Fulgazepam: A Fulgimide-Based Potentiator of $GABA_A$ Receptors

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Abstract

The γ-aminobutyric acid gated chloride channel represents the major mediator of inhibitory neurotransmission in the mammalian central nervous system and its dysfunction is related to severe diseases like epilepsy and depression, which can be relieved by the application of allosteric modulators. However, the drugs' potential side-effects limit their application for long-term treatment. Applying light as external stimulus to modify the pharmacophore's activity, as emerged in the field of photopharmacology, provides a non-invasive tool with high spatial and temporal resolution for the modulation of protein function. Herein, we report the design, synthesis, and biological evaluation of photochromic fulgimide-based benzodiazepine derivatives as light-controllable potentiators of $GABA_A$ receptors ($GABA_A$Rs). A photocontrolled potentiator of $GABA_A$Rs (Fulgazepam) has been identified that does not display agonist or antagonist activity and allows manipulating zebrafish larvae swimming.

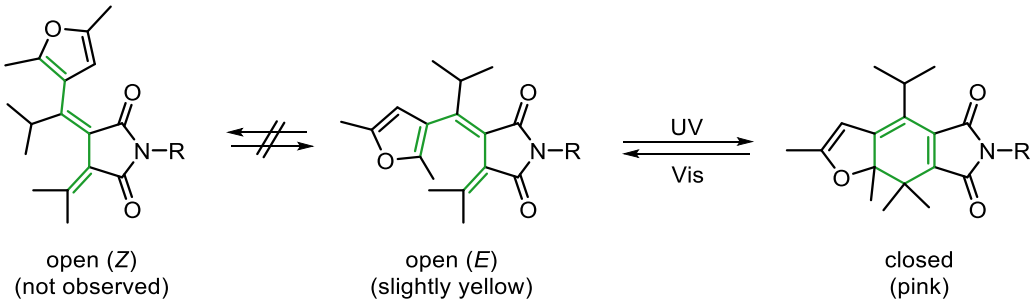
Introduction

In nature, various receptors and biochemical processes evolved provoked by light.[1,2] Photons as non-invasive, abundant input signal may trigger a system's response with high spatial and temporal resolution.[3,4] Their use as a control element for biological systems is highly suitable as light matches perfectly with fast signal transduction, especially of ion channels.[3-6] One profoundly investigated example is represented by γ-aminobutyric acid type A ($GABA_A$) gated chloride channels, the major mediators of inhibitory neurotransmission in the mammalian central nervous system. Besides $GABA_A$ receptors ($GABA_A$Rs) the Cys-loop family of pentameric ligand-gated ion channels also includes glycine, serotonin and nicotinic acetylcholine receptors.[7] $GABA_A$Rs display a broad variety regarding their subunit composition and connected physiological functions as cognition, learning, and memory.[8,9] Owing to their complexity, misfunction of these receptors leads to epilepsy, anxiety, depression and sleep disorders.[9] Ligand-gated chloride channels, such as GABA type A receptors ($GABA_A$Rs), mediate fast inhibition of neural activity and determine the bulk of synaptic transmission controlling all behavioural relevant circuitry.[10] Thus, $GABA_A$Rs represent an important drug target and object of current research aiming for suitable therapeutics for improved medical care. For instance, benzodiazepine-based pharmaceuticals act *via* allosteric modulation potentiating GABARs function. Despite successful clinical administration, improved drugs with reduced side effects or tools allowing further receptor investigation and mechanistic studies are desirable.[11-13]

Light allows superior spatiotemporal resolution and enables delimited manipulation onto protein targets. Several optogenetic strategies have been addressed to control inhibitory (GABA-releasing) neurons, giving much insight on brain-wide inhibitory circuits using photoswitchable tethered ligands (PTLs) – usually needing a genetically engineered cysteine near its GABA-binding site.[14,15] Another optogenetic approach for inhibitory circuit studies would be to use light-gated chloride anion channels[16,17] although improvement in cell level expression and channel conductance[18] must be addressed for map circuitry and *in vivo* purposes. Light-caged GABA compounds have shown a remarkable action onto neuronal spines and seizure control. Unfortunately, toxicity levels on neuronal culture must be corrected prior to *in vivo* applications.[19]

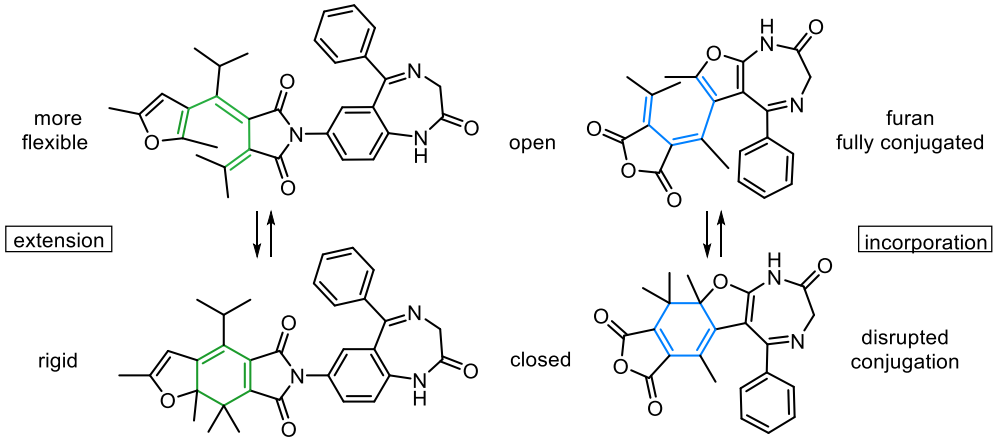
To overcome genetic manipulation, the use of exogenous proteins and non-toxic by-products, we focused on the advantages of photopharmacology to synthesize a photoswitchable modulator for endogenous neurotransmission, inactive prior to illumination and fully reversible with light. Photopharmacology allows orthogonal control to most cellular processes.[3-6] Triggered by illumination, a photochromic ligand may reversibly be interconverted between at least two isomeric states with different absorption spectra. Depending on the targeted field of application, various photochromic scaffolds emerged, amongst which azobenzenes, dithienylethenes, and fulgimides are the most prominent examples.[3-6,20] Our quest for photochromic derivatives of benzodiazepines has led to the serendipitous identification of a pore blocker of $GABA_A$ receptors (Azo-NZ1)[21], and a selective inhibitor of the structurally related glycine receptors (Glyght).[22] However, it has failed to preserve the allosteric potentiator profile of $GABA_A$Rs that is characteristic of benzodiazepines. Azobenzenes have been successfully used as photochromic scaffolds for biological applications[21-28] due to their synthetic accessibility and their large change in geometry and dipole moment upon switching.[3-6,20,29] However, several drawbacks limit their range of application. Their photoinduced *cis* isomer is thermally bistable and its half-life strongly solvent- and substitution-dependent.[30,31] Determined by the exact photochromic scaffold, incomplete photoconversion due to a substantial overlap of the absorption maxima of both isomers may be considered, but structural optimization (*e.g.*, arylazopyrazoles) for a better n-π^* and π-π^* band separation is possible.[32] Furthermore, the stability of azobenzenes towards glutathione reductase in biological systems is controversial.[33-35]

In contrast, dithienylethenes, fulgides and their fulgimide-named amide derivatives generally feature high photostationary states (PSS) with both photoisomers being thermally stable.[3,20] As dithienylethenes often lack of switching efficiency and stability in polar solvents due to a twisted intramolecular electron charge transfer,[36-38] fulgi(mi)des were chosen 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 (Scheme 1).[20,39] 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 and enabling a photopharmacological profile corresponding to a pure modulator, without agonist or antagonist[40,41] or antagonist activies[42] which could interfere with endogenous neurotransmission. 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 (Scheme 1).[43]



Scheme 1. Furan-fulgimide in its open and closed isomeric state interconvertible by irradiation with UV and visible light.[20,43]

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 recyclization allows the smooth introduction of amino-functionalized biomolecules.[20,43] Recently, few examples using fulgi(mi)des in a biological context are reported.[44-46] The transformation of a known ligand into a photoresponsive molecule is typically achieved 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 is merged with an amino-benzodiazepine under fulgimide formation (Scheme 2, left panel). A difference in activity arises from the different flexibility of both isomeric states. On the other hand, a functionalized diazepine was synthesized aiming for a photochromic benzodiazepine core (Scheme 2, right panel). 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 **9**, Scheme 4) was inactive in patch-clamp studies (data not shown) and the synthesis towards the photoswitch was not further pursued.

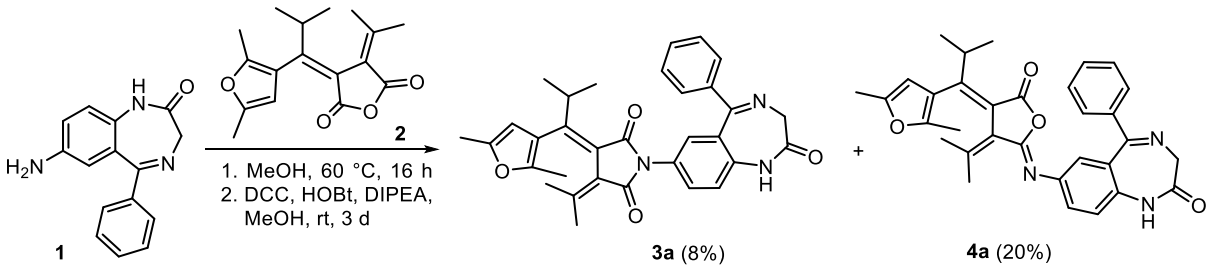


Scheme 2. Left: Pharmacophore nitrazepam and its extension towards a photochromic fulgimide. Right: Derivatization towards a photochromic diazepine fulgide hybrid.

Results and Discussion

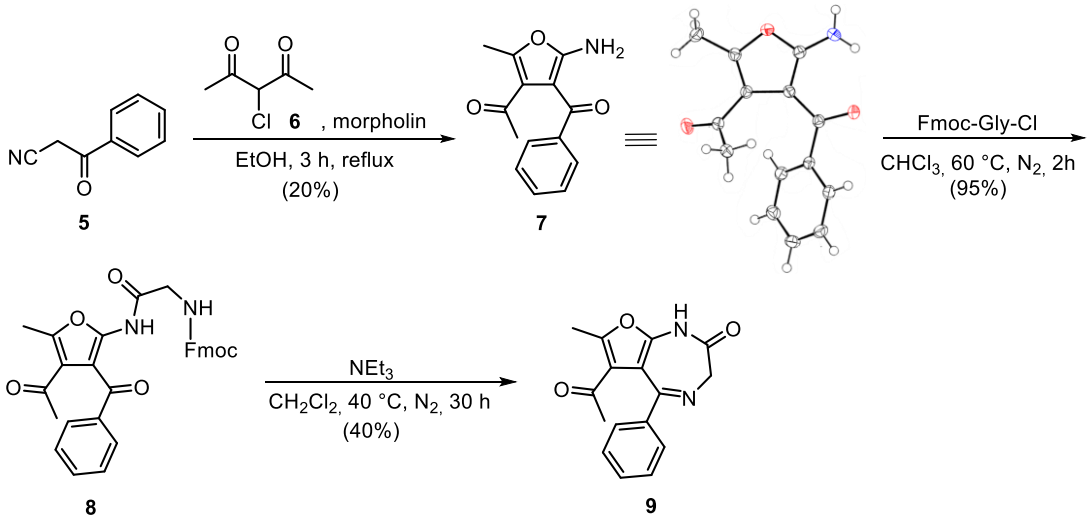
Synthesis

The reaction of furano-fulgide **2**[45] with amino-nitrazepam **1**[47] upon addition of dicyclohexylcarbodiimide (DCC), diisopropylethylamine (DIPEA), and 1-hydroxybenzotirazole (HOBt) in methanol afforded the desired benzodiazepine-furano-fulgimide **3a** and its iso-fulgimide derivative **4a**.



Scheme 3. Synthesis of fulgimide-nitrazepam **3a** and its iso-fulgimide derivative **4a**.

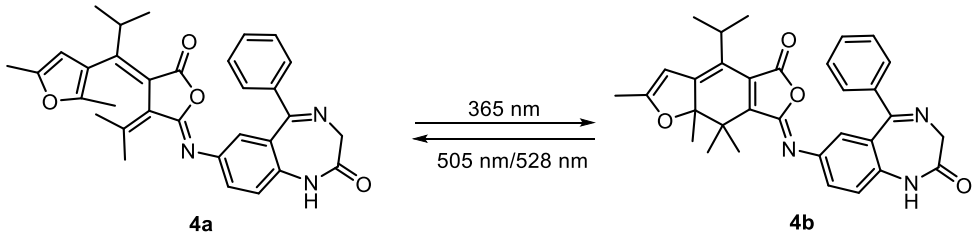
To obtain a photochromic pharmacophore core, we envisioned a functionalized diazepine derivative (**7**) providing an acetyl group in position 3 required for Stobbe[48-50] condensation towards fulgide formation and a methyl-group in position 2 beneficial for the fulgide's switching performance.[45] For diazepine formation, the highly functionalized precursor **7** requires in addition a primary amine in position 5 and a phenone substitution in position 4.[47] Based on the literature known Gewald-reaction[51] and screening of solvents and bases the desired functionalized furan **7** was obtained in good yield in a one-step synthesis starting from commercially available benzoylacetonitrile **5** and 3-chloroacetylaceton **6** (Scheme 4).[52,53] The following ring closure required for diazepine formation of **9** was performed in analogy to literature reports.[47]



Scheme 4. Synthesis of the highly functionalized furan **7** and its diazepine formation towards compound **9**.[37,44-46]

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 (Scheme 1). Only the *E* isomer undergoes a photocyclization reaction to the thermally stable closed isomer (Scheme 5). The colorless open isomers **3a** and **4a** were converted to their strongly colored ring closed isomers **3b** and **4b** upon irradiation with UV light of λ = 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 1). 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).



Scheme 5. Illumination induced ring-closing (**4b**) and ring-opening (**4a**) of iso-fulgimide **4** (Fulgazepam).

Figure 1 shows exemplarily the UV-Vis absorption spectrum and cycle performance ofisofulgimide**4** upon irradiation with 365 nm and 505 nm. Black arrows indicate the spectral evolution upon irradiation. Dotted black arrows label isosbestic points indicating a clear two component switching. After 10 s irradiation at λ = 365 nm the closed-PSS was reached and 93% of the closed-isomer accumulated. Quantitative reopening was achieved within 120s irradiation at λ = 505 nm or 528 nm, respectively. Both compounds show a high fatigue resistance over ten measured cycles upon alternate irradiation with 365 nm for closing and 528 nm for opening.

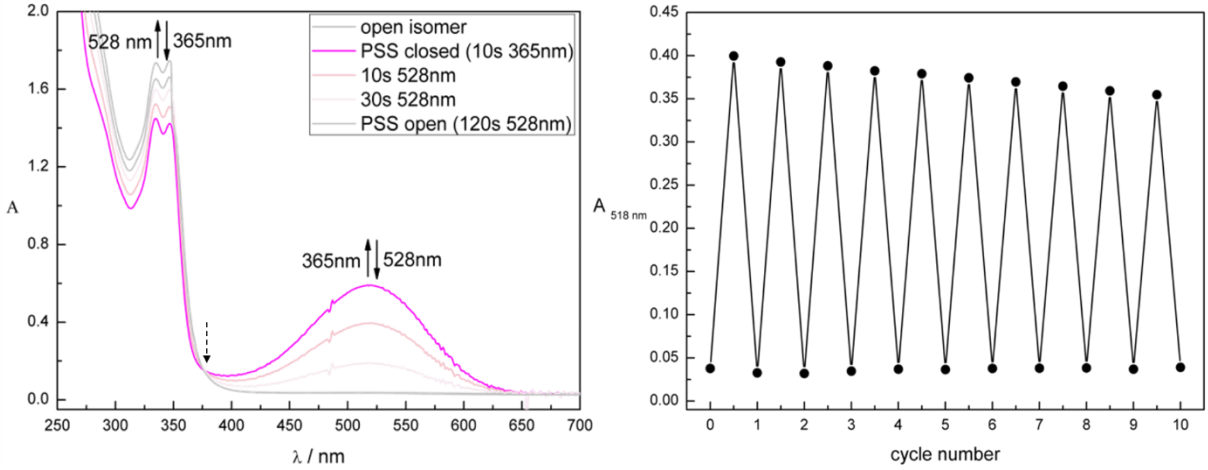


Figure 1. Photochromic properties of iso-fulgimide**4** measured 50 µM in DMSO. Left. Spectral evolution of **4a** (open isomer; grey spectrum) upon irradiation with 365 nm and re-opening of **4b** (closed isomer; purple spectrum) upon irradiation with 528 nm. Right. Cycle performance of **4** upon alternate irradiation with 365 nm (ring closing) and 528 nm (ring opening) detected at 518 nm (λ_{max} closed isomer).

The photochromic properties of compounds **3** and **4** measured 50 µM in DMSO are summarized in table 1. The photostationary states were determined via analytical HPLC measurement of an irradiated sample and detected at the wavelength of the isosbestic point.

Table 1. Photochemical properties of fulgimide-based benzodiazepine derivatives **3** and **4** measured 50 µM in DMSO at 25 °C. Cpd = Compound. PSS = Photostationary state.

Entry	Cpd	λ_{max} open [nm]	λ_{max} closed [nm]	Isosbestic point [nm]	PSS
1	**3**	-	521	375	95% closed (UV); 99% closed (green)
2	**4**	335,347	518	377	93% closed (UV); 99% closed (green)

In Vitro Patch-Clamp Testing

All experiments were performed on cells transiently expressing alpha1/beta2/gamma2 subunits of the $GABA_A$ receptor. This receptor possesses the canonical benzodiazepine allosteric site and its EC_{50} for GABA is about 8 µM.[54] 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, *i.e.* the concentration, which is below the EC_{50} (close to EC_3) and allows to observe allosteric potentiation of $GABA_AR$-mediated currents.[55]

Application of compound **4a** (open isomer) (10 µM) caused no significant effect on $GABA_A$-mediated currents, while application of **4b** (closed isomer), generated by pre-irradiation with UV light (365 nm), induced an increase of $GABA_A$-mediated current amplitudes (Figure 2A). Thus, two different isomers of compound **4** differently interact with $GABA_A$ receptors: being inactive in its open form and potentiatory in its closed form. Analysis of a series of dose-response curves established that the EC_{50} for **4b** was 13 µM (*n* = 6; Figure 2B).

Figure 2C demonstrates that UV irradiation can induce a live-time switching of compound's **4** conformation and a prominent increase of the amplitude of GABA-induced currents. In average during isomerization of 10 µM of **4a** into **4b** under UV irradiation the currents amplitude increased on 228±41% (Figure 2D; *n* = 11).

Compound **3a** in its open state co-applied with GABA (0.5 µM) induced a powerful potentiation of $GABA_AR$-mediated currents (Figure S3A). This potentiation was not sensitive to irradiation 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 on 292±65%, while 50 µM of **3a** increased the current amplitude on 544±107% (*n* = 11). The EC_{50} for **3a** was as well similar to the one of compound **4b** – it comprised 12 µM (Figure S3C, *n* = 11). The degree of the potentiation by **3a** markedly varied for different cells (*cf*. A and B in Figure S3). The similar feature was also characteristic for the action of **4b** on $GABA_ARs$. We suggest that this effect reflects the variability in the EC_{50} for GABA on different cells, as it has been shown that allosteric potentiation decreases with an elevation of the effective GABA concentration.[56]

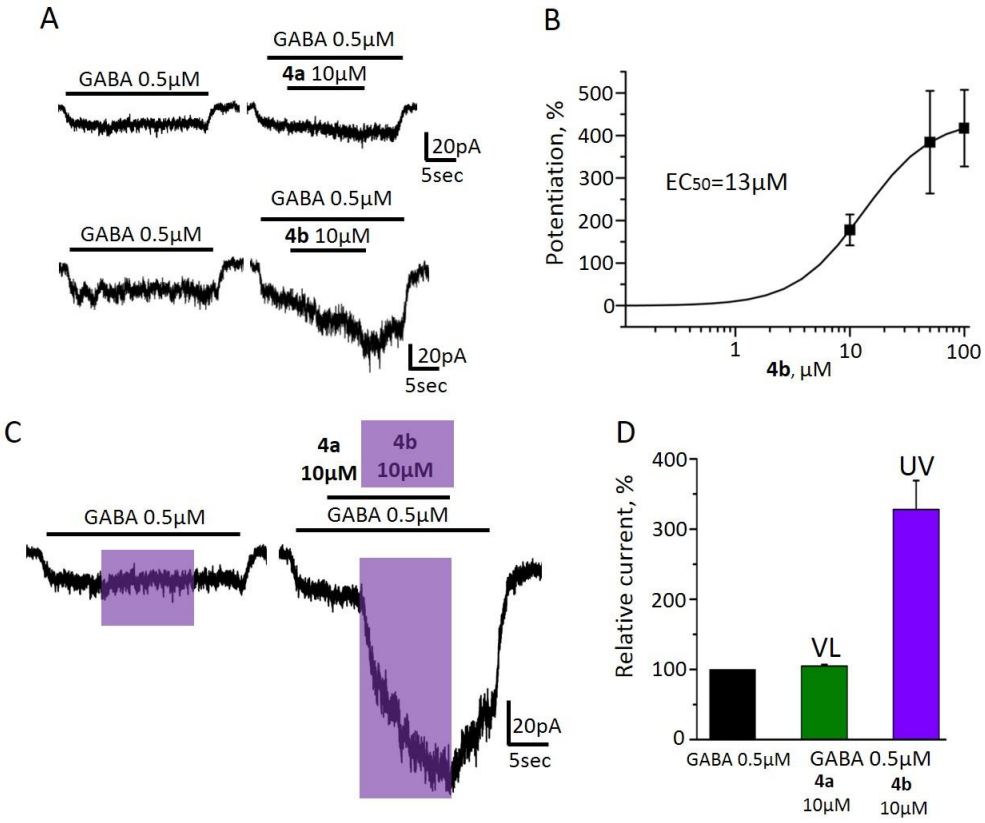


Figure 2. The effect of compounds **4a** and **4b** on $GABA_A$-mediated currents. (A) Upper panel: representative traces of currents induced by application of GABA 0.5 µM and by mixture of GABA 0.5 µM with **4a** 10 µM; lower

panel: representative traces of currents induced by application of GABA 0.5 µM and by mixture of GABA 0.5 µM with **4b** 10 µM. Durations of applications of GABA and compound **4** are indicated by black bars above the traces. (B) Cumulative dose-response curve for the compound **4b** (*n* = 6). (C) Representative traces demonstrating the effect of **4a** photoswitching on the amplitude of GABA-induced currents. On the left: current was induced by application of GABA 0.5 µM; on the right: at the same trace current was induced subsequently by GABA 0.5 µM, by mixture of GABA with **4a** 10 µM at visible light and upon irradiation with UV light (**4b**). Duration of UV irradiation is indicated by violet rectangle. Note the prominent increase of the GABA-induced current in the presence of **4** during irradiation with UV light, which triggered ring-closing (**4b**). (D) Cumulative graph representing mean relative amplitude of currents induced by application of GABA 0.5 µM (black column), GABA 0.5 µM + **4a** 10 µM (green column) and GABA 0.5 µM + **4b** 10 µM (violet column) upon irradiation with UV light (*n* = 11).

In Vivo Behavioral Studies

Behavioral analysis on zebrafish larvae show that fulgazepam **4** influences their behavior, which is most likely driven by isomerization and can be maintained for dark periods over time. As both compound states are stable in the dark, larvae behaviors could be studied using pre-illuminated compounds in dark and under direct illumination using 365 and 500 nm wavelengths. Pre-irradiated solutions showed a dose dependent difference on undisturbed larvae during the resting period, where **4b** at 100 µM concentration evoked an increase in swimming distance (Figure 3B, top). For all three concentrations of **4a**, UV illumination (isomerization to **4b**) showed a significant increase in motility, highly potentiated on their following dark periods and reduced to vehicle levels once they were illuminated with visible light (Figure 3A). This photoswitching behavior was also observed for all **4b** concentrations upon illumination, showing even higher swimming distances over larvae incubated with **4a** (Figure 3B, bottom) and were controlled with visible light. Therefore, these changes in larvae motility are triggered by conformational changes of compound **4** rather than by natural photoresponsive behaviors. An increase in larvae activity over vehicle levels is enhanced when **4b** increases and lowered to natural activities when **4a** is recovered.

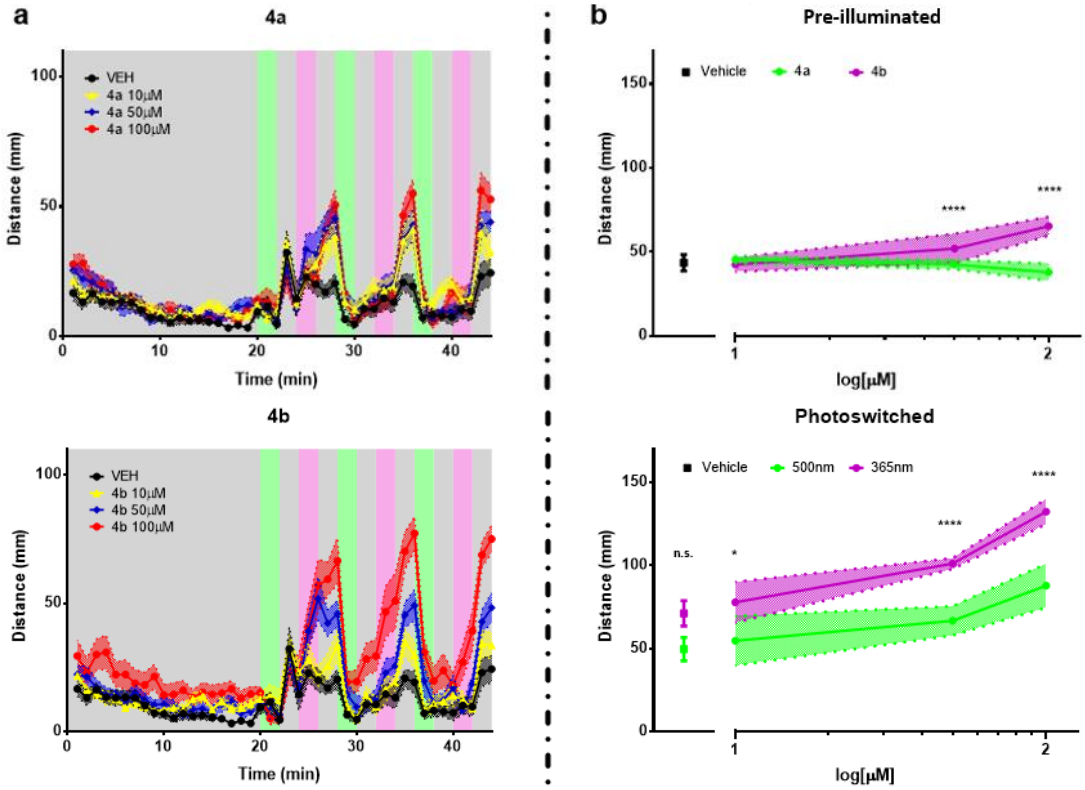


Figure 3. (a) One-minute trajectories of average swimming distances (*n* = 12 per treatment) are shown for vehicle (1% DMSO) and three different concentrations of compound **4** (**4a** (Top) and **4b** (Bottom)). For the first 20 minutes, larvae were undisturbed in complete darkness (Relaxation period, RP), therefore maintaining stable

states. Following RP larvae were illuminated with three consecutive cycles of visible light (500 nm) and UV (365 nm) with discrete dark between each wavelength and compound transit between both conformations. Colored areas show standard error of the mean (S.E.M.). (b) Top: Quantification of swimming distances over the last 5 minutes of the RP (darkness) from two independent experiments (*n* = 24 per treatment) for both pre-illuminated compounds **4a** (green trace) and **4b** (violet trace) and vehicle (1% DMSO). Bottom: Quantification of swimming distances over the light periods after UV illumination (violet traces) and visible light illumination (green trace) (*n* = 12 per treatment) for compound **4** and vehicle (1% DMSO). n.s. no significance, * p-value<0.05, ****p-value<0.0001. Colored areas show standard deviation (S.D.).

Conclusion

In summary, we successfully functionalized the benzodiazepine nitrazepam into a light-controllable molecule *via* extension by a photochromic fulgimide and report the first photochromic switch-on potentiator of $GABA_A$ receptors based on a fulgimide scaffold. The synthesized fulgimides **3** and **4** (Fulgazepam) displayed good photochromic properties and high photostationary states. Both fulgimides preserve the $GABA_A$ 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**) did not influence the amplitude of GABA-induced currents, while being switched to its closed form **4b** by UV irradiation resulted in a prominent potentiating effect. The open (**4a**) and closed (**4b**) conformation of iso-fulgimide **4** produced different behavioral outcomes on *Danio rerio* larvae. The ring-open isomer **4a** did not alter larvae swimming activities, neither with undisturbed long-term larvae nor upon irradiation cycles. *Vice versa*, the closed conformation **4b** produced an increase in larvae motility in a dose dependent manner during prolonged dark periods and under UV illumination. Hence, the photoswitching between both conformations of Fulgazepam **4** controls the behavior of larvae, producing high activity swimming upon UV illumination, which persists for continuous dark periods, and lowering it to control levels upon ring-opening *via* visible light illumination.

Here we have developed a novel compound to study and control $GABA_AR$ activity. Fulgazepam **4** possesses some unique characteristics as a direct result of its photochromic (fulgimide) and pharmacological (diazepam) moieties: (i) the fulgimide scaffold imparts complete reversible switching of the Fulgazepam conformation; (ii) both Fulgazepam states are stable and can be easily obtained by illumination with light of the appropriate wavelengths; (iii) Fulgazepam is a soluble photochromic compound successfully used to photocontrol of endogenous $GABA_ARs$ *in vitro* – in its closed form it is a pure potentiator of $GABA_ARs$ without agonist or antagonist activity; (iv) Fulgazepam allows to photocontrol the zebrafish behavior *in vivo*.

Acknowledgements

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