ABSTRACT: Glycine neurotransmission in the dorsal horn of the spinal cord plays a key role in regulating nociceptive signaling, but in chronic pain states reduced glycine neurotransmission is associated with the development of allodynia and hypersensitivity to painful stimuli. This suggests that restoration of glycine neurotransmission may be therapeutic for the treatment of chronic pain. Glycine transporter 2 inhibitors have been demonstrated to enhance glycine neurotransmission and provide relief from allodynia in rodent models of chronic pain. In recent years, photoswitchable compounds have been developed to provide the possibility of controlling the activity of target proteins using light. In this study we have developed a photoswitchable noncompetitive inhibitor of glycine transporter 2 that has different affinities for the transporter at 365 nm compared to 470 nm light.

KEYWORDS: Glycine transport, glycine transporter 2 inhibitor, photoswitchable inhibitor, ORG25543 congener, chronic pain, neuropathic pain

INTRODUCTION

Chronic neuropathic pain is a debilitating disease, which arises as a result of nerve damage and rewiring of circuits within the pain processing pathway. One of the major changes to the central nervous system during this process is an emerging predominance of excitatory tone in the spinal cord dorsal horn. Under normal nociception, excitatory glutamatergic neurons are controlled by a subpopulation of inhibitory glycinergic neurons in the dorsal horn. It has been shown in a rodent model of neuropathic pain that these inhibitory projections to radial neurons are diminished. Therefore, a loss of inhibitory inputs would allow for unregulated nerve firing in the ascending pain pathway, producing the battering of enhanced pain sensations characteristic of chronic pain.

Glycine concentrations within inhibitory synapses are tightly controlled by the glycine transporter, GlyT2, and may therefore be a promising target for therapeutics which would act to restore normal nociceptive control. Indeed, knockdown of GlyT2 via targeting siRNA to ~30% expression produces analgesia in rat models of pain. While partial knockdown of GlyT2 produces analgesia with no observable behavioral side effects, total gene knockout of GlyT2 in mice produces severe neuromotor symptoms, with death 2 weeks postnatal. Additionally, electrophysiological recordings from knockout GlyT2 mice show a reduction of postsynaptic glycinergic currents, which highlights the important role of GlyT2 for recycling of glycine into presynaptic vesicles. The GlyT2 knockout phenotype shows similarities with hyperkplexia, a severe neuromotor disease where strong tremor can be induced through touch, and subjects experience muscle rigidity and an inability to right themselves. Mutation of the human gene encoding GlyT2 (SLC6A5) is the most common presynaptic cause of hyperkplexia, with mutations altering glycine and/or ion binding sites, as well as affecting localization and expression in the plasma cell membrane.

ORG25543 (1) (Figure 1) is a GlyT2 inhibitor that ameliorates both hallmarks of neuropathic pain: hyperalgesia, the heightened pain response, and allodynia, an improper response to innocuous stimuli. Unfortunately, acute dosing in rodents produces excitotoxicity, hyperkplexia-like symptoms, and in severe cases death. Exposure of mouse spinal cord slices to ORG25543 for >10 min results in a long-term reduction of inhibitory postsynaptic currents, likely to be due to blockade of glycine recycling. Furthermore, glycinergic currents cannot be restored following washout of ORG25543 from oocytes expressing GlyT2, suggesting sustained, complete block of glycine transporters mimics the GlyT2 knockout phenotype. Following the abandonment of many GlyT2 inhibitor programs, Mingorance-Le Meur, Courade, and colleagues resurrected the ORG25543 scaffold and produced a series of analogues to examine the requirements for on-target...
controlled using certain light wavelengths. These light active compounds that possess a switch that can be precision sensitive moiety into a desired chemical structure produces chemical compounds, whereby incorporation of a photospatiotemporal control of cellular activity using light-sensitive binding and their e developed, greater insight into the mechanism of reversible order for ORG25543-based GlyT2 inhibitors to be further should be obtained.

porters. Synaptic transmission, such as ion channels and trans... electronic properties of the azobenzene substituents and the isomer from between milliseconds to days depending on the... the diazo N bond upon irradiation with UV or blue visible light21 (Figure 2). Azobenzenes are metabolically stable, and organisms. The azobenzene moiety is one of the most widely used photoswitches, where incorporation produces a molecule that can undergo cis-to-trans isomerization around the d... produced photochromic compound 3. Azobenzene is well suited to azologization because it contains a benzyl phenyl ether moiety that is structurally homologous to azobenzenes in the trans configuration. In this study we have produced the photochrome azobenzene 3 that was derived from direct azologization of GlyT2 inhibitor 2 (Figure 2). Azobenzene 3 readily photoswitches between the Z and E geometric isomers upon irradiation with λ = 365 nm or λ = 470 nm light. We examined the inhibitory activity of both geometric isomers (E)-3 and (Z)-3 at GlyT2 and investigated their reversibility and mechanisms of inhibition.

■ RESULTS AND DISCUSSION

Chemistry. The synthesis of diazo photochromic compound 3 begins with the preparation of 1-(aminomethyl)-N,N-dimethylcyclohexan-1-amine (6), which is presented in Scheme 1. Amino nitrile 5 was manufactured via a Strecker condensation reaction between cyclopentanone (4) and dimethylamine hydrochloride in the presence of KCN. Nitrile 5 was subsequently reduced to diamine 6 using LiAlH4. The high volatility of 6 prohibited in vacuo concentration and isolation of it upon extraction into Et2O during the workup. Therefore, crude 6 was used in the subsequent peptide coupling reaction as a solution in Et2O, with an estimated concentration of ~0.49 M as per the theoretical yield for the LiAlH4 reduction.

The next phase of the synthesis of azo-compound 3 is shown in Scheme 2. Aniline 7 underwent a Baeyer-Mills coupling reaction with commercially available nitrosobenzene in HOAc at 40 °C to afford (E)-8. Only one product was observed for this reaction by LC-MS (data not shown) and 1H NMR analysis, which was presumed to be the more thermodynamically stable trans geometric isomer (E)-8. Saponification of the methyl ester of (E)-8 with 2 M NaOH gave the corresponding carboxylic acid (E)-9, which underwent a subsequent peptide coupling reaction with previously described amine 6 in the presence of HBTU and i-Pr3NEt in THF. This reaction produced photochromic compound 3 as an orange solid, which
was found to be a ∼1:1 mixture of E and Z isomers. Repeated attempts to isolate and independently characterize (E)-3 and (Z)-3 were deemed futile as both geometric isomers quickly interconverted upon purification and in the presence of visible light to give a nearly 1:1 mixture of both compounds. The LC-MS, HPLC, and 1H NMR data confirmed that compound 3 is a mixture that contains only (E)-3 and (Z)-3, and the HPLC data indicates an overall purity for 3 to be at 99.76%.

**Azo-Compound 3 Is Photoswitchable.** The photoswitchable properties of azo-compound 3 in solution were investigated using UV/vis spectroscopy. Phosphate-buffered saline (PBS) buffered to pH 7.4 was used as the solvent to reflect the conditions of the GlyT2 inhibition assay. The UV/vis spectrum of a solution of 3 preirradiated with 470 nm light displayed a strong UV absorption band at 316 nm and a weaker absorption band at 290 nm which arises from the π* π transition in (E)-3 (Figure 3A, blue line). These absorptions arise from π-π* and n-π* transitions in trans-azobenzenes, respectively,21 and confirm the presence of (E)-3 in the solution. Irradiation of the sample with 365 nm UV light produced a change in the UV/vis spectrum toward an absorption profile characteristic of cis-azobenzenes (Figure 3A, purple line). Thus, the strong band at 316 nm was replaced with a weaker absorption band at 290 nm which arises from π-π* transition in (Z)-3. Subsequent irradiation of the solution with 470 nm blue light caused the UV/vis spectrum to return to that of the trans-azobenzene (E)-3 (Figure 3A, blue line). We also studied the thermal relaxation of (Z)-3 because cis-azobenzenes thermally isomerize to their more stable trans configuration in the absence of light. A solution of (Z)-3, formed by preirradiation with 365 nm light, was left in the dark and monitored by UV/vis spectroscopy every 12 h. As shown in Figure 3B, the characteristic cis-azobenzene absorption band at 290 nm decreased and was replaced with the 316 nm trans-azobenzene absorption band, and complete thermal relaxation to (E)-3 occurred in 84 h. Collectively, these experiments show that azo-compound 3 is readily and reversibly photoswitchable and can be isomerized in real-time biological assays.

**Activity of Azo-Compound 3 at GlyT2.** Glycine transport by GlyT2 is coupled to the cotransport of 3 Na+ and 1 Cl−, which generates an electrogenic process that can be measured using the two-electrode voltage clamp technique with *Xenopus laevis* oocytes expressing GlyT2. Under this experimental design, ORG25543 and analogue 2 have previously been shown to inhibit GlyT2 with potencies of 20 nM and 100 nM, respectively.16 Inhibition by ORG25543 was irreversible and was maintained for 10 min after cessation of application, while 2 is readily reversible. We first confirmed their actions on GlyT2 by demonstrating a comparable dose dependent reduction of glycine transport currents, with IC50 values of 3.76 (2.36−6.01) nM and 48.5 (42.6−55.2) nM (Figure 4A). We also observed restoration of glycine transport currents following reversible binding of 2 but no reversibility of ORG25543, even after 30 min of washout (Figure 4B).

Following validation of the activity of the parent compounds in our assay, compound 2 was chosen as the base compound for generating the photoswitchable analogue, 3, because of its reversibility. Compound 3 was examined for the ability of each photoswitchable isomer to inhibit GlyT2. An external light source with a liquid light guide was connected to tubes of recording buffer containing 3, and photoswitching in real-time was achieved by alternating between λ = 365 nm and λ = 470 nm. We began by preirradiating 3 with λ = 470 nm light to ensure 3 remained in its thermally relaxed trans configuration state ((E)-3). Perfusion of GlyT2 expressing oocytes with buffer containing glycine produced inward currents that were blocked by (E)-3 (Figure 5A). Once a steady state of inhibition was reached, the light was switched to λ = 365 nm, and an increase in transport current was observed, indicating GlyT2 was less sensitive to inhibition by the cis isomer ((Z)-3). The light was then switched back to λ = 470 nm, and (E)-3 produced the same level of inhibition compared to the preirradiated analogue, demonstrating real-time isomerization could be achieved in this electrophysiological assay. The recording chamber was then washed for 10 min, and the assay was repeated but beginning with preirradiated (Z)-3, showing that comparable levels of inhibition are achieved regardless of the order of photoswitching (Figure 5A). Following the inhibitory assay, the oocyte was again washed, and glycine was reapplied to show that currents could be restored. In contrast to 1, inhibition by 3 is fully reversed after 5 min, and glycine transport currents were restored to

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**Scheme 2**

![Scheme 2](https://example.com/scheme2.png)

Reagents and conditions: (a) nitrosobenzene, HOAc, 40 °C, 48 h; (b) aqueous 2 M NaOH, CH3OH, THF, rt, 16 h; (c) 6, HBTU, 1-Pr2NEt, THF, 0 °C to rt, 16 h.
over 84 h. Recorded at 12 h intervals. Complete isomerization to \((E)\)-\(E\)-3 was formed. The solution was irradiated with 470 nm blue light for 15 min to ensure the corresponding UV–visible absorption spectrum was measured (blue line). \((E)\)-3 was then irradiated with 365 nm UV-A light in 5 s intervals until no further change in absorbance was observed (purple line), and \((Z)\)-3 was formed. The solution was irradiated with 470 nm light, and \((Z)\)-3 isomerized back to the \(trans\) isomer \((E)\)-3 (blue line).

Figure 3. Photoisomerization of azo-compound 3. A. A solution of azo-compound 3 in PBS (pH 7.4) was irradiated with 470 nm blue light for 15 min to ensure 3 was in the \(trans\) configuration, and the corresponding UV–visible absorption spectrum was measured (blue line). \((E)\)-3 was then irradiated with 365 nm UV-A light in 5 s intervals until no further change in absorbance was observed (purple line), and \((Z)\)-3 was formed. The solution was irradiated with 470 nm light, and \((Z)\)-3 isomerized back to the \(trans\) isomer \((E)\)-3 (blue line). B. Thermal relaxation of \((Z)\)-3. Compound 3 was isomerized to \((Z)\)-3 with 365 nm UV-A light and then allowed to thermally relax to \((E)\)-3 in the dark over an 84 h time period. UV–vis spectra were recorded at 12 h intervals. Complete isomerization to \((E)\)-3 occurred over 84 h.

preinhibition levels (Figure 5C). Compound 3 was also tested on oocytes expressing GlyT1 and was found to have no activity at either 365 or 470 nm (Figure 5B).

To determine the IC\(_{50}\) values for each isomer, increasing concentrations of 3 irradiated with either \(\lambda = 365\) nm or \(\lambda = 470\) nm light were applied to GlyT2 expressing oocytes to block currents generated by 3, 10, 30, 100, 300, and 1000 \(\mu\)M glycine (Table 1, Figure 5B – representative concentration response curves at 30 \(\mu\)M glycine). The IC\(_{50}\) values were consistent at each glycine concentration, and average IC\(_{50}\) values were calculated to be 9.94 \(\mu\)M and 5.36 \(\mu\)M for \((Z)\)-3 and \((E)\)-3, respectively. Both isomers of 3 are active inhibitors of GlyT2, with the \(trans\) configuration possessing a marginally higher potency than \(cis\). While both isomers are active, they are >110-fold less potent than the nonazologized parent analogue, 2. This observed loss in potency may be attributed to one of the following factors (or any combination thereof): 1) diminished affinity may result from an inability of the azobenzene appendage of \((E)\)-3 or \((Z)\)-3 to adopt a putative bioactive conformation proposed for the benzyl ether appendage of ORG25543\(^{15}\) due to potential preferred orientations between the adjacent diazo and methyl ether groups,\(^{26,27}\) 2) the electron withdrawing diazo bridge may also impart unfavorable electronic effects on the pendant phenyl ring,\(^{28}\) which could lead to a loss in potency due to potentially diminished \(\pi–\pi\) stacking or \(\pi\)-cation binding interactions, and 3) the azobenzene group may be projecting into a hydrophobic binding pocket and may not be well-tolerated due to the increased polarity of the diazo bridge relative to the benzyl ether tethers of ORG25543 and compound 2.

In order to determine the mechanism of glycine transport inhibition at GlyT2, glycine concentration response curves were performed in the presence of increasing concentrations of \((Z)\)-3 or \((E)\)-3 (Figure 6A,B, Table 2). EC\(_{50}\) values for each concentration of 3 at both \(\lambda = 365\) and 470 nm are unchanged, while \(I_{\text{max}}\) decreased as the concentration of 3 increased, which suggests that neither isomer of 3 is competing for the glycine binding site. Additionally, Eadie-Hofstee plots of the data confirm 3 is a noncompetitive inhibitor (Figure 6C,D). Inhibitors of the SLC6 family of transporters usually bind in either the central substrate site and compete for glycine or their binding site lies at a wider point of the transporter cavity in a location referred to as either the “substrate S2” site or “allosteric” site. For the related serotonin transporter, the transport blocker \((\text{5})\)-citalopram can bind in either the central substrate site or the allosteric site to inhibit serotonin reuptake.\(^{29}\) GlyT2 possesses the smallest central substrate site of the SLC6 family and cannot transport any other substrates other than glycine.\(^{30}\) GlyT2 also has no known substrate site or the allosteric site to inhibit serotonin transport blocker (\(\text{5}\))-citalopram can bind in either the central substrate site or the allosteric site to inhibit serotonin reuptake.\(^{29}\) GlyT2 possesses the smallest central substrate site of the SLC6 family and cannot transport any other substrates other than glycine.\(^{30}\) GlyT2 also has no known

Figure 4. ORG25543 (1) and compound 2 inhibit glycine evoked currents. A. Increasing concentrations of 1 and 2 were applied to oocytes expressing GlyT2, to reduce glycine transport currents. B. Following cessation of inhibition by IC\(_{50}\) concentrations of 1 (4 nM) and 2 (50 nM) (time 0), oocytes were washed with ND96 for 30 min, and glycine was reapplied at 5 min intervals to compare currents to preinhibition levels (Gly).
transport currents. Transport currents were normalized to the current produced by glycine alone. The closely related GlyT1 transporter was tested was reapplied at 5 min intervals to establish reversibility of each isomer. Shown here is 10

Then the inhibition was tested again with the reverse order of photoswitching. Following 5 min of washing, glycine was reapplied to monitor the switched back to

nm to shift the conformation to

and irradiation was continued until a new stable point of inhibition was reached. Lastly, the light was switched back to

nm to ensure levels of original inhibition could be restored. The oocyte was washed for 10 min while 3 was preirradiated. Then the inhibition was tested again with the reverse order of photoswitching. Following 5 min of washing, glycine was reapplied to monitor the reversibility.

Increasing concentrations of

and

nm irradiated 3 were applied to oocytes expressing GlyT2 to reduce 30 μM glycine transport currents. Transport currents were normalized to the current produced by glycine alone. The closely related GlyT1 transporter was tested at the highest concentration of both isomers of 3 (open circles). C. Following inhibition (time 0), oocytes were washed for 5 min, and then glycine was reapplied at 5 min intervals to establish reversibility of each isomer. Shown here is 10 μM inhibition of 300 μM glycine.

Table 1. IC50 Values for Inhibition of GlyT2 by (Z)-3 and (E)-3

| glycin (μM) | 365 nm (Z)-3 IC50 | 470 nm (E)-3 IC50 |
|------------|-------------------|-------------------|
| 10         | 11.1 (2.37–167) n = 4 | 4.84 (0.789–26.0) n = 4 |
| 30         | 12.3 (7.81–20.5) n > 4 | 5.77 (3.18–10.5) n > 4 |
| 100        | 10.6 (4.81–26.5) n = 4 | 4.49 (2.06–9.55) n = 4 |
| 300        | 9.25 (3.74–26.6) n > 5 | 7.12 (2.95–18.7) n > 5 |
| 1000       | 6.44 (2.77–15.6) n > 3 | 4.60 (1.99–10.1) n > 3 |

“Increasing concentrations of (Z)-3 or (E)-3 were applied to oocytes expressing GlyT2 in the presence of 3, 10, 30, 100, 300, and 1000 μM glycine. IC50 values for each isomer were calculated at each concentration of glycine and are shown as mean and 95% confidence interval. The IC50 values for 3 μM glycine could not be accurately calculated and are omitted from the table.

competitive inhibitors. 3 is therefore unlikely to bind in this region and, rather, may bind in the allosteric site. It is striking that both isomers of 3 inhibit GlyT2, which further suggests that the binding site is a larger, flexible region of the protein.

CONCLUSIONS

In this study, we synthesized azo-compound 3 based on the chemical structures of the GlyT2 inhibitors, ORG25543 (1) and compound 2. Azo-compound 3 was shown to exhibit photocontrolled isomerization and to possess photoswitchable inhibition of GlyT2. Both (Z)-3 and (E)-3 were active, with the trans conformation being 2-fold more potent. Despite their differences, it is apparent that both isomers can be accommodated in the binding site, which suggests there is conformational flexibility in this binding region which could inform the design of new inhibitors. We also demonstrate that both conformations of 3 are noncompetitive inhibitors of GlyT2 and are readily reversible, an important consideration when developing GlyT2 targeting inhibitors to treat chronic pain. At this stage the precise location of the binding site for compound 3, which presumably overlaps with the site for ORG25543, is not well-defined, and further work is required to define specific interactions. This information together with the photoisomerization properties will help to design optimal inhibitors for the control of GlyT2 activity. Furthermore, it is anticipated that a photosensitive GlyT2 inhibitor could be a useful tool for further biological studies focused on identifying the optimal level of GlyT2 inhibition required for treatment of chronic pain conditions. Such compounds may also prove useful in understanding physiological mechanisms of on-target side effects and hyperekplexia-like symptoms. For example, by titrating inhibition at GlyT2 expressing synapses using photoswitching in real-time, the effects on vesicle refilling and recycling could be examined. Future development of photoswitching GlyT2 inhibitors to produce isomers with varying rates of reversibility would be valuable to understand how to produce inhibitors for the treatment of chronic pain while avoiding adverse side effects.

METHODS

Chemistry. General Chemistry. All reactions were performed under a dry atmosphere of nitrogen unless otherwise specified. Indicated reaction temperatures refer to the reaction bath, while room temperature (rt) is noted as 25 °C. Commercial grade reagents and anhydrous solvents were used as received from vendors, and no attempts were made to purify or dry these components further. Removal of solvents under reduced pressure was accomplished with a

Figure 5. Activity of 3 at GlyT2 expressing oocytes. A. Current trace of 10 μM 3 mediated inhibition of 300 μM glycine transport by GlyT2. 300 μM glycine was applied to oocytes expressing GlyT2 to produce an inward current. 3 was irradiated with λ = 470 nm light to preserve the thermally relaxed trans state ((E)-3) and was then coapplied with glycine until stable inhibition was reached. 3 was then immediately irradiated with λ = 365 nm to shift the conformation to cis ((Z)-3), and irradiation was continued until a new stable point of inhibition was reached. Lastly, the light was switched back to λ = 470 nm to ensure levels of original inhibition could be restored. The oocyte was washed for 10 min while 3 was preirradiated. Then the inhibition was tested again with the reverse order of photoswitching. Following 5 min of washing, glycine was reapplied to monitor the reversibility. B. Increasing concentrations of λ = 365 and 470 nm irradiated 3 were applied to oocytes expressing GlyT2 to reduce 30 μM glycine transport currents. Transport currents were normalized to the current produced by glycine alone. The closely related GlyT1 transporter was tested at the highest concentration of both isomers of 3 (open circles). C. Following inhibition (time 0), oocytes were washed for 5 min, and then glycine was reapplied at 5 min intervals to establish reversibility of each isomer. Shown here is 10 μM inhibition of 300 μM glycine.
Figure 6. (Z)-3 and (E)-3 reduce concentration-dependent glycine currents. A. Concentration-dependent glycine currents were measured in the presence of increasing concentrations of (Z)-3. B. Concentration-dependent glycine currents were measured in the presence of increasing concentrations of (E)-3. Values shown are means ± SEM. C and D. Eadie-Hofstee plots from each indicate both isomers inhibit GlyT2 noncompetitively.

Table 2. EC50 and I max Values for Glycine at GlyT2 in the Presence of cis- and trans-3

| Azo-compound (μM) | 365 nm (Z)-3 | 470 nm (E)-3 |
|-------------------|-------------|-------------|
| 0                 | 14.3 ± 1.89 μM > 4 | 14.3 ± 1.89 μM > 4 |
| 1                 | 12.77 ± 1.46 μM > 4 | 12.7 ± 2.04 μM > 4 |
| 3                 | 14.5 ± 2.59 μM > 4 | 15.3 ± 3.16 μM > 4 |
| 10                | 17.2 ± 3.53 μM > 3 | 16.8 ± 5.43 μM > 3 |
| 30                | 36.0 ± 17.5 μM > 3 | 14.8 ± 8.57 μM > 3 |

aGlycine concentration-dependent currents were measured in the presence of 3 and fit to the Michaelis–Menten equation to calculate EC50 values which are shown as mean ± SEM. I max values were normalized to the I max of glycine transport in the absence of inhibitor and are shown as mean ± SEM.

Buchi rotary evaporator at approximately 28 mmHg pressure using a Teflon-linked KNF vacuum pump. Thin layer chromatography was performed using 1’ × 3’ AnalTech No. 02521 silica gel plates with a fluorescent indicator. Visualization of TLC plates was made by observation with either short wave UV light (254 nm lamp), 10% phosphomolybdic acid in ethanol, or in iodine vapors. Preparative thin layer chromatography was performed using Analtech, 20 × 20 cm, 1000 μm preparative TLC plates. Flash column chromatography was carried out using a Teledyne Isco CombiFlash Companion Unit with RediSep Rf silica gel columns. Proton NMR spectra were obtained on a 400 MHz Varian Nuclear Magnetic Resonance Spectrometer, chemical shifts (δ) are reported in parts per million (ppm), and coupling constant (J) values are given in Hz, with the following spectral pattern designations: s, singlet; d, doublet; t, triplet, q, quartet; dd, doublet of doublets; m, multiplet; br, broad. Tetramethylsilane was used as an internal reference. Mass spectroscopic analyses were performed using ESI ionization on a Waters AQUITY UPLC MS single quadrupole mass spectrometer. High pressure liquid chromatography (HPLC) purity analysis was performed using a Waters Breeze 2 HPLC system with a binary solvent system A and B using a gradient elusion [A, H2O with 0.25% TFA; B, CH3CN with 0.25% TFA] and flow rate = 1 mL/min, with UV detection at 254 nm (system equipped with a photodiode array (PDA) detector). The purity of all tested compounds was >95% as confirmed by reverse phase analytical HPLC and 1H NMR.

(E)-N-(1-(Dimethylamino)cyclohexyl)methyl)-3-methoxy-4-(phenyldiazenyl)benzamide (E)-3 and (Z)-N-(1-(Dimethylamino)cyclohexyl)methyl)-3-methoxy-4-(phenyldiazenyl)benzamide (Z)-3. Step A: To a 0 °C cooled solution of cyclohexanone (4, 5.0 g, 50.0 mmol) in H2O (50 mL) was added dimethylamine hydrochloride (4.10 g, 50.0 mmol) and KCN (3.30 g, 50.0 mmol). The resultant mixture stirred for 18 h while gradually warming to rt. The mixture was diluted with additional H2O (100 mL) and then extracted with EtO (3 × 100 mL). The combined organic extracts were washed with brine, dried over Na2SO4, filtered, and concentrated under reduced pressure to give 1-(dimethylamino)cyclohexane-1-carbonitrile (5, 8.0 g crude); ESI MS m/z = 153 [M + H]⁺.

Step B: To a 0 °C cooled solution of 1-(dimethylamino)cyclohexane-1-carbonitrile (5, 7.5 g, 49.0 mmol) in EtO (50 mL) was added LiAlH4 (2.40 g, 64.0 mmol) portionwise. The resultant mixture stirred for 18 h while gradually warming to rt under an atmosphere of N2. The mixture was then cooled back to 0 °C, carefully quenched with aqueous 2 M NaOH (3 mL), and filtered through a pad of Celite. The aqueous filtrate was extracted with EtO (3 × 100 mL), and the combined organic extracts were dried over Na2SO4 and filtered. Due to the high volatility of 1-(aminomethyl)-N,N-dimethylcyclohexan-1-amine (6), the resulting solution contain-
ing the crude product in EtO was not concentrated under reduced pressure and was instead used as is in the next step with an estimated concentration of 7.6 g of 6 (theoretical yield) in 100 mL of EtO

Step C: A mixture of methyl 4-amino-3-methoxybenzoate (7, 1.0 g, 5.52 mmol) and nitrosobenzene (0.77 g, 7.18 mmol) in HOAc (15 mL) was heated at 40 °C for 16 h. The reaction was allowed to cool to rt, then carefully quenched with H2O (200 mL), and neutralized to pH = 7 with aqueous saturated NaHCO3 solution (5 mL). The aqueous mixture was extracted with EtOAc (3 × 100 mL). The aqueous mixture was extracted with EtOAc (3 × 20 mL), and the combined organic extracts were washed with brine, dried over anhydrous Na2SO4, and concentrated under reduced pressure. The resulting residue was chromatographed over silica gel (Isco CombiFlash Companion unit, 12 g Redisep column, 0–30% EtOAc in hexanes) to give methyl (E)-3-methoxy-4-((phenylazanyl)benzoate ((E)-8) as an orange solid (1.4 g, 94%); 1H NMR (400 MHz, DMSO-d6) δ 7.84–7.82 (m, 2 H), 7.69 (s, 1 H), 7.61–7.51 (m, 5 H), 3.97 (s, 3 H), 3.85 (s, 3 H); ESI MS m/z = 271 [M + H]+.

Step D: To a solution of methyl (E)-3-methoxy-4-((phenylazanyl)benzoate ((E)-8) (1.4 g, 5.19 mmol) in a 1:4 mixture of THF (10 mL) and CH2OH (40 mL) was added aqueous 2 N NaOH (13 mL, 260.00 mmol). The mixture stirred for 16 h at rt. The mixture was concentrated under reduced pressure, and the crude residue was diluted with H2O (50 mL) and neutralized to pH = 7 with aqueous 2 N HCl. The aqueous mixture was extracted with EtOAc (3 × 20 mL), and the combined organic extracts were washed with brine, dried over anhydrous MgSO4 and concentrated under reduced pressure to give (E)-3-methoxy-4-((phenylazanyl)benzoic acid ((E)-9) as an orange solid (680 mg, 52% yield); 1H NMR (400 MHz, DMSO-d6) δ 7.84–7.82 (m, 2 H), 7.70 (s, 1 H), 7.61–7.50 (m, 5 H), 3.97 (s, 3 H); ESI MS m/z = 257 [M + H]+.

Step E: To a solution of (E)-3-methoxy-4-((phenylazanyl)benzoic acid ((E)-9) (0.20 g, 0.78 mmol), HBTU (0.44 g, 1.17 mmol), and i-Pr2NEt (0.40 mL, 2.34 mmol) in THF (16 mL) was added an ~0.49 M solution of 1-(aminomethyl)-N,N-dimethycyclohexan-1-amine in EtO (6, 15.9 mL, 7.81 mmol). The mixture stirred at rt for 18 h under an atmosphere of N2. The mixture was concentrated under reduced pressure, and the resulting residue was chromatographed over silica gel (Isco CombiFlash Companion unit, 12 g Redisep column, 0–10% CH2OH in CH2Cl2) to afford a mixture of isomers (E)-N-((1-(dimethylamino)cyclohexyl)methyl)-3-methoxy-4-((phenylazanyl)benzamide ((E)-3) and (Z)-N-((1-(dimethylamino)cyclohexyl)methyl)-3-methoxy-4-((phenylazanyl)benzamide ((Z)-3) as an orange solid (121.0 mg, 40%). The individual geometric isomers (E)-3 and (Z)-3 readily interconverted and could not be isolated separately and independently characterized. The HPLC and 1H NMR data generated indicates a nearly 1:1 mixture of (E)-3 and (Z)-3; 1H NMR (400 MHz, DMSO-d6) δ 8.76–7.82 (m, 3 H), 8.42 (brs, 1 H), 8.11 (brs, 1 H), 7.83 (d, J = 5.6 Hz, 2 H), 7.66 (s, 1 H), 7.57–7.56 (m, 4 H), 7.35–7.12 (m, 3 H), 6.81 (d, J = 7.6 Hz, 1 H), 6.74 (d, J = 8.4 Hz, 1 H), 3.99 (s, 3 H), 3.61–3.53 (m, 2 H), 3.12–3.06 (m, 2 H), 2.80–2.79 (m, 4 H), 2.75–2.74 (m, 3 H), 1.64–1.54 (m, 10 H); ESI MS m/z = 395 [M + H]+; HPLC 99.76% total purity (AUC), peak 1 tR = 12.06 min (42.57%), peak tR = 12.79 min (57.19%).

UV–Visible Spectroscopy. Isomerization of azo-compound 3 was monitored using a Cary 60 v2.0 UV–visible spectrophotometer (Agilent). A solution of azo-compound 3 was made in phosphate-buffered saline (PBS; pH 7.4) to a final concentration of 20 ppm (20 μg/mL). An initial baseline absorbance of this solution was determined at time, t = 0. The azo-compound 3 solution was irradiated with λ = 470 nm blue light using an external light source (pE200-CooLED) at an intensity of 0.02 mW/cm² for 15 min to ensure that the entire solution was in the trans configuration; the corresponding absorbance was determined. Subsequently, the azo-compound 3 solution was irradiated with λ = 365 nm UV-A light using the same light source at the same intensity in 5 s intervals, and the corresponding absorbance was simultaneously determined using a start (800 nm)/stop (200 nm) measurement protocol (scan rate = 24000 nm/min; data interval = 5 nm; average time = 0.0125 s) until no further change in absorbance was observed, i.e., the trans isomer was completely isomerized to the cis isomer. The azo-compound 3 solution was then irradiated with λ = 470 nm blue light using the same light source at the same intensity in 5 s intervals, and the corresponding absorbance was simultaneously determined using the same protocol. The reaction was monitored until no further change in absorbance could be observed.

For the thermal relaxation study, a solution of 3 in a well-sealed cuvette was irradiated with 365 nm UV light for 5 min. The resulting solution of (Z)-3 was then placed in a Cary 60 v2.0 UV–visible spectrophotometer that was set to record UV–vis spectra at 12 h time intervals. The solution was left in the dark until no further changes in the UV/vis spectra were observed.

Electrophysiology. Human GlyT2a DNA subcloned into the plasmid oocyte transcription vector was linearized with SpeI (New England Biolabs (Genesearch), Arundel, Australia), and RNA was transcribed using t7 RNA polymerase (mMessage mMachine kit, Ambion, TX). RNA encoding the transporter was then injected into defolliculated Xenopus laevis oocytes with a Drummond Nanoinject (Drummond Scientific Co., Broomall, PA). The oocytes were then stored at 16–18 °C for 2–5 days in ND96 solution (96 mM NaCl, 2 mM KCl, 1 mM MgCl2, 1.8 mM CaCl2, 5 mM HEPES, pH 7.55), supplemented with 2.5 mM sodium pyruvate, 0.5 mM theophylline, 50 μg/mL gentamicin, and 100 μM/L tetracycline, until transporter expression was sufficient to measure transport currents.

Oocytes were held at ~60 mV, and glycerine transport currents were measured using a Geneclamp 500 amplifier (Axon Instruments, Foster City, CA) with a Powerlab 2/20 chart recorder (ADInstruments, Sydney, Australia), interfaced with chart software (ADInstruments).

Isomerization was achieved using an external light source (pE200-CooLED) with a liquid light guide directed over the recording solution to maximize the intensity of light incident. All experiments were carried out in a dark, enclosed, Faraday cage to minimize background interference.

Increasing concentrations of azo-compound 3 (1, 3, 10, 30 μM) were coapplied to oocytes in the presence of a range of glycine concentrations (3, 10, 30, 100, 1000, 3000 μM). At each concentration, the light source was switched from 470 to 365 nm and then 365 to 470 nm, to generate plateau values of inhibition that were distinct for each isomer. Following inhibition, glycerine was reapplied to determine the reversibility of azo-compound 3. Currents were normalized to the maximal current produced by glycerine in the absence of any inhibitor. Data were analyzed using GraphPad Prism 7.02 (GraphPad Software, San Diego, CA). Glycine concentration responses were fit to the Michaelis–Menton equation, I = ([Gly] · [Ic]/[E0 + [Gly]], and then transformed using an Edie-Hofste plot. Inhibitor concentration responses were fit by the method of least-squares using Y = bottom + (top–bottom)/(1 + (X − log IC50)), where X is log(azo-compound 3) (μM), Y is the current normalized to glycine in the absence of inhibitor, and top and bottom are the maximal and minimal plateau responses, respectively.

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S.N.M. and Su.Sa. designed and conducted experiments with GlyT2 expressing Xenopus laevis oocytes, analyzed data, and wrote the manuscript. R.J.V., A.R., and C.C. designed and synthesized compounds. Su.Sh. and T.R. characterized the photoswitchable properties of the compound. R.J.V. helped synthesized compounds. S.N.M. and Su.Sa. designed and conducted experiments with Xenopus laevis oocytes, analyzed data, and wrote the manuscript. R.J.V., C.C., and Su.Sa. obtained funding to support the work.

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Notes

The authors declare no competing financial interest.

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Abbreviations

GlyT2, glycine transporter 2; THF, tetrahydrofuran; Et$_2$O, diethyl ether; EtOAc, ethyl acetate; CH$_3$OH, methyl alcohol; i-Pr$_2$NMe$_2$, N,N-diisopropylethylamine; NaOH, sodium hydroxide; LiAlH$_4$, lithium aluminum hydride; CH$_3$Cl, dichloromethane; rt, room temperature; KCN, potassium cyanide; HBTU, (2-(1-methoxy-1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOAc, acetic acid; 10% Pd/C, 10% palladium on carbon (wt./wt.); CH$_3$I, methyl iodide; K$_2$CO$_3$, potassium carbonate; CH$_3$CN, acetonitrile; TFA, trifluoroacetic acid.

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