dark followed by in situ illumination using 365 and 500 nm wavelengths (Figure 3). Pre-illuminated compound 4b altered in a dose dependent manner the behavior of undisturbed larvae. In particular, 100 μM 4b evoked an increase in swimming distance (Figure 5B, top). The effect of subsequent illumination was also studied. For all concentrations of 4a, UV light (hence, photoconversion to 4b) significantly increased larvae motility, and this effect was potentiated during the following dark period and reduced to vehicle levels upon illumination with visible light (Figure 5A and Figure 5B, bottom). Therefore, these changes in larvae motility are triggered by conformational changes of compound 4 rather than by natural photoresponsive behaviors. A significant increase in larvae activity, above vehicle levels, is observed when 4b isomerisation is potentiated (during and after UV illumination) and lowers to natural activity when 4a is recovered with green illumination independently of the initial Fulgazepam state that is administered to larvae.

In summary, we have achieved the functionalization of the benzodiazepine nitrazepam via extension by a fulgimide and report a new photochromic potentiator of GABA\textsubscript{A}Rs. The synthesized fulgimides 3 and 4 (Fulgazepam) display good photochromic properties and high photostationary states. Both fulgimides preserve the GABA\textsubscript{A}R potentiator behavior that is characteristic of benzodiazepines, indicating that it is a pharmacologically tolerable substitution, in contrast to azobenzenes at the same position. Remarkably, both fulgimides are photochromic but only Fulgazepam (4) enables controlling the pharmacological activity with light. The open conformation of Fulgazepam (4a) does not influence the amplitude of GABA\textsubscript{A}R currents in vitro, while switching to its closed form 4b with UV light strongly potentiates them. The open (4a) and closed (4b) conformation of iso-fulgimide 4 produce different behavioral outcomes in zebrafish larvae. The ring-open isomer 4a does not alter larvae swimming activities, neither applied directly nor obtained by illumination cycles, and the closed conformation 4b increases larvae motility in a dose dependent manner both during prolonged dark periods and under UV illumination. Hence, Fulgazepam photoswitching reversibly controls the behavior of larvae, producing high activity swimming upon UV illumination, which persists for continuous dark periods, and reducing activity to control levels with visible light illumination.

GABA\textsubscript{A}Rs mediate fast inhibition of neural activity and are determinant in cognition, learning, and memory.\textsuperscript{36–38} Malfunction of these receptors leads to epilepsy, anxiety, depression and sleep disorders.\textsuperscript{39} Clinical treatments with GABA\textsubscript{A}R modulators have limited efficacy and adverse side effects,\textsuperscript{39,40} which...
could be alleviated by targeting drug action at specific circuits or locations.

We have recently developed an azobenzene-nitrazepam based compound (Azo-NZ1) that allows photo-modulation of GABA\(_A\) receptors. In trans-configuration, this compound selectively interacts with the chloride-permeable channel causing inhibition of GABA-induced currents, while UV-induced transition to the cis-state results in channel unblocking and restoring the current amplitude. Such unexpected action for a derivative of benzodiazepine (a GABA\(_A\) receptor potentiator) was explained by molecular docking calculations and mutagenesis analysis indicating that the 2' residue of the channel-forming transmembrane TM2 domain is the target of Azo-NZ1 blocking action.

In contrast to Azo-NZ1, the GABA\(_A\)R modulator presented here (Fulgazepam) does produce potentiation of GABA-induced currents. This GABA\(_A\)R photoswitch displays unique characteristics as a direct result of its photochromic (fulgimide) and pharmacological (diazepam) moieties: (i) the fulgimide scaffold imparts complete reversible switching of Fulgazepam’s conformation; (ii) both Fulgazepam states are stable and can be readily obtained by illumination with light of the appropriate wavelengths; (iii) Fulgazepam is sufficiently soluble in aqueous solution and effectively photocontrols endogenous GABA\(_A\)Rs in vitro—in its closed form it is a pure potentiator of GABA\(_A\)Rs without agonist or antagonist activity; (iv) Fulgazepam does not display toxicity in zebrafish and allows controlling their behavior with light. These outstanding molecular properties enable dissecting the mechanisms of GABAergic neurotransmission at high spatiotemporal resolution, and pursuing novel phototherapies based on localized, acute, and reversible neuroinhibition.

### Experimental Section

All animal experiments were conducted according to the EU Directive 2010/63/EU on the protection of animals used for scientific purposes. According to this directive, zebrafish are considered vertebrates and therefore subject to legislation governing animal testing, but embryos and non-independently feeding form larvae are excepted.

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Conflict of interest

The authors declare no conflict of interest.

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