

Synthetic photoswitchable neurotransmitters based on bridged azobenzenes

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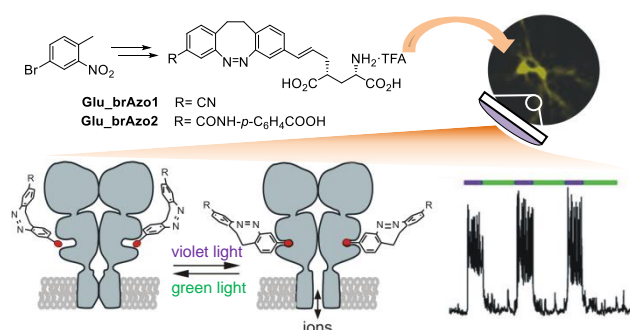
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Supporting Information Placeholder



ABSTRACT: Photoswitchable neurotransmitters of ionotropic kainate receptors were synthesized by tethering a glutamate moiety to disubstituted C2-bridged azobenzenes, which were prepared through a novel methodology that allows access to diazocines with higher yields and versatility. Because of the singular properties of these photochromes, photoisomerizable compounds were obtained with larger thermal stability for their inert *cis* isomer than for their biological activity *trans* state. This enabled selective neuronal firing upon irradiation without background activity in the dark.

By enabling remote manipulation of neuronal signaling with light, optogenetics¹ and photopharmacology² have revolutionized neuroscience and neurobiology. Neural receptors responding to glutamate (GluRs), the primary excitatory neurotransmitter, are one of the major targets in these fields, since they regulate several key processes in the nervous system and are related to numerous diseases.³ As such, a plethora of photopharmacological tools have been developed for light-gating GluRs,⁴ among which photochromic ligands (PCLs)⁵⁻¹² are often preferred because they combine (a) the advantages of small-molecule, freely diffusing drugs with (b) the capacity for reversibly photoswitching their activity without by-product generation nor modification of native receptors.^{2,4}

The major strategy employed to derive PCLs relies on introducing an azoaromatic photoswitch into the structure of well-known, biologically-active ligands.^{2,4} Upon *trans*-*cis* photoisomerization,¹³ a geometrical change is induced in these compounds that alters their interaction with the receptor. Typically, the more extended configuration of the *trans* isomer favors such interaction, while affinity is reduced for the folded *cis* state due to steric effects (i.e. *trans*-active PCLs).^{2,4} Because of the inherent photochemical properties of azo-aromatic compounds,¹³ this imposes a severe limitation to most PCLs developed to date for GluRs⁵⁻¹¹ (and other receptors²): they are active in the dark, where they lie in the more stable *trans* state. Consequently, these compounds elicit strong tonic responses

in the absence of illumination (e.g. when acting as receptor agonists), which drastically hampers their use. This is the case of **GluAzo** (Figure 1a), which is a *trans*-active, *trans*-stable partial agonist of ionotropic kainate receptors GluK1 and GluK2,^{5,14,15} two of the principal GluRs mediating excitatory neurotransmission in the central nervous system.³

To overcome this obstacle while preserving the main design principles behind azo-based PCLs, bridged azobenzenes (brAzo) such as diazocines (C2 bridge, Figure 1b) could be used as photochromes, since they (a) should also favor *trans*-active behavior by switching between extended *trans* and bent *cis* configurations, but (b) exhibit *cis* thermal stability.¹⁶⁻²³ This combination of properties should therefore allow direct administration of the inert *cis* form of the PCL, which could then be selectively photoactivated. In addition, diazocines isomerize with visible light,¹⁶⁻²³ which is a further advantage with respect to common UV-responding azoaromatic compounds.¹³ However, the limited synthetic accessibility and versatility of these photochromes has so far limited their application to the photocontrol of biological systems.^{22,23b} Actually, their use in photopharmacology to modulate the activity of *N*-methyl-D-aspartate (NMDA) receptors and potassium ion channels has only been reported very recently,²⁴ for which a low-yield 4-mono-substituted diazocine previously described was employed.²³

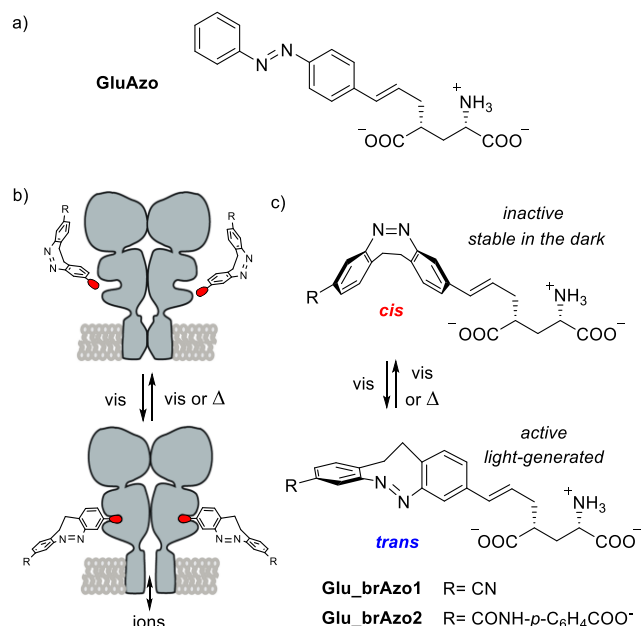


Figure 1. (a) Structure of **GluAzo.5** (b) Strategy proposed to prepare *trans*-active, *cis*-stable PCLs based on C2-bridged azobenzenes for the light-control of neuronal membrane receptors GluK1 and GluK2. (c) Photoisomerization of **Glu_brAzo1-2**.

In this work we aim to apply this strategy to photocontrol GluK1 and GluK2, while broadening its scope by developing diazocine-based PCLs with larger yields and multiple functionalization sites to favor chemical versatility. To reach this goal our attention focused on 3,3'-disubstituted diazocines,^{17,18,21} because very inefficient syntheses have been reported to date for 4,4'-disubstituted analogues (< 1% yield²²). Based on these photochromes and the structure of **GluAzo**,⁵ we designed **Glu_brAzo2** as the first *trans*-active, *cis*-stable agonist of GluK1 and GluK2 by tethering a diazocine unit to a biologically active glutamate moiety through a vinyl linker (Figure 1c). In addition, we took advantage of the disubstitution pattern of the photochrome to introduce a lateral ionic, bulky group for (a) enhancing solubility in aqueous media, and (b) further hindering the interaction of the *cis* isomer with the receptor by increasing the steric congestion around the glutamate moiety. The latter should boost the difference in activity between the two states of the PCL, a required feature given the moderate photoconversions of most functionalized diazocines.^{17,18,20-23} To evaluate this effect, **Glu_brAzo1** lacking the additional bulky substituent was also synthesized (Figure 1c).

To validate our design principles, we conducted molecular docking simulations for the two isomers of **Glu_brAzo1-2** on kainate receptors. Our attention particularly focused on GluK2, since (a) it presents a narrower binding cavity that imposes larger steric constraints to ligands,²⁵ and (b) the crystallographic structure of its ligand-binding domain after complexation with *trans*-**GluAzo** is available.¹⁵ Dockings were computed on this structure keeping the protein rigid, while the initial geometries of the PCLs were optimized at the B3LYP/6-31G(d) level. In all the cases, the best docking solutions placed the glutamate moiety of the ligands in a very similar position as with *trans*-**GluAzo**,²⁶ thus suggesting analogous interaction with the receptor via hydrogen bonds. However, clear differences were observed for the binding arrangement of each ligand (Figure 2 and S1), which led to different

complexation energies (in Chemscore units²⁷): 41.2, 37.0, 48.0, 37.3 for *trans*-**Glu_brAzo1**, *cis*-**Glu_brAzo1**, *trans*-**Glu_brAzo2** and *cis*-**Glu_brAzo2** respectively. Importantly, these figures indicate larger affinity of the *trans* isomers of **Glu_brAzo1-2** to GluK2, thus preserving the targeted *trans*-active behavior of **GluAzo**. By contrast, they do not support our hypothesis that the introduction of an ionic, bulky group in **Glu_brAzo2** should decrease the binding efficiency of the *cis* isomer with respect to less hindered **Glu_brAzo1**. It must be noted, however, that this is compensated by the higher complexation energy calculated for *trans*-**Glu_brAzo2** relative to *trans*-**Glu_brAzo1**, which arises from the additional attractive hydrogen bonding and lipophilic interactions formed between its bulky group and the receptor. Therefore, an enhanced contrast in biological activity is indeed to be expected between the two states of **Glu_brAzo2**, as originally designed.

Based on our experience in the synthesis of C2-bridged azobenzenes¹⁶⁻²¹ and light-responsive glutamate ligands,²⁸⁻³⁰ we devised a linear sequence to prepare **Glu_brAzo1-2** by Heck coupling reaction between their constituting units: a 3,3'-disubstituted diazocine and previously reported, protected glutamate derivative **1**²⁹ (Scheme 1). The preparation of the photochromic unit started from commercially available 4-bromo-2-nitrotoluene, **3**, which was subjected to deprotonation with potassium *tert*-butoxide and further oxidation with bromine to afford dinitro derivative **4**. When attempting common reductive ring-closing conditions on this intermediate that had been previously reported for the synthesis of diazocines (e.g. Zn, Ba(OH)₂),¹⁶⁻²¹ formation of the desired bridged azobenzene was only observed with very low yields (< 14%). This prompted us to develop a new methodology for the azocyclization process, for which we explored Mills coupling reaction. With this aim, we first reduced the nitro groups of **4** using sodium sulphide,³¹ which delivered diamine **5** in 90% yield. Then, oxidative coupling of **5** was undertaken in the presence of Oxone^{®32} in glacial acetic acid³³ at room temperature for 1 h, which nicely furnished dibromosubstituted diazocine **6** in good yield (40%). According to this result, Oxone[®]-mediated oxidation of one of the aniline moieties to provide the corresponding nitrosoarene followed by condensation with the other, unaltered aniline emerges as a novel one-pot synthesis of diazocines with reproducible yields.

Azobenzene **6** was next monocyated under standard Rosenmund-von Braun conditions³⁴ to obtain common asymmetric diazocine **7** in 78% yield (based on recovered **6**). This

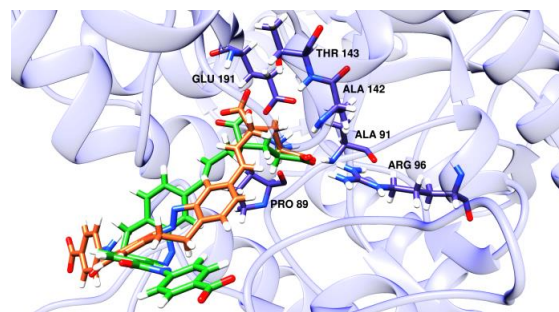
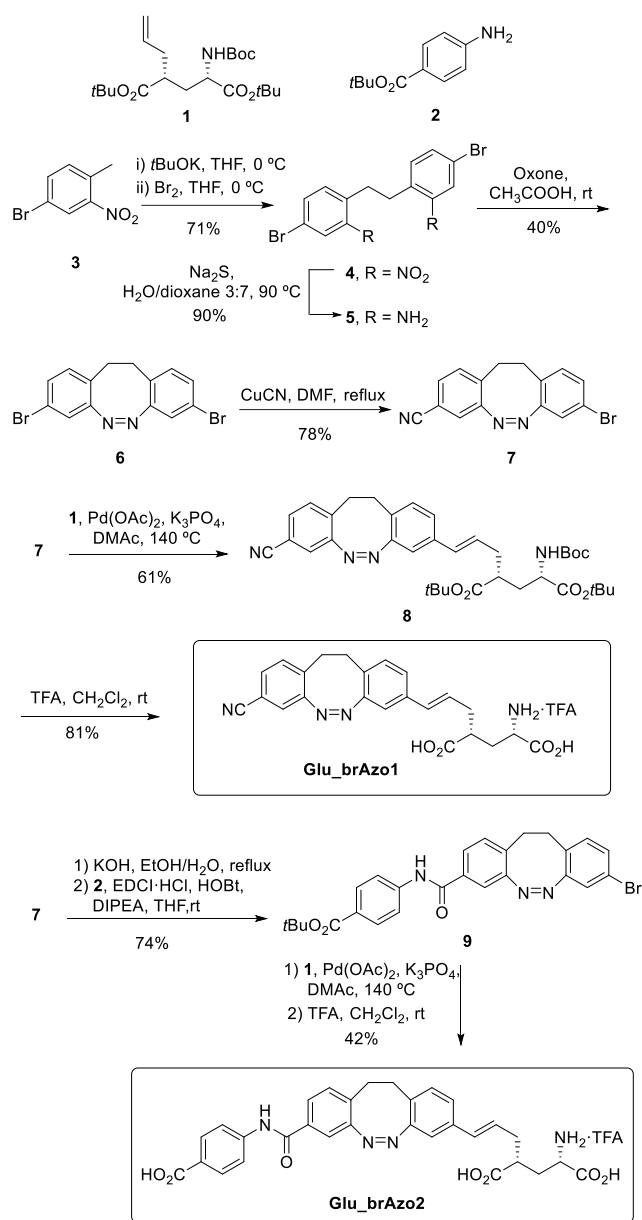


Figure 2. Best docking solutions for *trans*-**Glu_brAzo2** (orange) and *cis*-**Glu_brAzo2** (green) in GluK2. The protein residues interacting with the glutamate group of both ligands are also indicated. Oxygen, nitrogen and hydrogen atoms are depicted in red, blue and white, respectively.

Scheme 1. Synthesis of Glu_brAzo1 and Glu_brAzo2



compound was directly coupled to glutamate **1** under Palladium catalysis, for which proper selection of the base and the solvent was found to be fundamental. After several tests, the reaction was performed with K_3PO_4 and N,N -dimethylacetamide^{3,5} at 140°C under argon in the presence of 0.1 mol% $\text{Pd}(\text{OAc})_2$, which delivered **8** in 61% yield. Finally, acid removal of the protecting groups gave the target compound **Glu_brAzo1** as its monotrifluoroacetate salt in good yield. For **Glu_brAzo2**, intermediate **7** was hydrolyzed in basic medium to give the corresponding acid, which was then tethered to previously prepared amine **2** using carbodiimide coupling reagent EDCI-HCl along with HOBt to afford **9** in 74% yield for the two steps. Heck reaction of this compound with **1** under the aforementioned conditions and subsequent removal of the protecting groups finally delivered the target PCL. It must be noted that, despite its larger structural complexity, **Glu_brAzo2** was obtained with an overall yield that is ca. 2- to 10-fold larger than those recently reported for diazo-

cine-based PCLs,²⁴ thus paving the way for the general application of this strategy in photopharmacology.

By comparison with previous data reported for diazocines,¹⁶⁻²¹ NMR and UV-vis absorption analysis confirmed obtainment of the *cis* isomer of **Glu_brAzo1-2** due to their larger stability. In addition, upon irradiation of their $n-\pi^*$ absorption band with violet light ($\lambda_{\text{abs,max}} \sim 395$ nm, $\lambda_{\text{exc}} = 405$ nm), spectral changes were observed indicative of *cis-trans* isomerization (Figures 3a and S2-S4). This is the case of the new red-shifted absorption band found at $\lambda_{\text{abs,max}} \sim 480$ nm, which is distinctive of the *trans* isomer of diazocines^{16-18,20-23} and allowed reverting the isomerization process by green light illumination ($\lambda_{\text{exc}} = 532$ nm, Figure 3a and S2-S3 and S5). Further characterization of **Glu_brAzo1-2** revealed that they undergo *cis-trans* photoisomerization with moderate quantum yields ($\Phi_{\text{cis-trans}} \sim 0.1$) and efficiencies in aqueous media (Table S1), which resulted in photostationary state mixtures (PSS_{*cis-trans*}) containing 47% and 60% of their *trans* isomer, respectively. By contrast, *trans-cis* photoconversion proceeded quantitatively with high quantum yields ($\Phi_{\text{trans-cis}} \sim 0.9$), thus ensuring fast light-induced recovery of *cis*-**Glu_brAzo1-2** (Table S1) and repetitive photo-switching without degradation (Figure S6). Back-isomerization of the *trans* state of these compounds was also found to occur in the dark, though at a very much longer time scale ($t_{1/2} \sim 4$ h at 298 K in aqueous media, Figure S7 and Table S1).

To assess the capacity of **Glu_brAzo1-2** to light-gate GluRs, whole-cell voltage clamp recordings were conducted on HEK293 cells expressing GluK1 or GluK2, whose activation causes channel opening and ion flux across the membrane (Figure 1b).^{3,5} After perfusion of **Glu_brAzo1-2**, clear changes in the currents evoked in these cells were observed upon irradiation, which were consistent with the different absorption properties of their *cis* and *trans* isomers (Figure 3b and S8-S10). In particular, maximal inward currents arising from

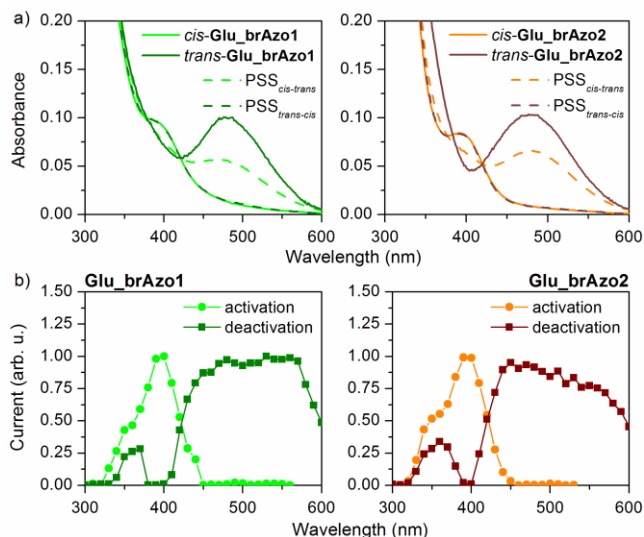


Figure 3. (a) Absorption spectra of both isomers of **Glu_brAzo1** and **Glu_brAzo2** in PBS:DMSO 1:1, as well as of the PSS mixtures obtained under *cis-trans* ($\lambda_{\text{exc}} = 405$ nm) and *trans-cis* ($\lambda_{\text{exc}} = 532$ nm) photoisomerization. (b) Activation and deactivation spectra of GluK2 in HEK293 cells after perfusion of **Glu_brAzo1** or **Glu_brAzo2** and irradiation to induce *cis-trans* (for activation) and *trans-cis* (for deactivation) photoisomerization.

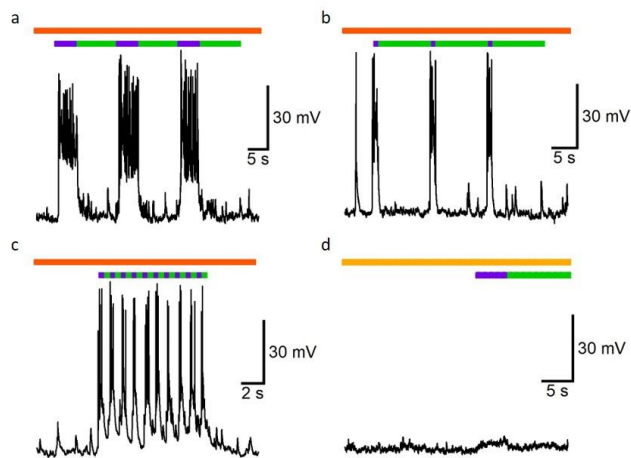


Figure 4. Whole-cell current clamp recordings of rat hippocampal neurons in culture after perfusion of **Glu_brAzo2** (30 μ M) and irradiation at $\lambda_{exc} = 390$ (purple) and 530 nm (green). Successful neuron activation was observed with 390 nm light pulses of (a) 5 s, (b) 1 s, and (c) 0.5 s (1 Hz stimulation), while it was inhibited upon perfusion of DNQX (10 μ M). Neurons were current clamped at -60 mV and, to truly report on their GluK2-mediated gating and connectivity, no Concanavalin A was used to prevent receptor desensitization after activation.

receptor activation were measured at $\lambda_{exc} = 390$ -400 nm (i.e. when inducing *cis-trans* photoisomerization of the PCLs). Minimal signals due to receptor deactivation were instead detected in the dark (i.e. for *cis-Glu_brAzo1-2*) and at $\lambda_{exc} > 450$ nm (i.e. upon *trans-cis* photoisomerization). As predicted by our molecular docking calculations, preferential interaction between the glutamate agonist ligand and GluK1/GluK2 was therefore observed for the *trans* state of **Glu_brAzo1-2**, which allowed light-induced manipulation of these receptors.

The *trans*-active behavior of **Glu_brAzo1-2** was further demonstrated when measuring their dose-response curves with GluK1 and GluK2 in the dark and at $\lambda_{exc} = 390$ (10-300 μ M, Figure S11). In all the cases, larger inward current signals were retrieved for the PSS_{*cis-trans*} mixture generated upon irradiation of the initial *cis* isomer, though with (a) low-to-moderate photoinduced modulation (ca. 10-35% current increase with respect to the dark) and (b) maximal values that were only about 20-40% of those evoked by free glutamate at 300 μ M. Three main factors should account for these results: incomplete photoconversion into the active *trans* isomer, as observed in solution (< 60%, Table S1); limited modification of the glutamate-binding site affinity upon *cis-trans* isomerization; and the steric effects imparted by the appended diazocine group with respect to free glutamate even in its less-hindered *trans* configuration, which are of especial importance for GluK2 interaction owing to its narrower ligand binding cavity.²⁵ Actually, the latter accounts for the lower inward current signals with higher light-induced selectivity obtained for this receptor. However, it did not prevent **Glu_brAzo2** bearing a bulkier terminal group to (a) show higher activity (ca. 2-fold increase with respect to **Glu_brAzo1** for GluK2), as anticipated by our molecular docking simulations, which (b) is further modulated upon photoisomerization, as pursued in our initial design.

Glu_brAzo1-2 were finally tested as photoswitchable neurotransmitters in hippocampal neurons where GluK2 is highly expressed.³⁶ Because of their *trans*-active, *cis*-stable behavior, physiological behavior was not altered upon perfusion of **Glu_brAzo1-2** in the dark, a clear advantage over most PCLs reported for GluRs to date such as **GluAzo**.⁵⁻¹¹ Neuronal firing was instead selectively induced upon *cis-trans* photoisomerization of **Glu_brAzo2** at relatively low concentrations (30 μ M, Figure 4a-c) and weak light intensities (22.0 and 47.4 μ W mm^{-2} at $\lambda_{exc} = 390$ and 530 nm, respectively). As a result, sequential and sustained trains of action potentials could be triggered with **Glu_brAzo2** by consecutively switching between violet and green light illumination, even at low irradiation powers and high excitation frequencies (up to 1 Hz, Figure 4c). Interestingly, this photoinduced behavior was inhibited by addition of DNQX,³⁷ a well-known antagonist of kainate and AMPA GluRs (Figure 4d), which further demonstrates that the efficient photoactivation of hippocampal neurons accomplished with **Glu_brAzo2** arose from its light-dependent interaction with GluK2. Noticeably, this clean physiological effect was achieved despite the limited activity modulation measured upon **Glu_brAzo2** photoisomerization in GluK2-expressing HEK293 cells (ca. 20% at 30 μ M concentration), while it could not be reproduced with the slightly less efficient **Glu_brAzo1** agonist (Figure S12). We ascribe this situation to the well-known nonlinear behavior of neuronal signaling, since ligand interaction with a minimum fraction of glutamate receptors is needed to overcome the depolarization threshold required to initiate an action potential.⁵ In our case, such threshold was only surpassed with *trans-Glu_brAzo2*, probably due to its larger affinity for GluK2, as suggested by our measurements in cultured cells.

In conclusion, we demonstrated in this work the potential of C2-bridged azobenzenes for the preparation of azo-based photochromic ligands capable of light-controlling neural receptors, which preserve the larger activity typically observed for their *trans* state while ensuring larger stability for the inert *cis* isomer. By introducing a novel synthetic methodology for the preparation of 3,3'-disubstituted diazocines based on an Oxone[®]-mediated intramolecular azocyclization reaction, the desired ligands could be obtained with higher yields and larger versatility, thus enabling fine-tuning of their light-dependent biological response. As a proof of concept, this strategy was applied to the preparation of new photoswitchable neurotransmitters for the light-induced operation of kainate glutamate receptors, with which we achieved selective neural firing upon irradiation without background activity in the dark.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

General methods and materials, synthetic procedures, and additional computational, photochemical and electrophysiological data (PDF)

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Notes

The authors declare no competing financial interests.

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