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ARTICLE

A photoswitchable CENP-E inhibitor with single blue-green light to control chromosome positioning in mitotic cells

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The reversibly photoswitchable chemical tools have aided in the development of novel approaches in the biomedical field. The visible region of light should be ideal for the biological application of this approach because of its low phototoxicity and deep penetration depth compared to ultraviolet light. Herein, we report a photoswitchable centromere-associated protein E (CENP-E) inhibitor, which is controllable with low-energy blue-green light (around 500 nm) illumination. This photoswitchable tool enabled us to control CENP-E-driven chromosome movements and positioning at subcellular resolutions with low phototoxic effects. This study can contribute to the development of a unique technique for chromosomes engineering.

Introduction

In photopharmacology, light is used as a non-invasive external stimulus for the remote activation or inactivation of biological functions with high spatiotemporal resolution.¹ Photopharmacological strategies have been increasingly focused on reversibly controllable chemical tools containing azobenzene derivatives as the photoresponsive chromophores. Azobenzene displays the reversible *cis-trans* photoisomerization upon illumination by the corresponding wavelength of light, which is generally ultraviolet light and visible light. However, the use of ultraviolet light (especially at 300–400 nm) for photoisomerization of the *trans* to the *cis* isomer of typical azobenzene derivatives can be harmful for the biological system.² To overcome the drawbacks of using ultraviolet light, several approaches have been reported.³ For instance, tetra-*ortho*-substituted azobenzenes with well-separated absorption spectra have been developed, enabling the control of biofunctions using visible light sources.⁴ Other strategies have also been extensively developed based on the heteroaromatics⁵, push-pull azo derivatives⁶, two-photon excitation⁷, sensitized switching⁸ etc.

Previously, we reported two different types of photopharmacological tools **1**⁹ and **2**¹⁰ (Fig. 1a) targeting centromere-associated protein E (CENP-E)¹¹. CENP-E can guide the mitotic chromosomes from mitotic poles to the cell equatorial plane (metaphase plate) along the spindle

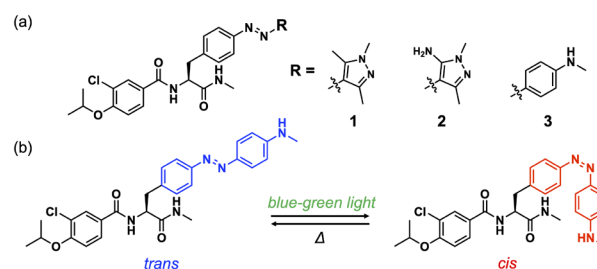


Fig. 1. Design of the photoswitchable CENP-E inhibitor controlled using single visible light. (a) Photoswitchable CENP-E inhibitors **1**, **2**, and **3**. (b) Photoisomerization and thermal relaxation of **3** with 488 nm light.

microtubules. To regulate the CENP-E activity in a reversible manner, we developed the original photoswitchable CENP-E inhibitor **1** based on bistable *cis* and *trans* photoisomerization in a typical arylazopyrazole photoswitch¹². Indeed, **1** is powerful for elucidating the essential functions of CENP-E in mitotic progression, but the simultaneous application of the intrinsically harmful ultraviolet light at 365 nm and the visible light at 510 nm have set the intricate optical system. To simplify the optical system for the light illumination and enable the precise engineering of CENP-E-driven chromosome positioning, a photoswitchable CENP-E inhibitor **2** using a single wavelength (405 nm) of visible light was recently developed through the incorporation of the amino group ($-NH_2$) of the pyrazole unit in **1**. Generally, azobenzene derivatives with strong electron-donating groups, such as amino ($-NH_2$) have demonstrated the significantly increased rates of *cis* to *trans* thermal back reactions.¹³ Thus, once **2** was isomerized from *trans* isomer by illumination of violet light at 405 nm, the resulting *cis* isomer of **2** instantly underwent the thermal relaxation reaction to the *trans* isomer and had a lifetime of 0.66 msec. However, the prolonged illumination time of violet light at 405 nm still can

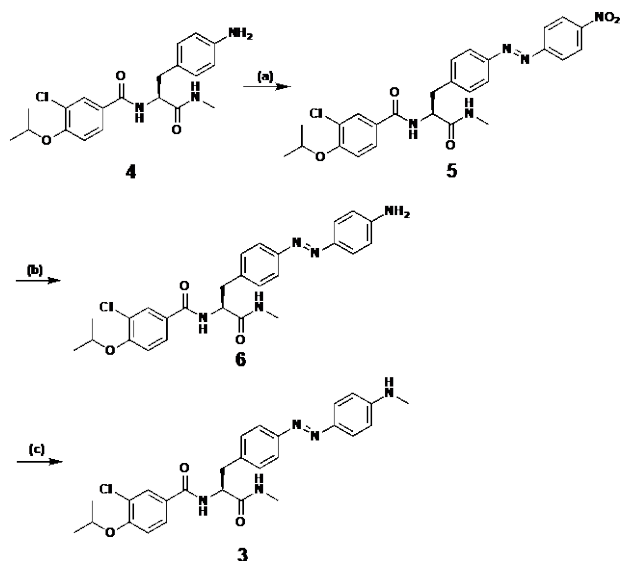
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Scheme 1. Synthetic scheme of the photoswitchable CENP-E inhibitor **3**. Conditions: (a) 4-Nitroaniline, acetic acid, (b) Sodium sulfide, THF, EtOH, (c) Paraformaldehyde, NaOMe, NaBH₄, MeOH.

induce the phototoxic effects in cells^{10,14}, although it is much less than ultraviolet light. Thus, we developed a photoswitchable CEN-P inhibitor using blue-green visible light, which has a longer wavelength than violet light.

In this report, we describe the development of photoswitchable CENP-E inhibitor **3** (absorption maximum 407 nm), which uses a single wavelength (around 500 nm) of visible light and provides a 34 nm-redshift compared with **2** (absorption maximum 373 nm). We clearly demonstrate that the photoswitchable CENP-E inhibitor **3** can regulate CENP-E activity with continuous illumination of less phototoxic blue-green light and thus, **3** can be used for precisely regulating CENP-E-guided chromosome movement and its positioning in live mitotic cells in a photocontrolled manner.

Results and discussion

To develop the photoswitchable CENP-E inhibitor with a longer wavelength of visible light, the aminopyrazole unit in **2** was altered into an aniline unit having higher aromaticity. A mono-methyl group was also introduced on the nitrogen atom of the aniline unit, providing a photochromic moiety with longer wavelength absorption.¹⁵ To chemically synthesize **3** (Scheme 1), the azo group (-N=N-) in **5** was formed by the reaction of amino group-substituted phenylalanine derivative **4**⁹ with 4-nitroaniline¹⁶ in the presence of acetic acid. After the reduction of the nitro group in **5** with sodium sulfide, aminoazobenzene derivative **6** was obtained. The reductive amination of **6** with paraformaldehyde afforded the mono methylated product **3**. All compounds were carefully characterized by ¹H-nuclear magnetic resonance (NMR), ¹³C-NMR and high resolution mass spectroscopy (Fig. S1-S6).

The photophysical properties of **3** in aqueous solution were compared with **2** (Fig. 2a). As expected, the absorption

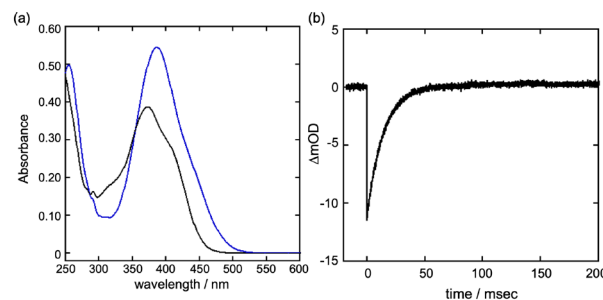


Fig. 2. The photophysical properties of **3**. (a) Absorption spectra of **2** (20 μ M, black) and **3** (20 μ M, blue) in an aqueous solution (acetonitrile to BRB80 buffer ratio (v/v) of 1/1) without light illumination. The molar extinction coefficients of **3** were 25,800 M⁻¹cm⁻¹ at 407 nm, 10,500 M⁻¹cm⁻¹ at 470 nm, 4,600 M⁻¹cm⁻¹ at 488 nm and 250 M⁻¹cm⁻¹ at 532 nm. (b) Transient absorbance changes of **3** (20 μ M) were detected at 407 nm upon excitation using 532 nm laser light with half bandwidth of 0.17 nm. BRB80 buffer containing 80 mM PIPES (Piperazine-1,4-bis(2-ethanesulfonic acid)), 1 mM MgCl₂, and 1 mM EGTA (ethylene glycol tetraacetic acid) (pH 6.9).

spectrum of **3** (absorption maximum 407 nm) in absence of light illumination exhibited absorption above 450 nm and a molar extinction coefficient greater than that of **2** (absorption maximum 373 nm). Due to the fast thermal relaxation of the *cis* isomer of **3**, it was not possible to detect slight changes (if any) in the absorption spectrum upon light illumination at 470 nm, using a standard UV-Vis spectrometer. Laser flash photolysis with 532 nm excitation was carried out to estimate the lifetime of the *cis* isomer of **3** in aqueous solution, which was found to be 14 msec (Fig. 2b). This lifetime of **3** was approximately 2.4×10^7 -fold shorter than that of (92.5 h)⁹ and 25-fold longer than that of **2** (0.57 msec)¹⁰. These unique photophysical properties suggested that **3** functioned as the photoswitchable CENP-E

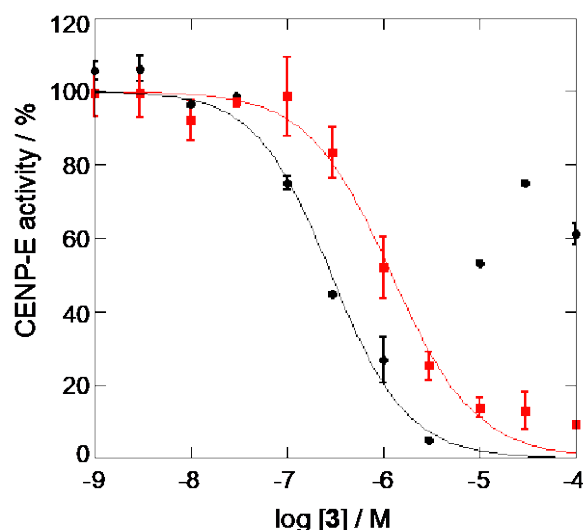


Fig. 3. *In vitro* ATPase assay with purified CENP-E in BRB80 buffer using **3** with dimethyl sulfoxide (DMSO, < 0.5%) as a co-solvent. A light stimulus at 470 nm was used to induce the *cis* isomer. Black circle: **3** in absence of light illumination. The fitting was done with the first eight data points and the other three are off because of the aggregation of **3** above 10 μ M concentration. Red square: **3** with light illumination at 470 nm. Error bars represent the standard errors from triplicate experiments.

inhibitor with single wavelength (> 450 nm) of light, as the *cis*-**3** could rapidly undergo the thermal relaxation reaction to form the *trans* isomer.

To validate the inhibitory activity of **3** to the purified CENP-E, ATPase assay was performed using the similar method of our previous report¹⁰. In the absence of light illumination, **3** showed the moderate CENP-E inhibition (half maximal inhibitory concentration; $IC_{50} = 0.28 \mu\text{M}$), which has lower potency than the photoswitchable CENP-E inhibitor **2** ($IC_{50} = 24 \text{ nM}$) (Fig. 3). This low potency is probably because the photoswitchable CENP-E inhibitor with a bulky photoswitch was unfavored for CENP-E inhibition⁹. The samples with the continuous light illumination at 470 nm exhibited the less inhibition property ($IC_{50} = 1.2 \mu\text{M}$) than the samples in the absence of light illumination. This 4.3-fold change in CENP-E inhibition of **3** made it evident that it was controllable using a blue-green light. Intriguingly, the inhibition properties of more than $10 \mu\text{M}$ of **3** in the absence of light illumination were observed to be attenuated to approximately 50% CENP-E activity. Therefore, we hypothesized that this attenuation of the inhibitory activity was due to the self-assembly of **3** in BRB80 buffer. The self-assembling property is one of the key factors in the inhibitor development as demonstrated in the several reports¹⁷. The absorption spectra were measured at different concentrations of **3** in the BRB80 buffer, which indicated that the critical self-assembly concentration of **3** was estimated to be $7 \mu\text{M}$ (Fig. S7a). In sharp contrast, no self-assembling tendency was detected with **3** in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and can be used for cell experiments even at a concentration of $100 \mu\text{M}$ (Fig. S7b). This encouraged us to explore the in-cell availability of **3**.

The feasibility of the photoswitchable CENP-E inhibitor **3** in HeLa cells was explored by the immunofluorescence method using blue-green light stimulus. Cells were incubated with **3** in the presence of a proteasome inhibitor (MG-132) for the prevention of the anaphase onset,¹⁸ which can facilitate the chromosome analysis during metaphase (Fig. 4a). In the absence of light illumination, $6.0 \mu\text{M}$ of **3** induced massive amounts of mitotic chromosomes and CENP-E were accumulated at the surroundings of mitotic spindle poles. This cellular phenotype significantly agreed with the CENP-E malfunction.¹¹ By contrast, under the condition with the continuous light illumination at 470 nm, the positions of chromosomes and CENP-E were restored at the metaphase plate even in the presence of **3**. More importantly, the intensity ($10 \text{ mW}/\text{cm}^2$) and duration (60 min) of light illumination at 470 nm in this experiment did not affect the functioning of CENP-E, while the same experiment with 405 nm light induced CENP-E malfunction and cell shrinkage from severe phototoxicity (Fig. 4b). This is because the reactive oxygen species (ROS) produced by the light illumination at 405 nm, which was measured with a ROS-reactive fluorescent probe (Fig. S8), can afford the oxidative damages to CENP-E. The phototoxic effect can be overcome by undertaking our strategy of using a longer wavelength of light for the external stimulus. We also quantified the mitotic cells containing aligned/misaligned chromosomes in **3**-treated cells in the presence and absence of light illumination

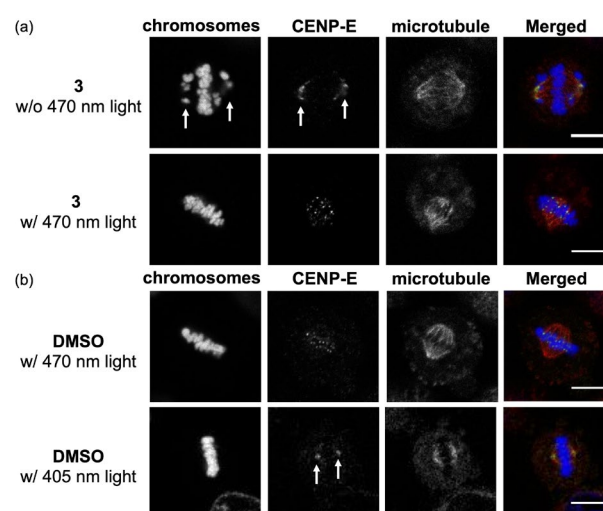


Fig. 4. Immunofluorescence imaging of HeLa Kyoto cells using 4',6-diamidino-2-phenylindole (DAPI), and antibodies against CENP-E and microtubules. (a) **3** ($6.0 \mu\text{M}$) with DMSO (0.1%) as a cosolvent-treated cell with or without light illumination at 470 nm ($10 \text{ mW}/\text{cm}^2$) for 1 h. (b) DMSO (0.1%)-treated cell with light illumination at 470 nm ($10 \text{ mW}/\text{cm}^2$, upper) or 405 nm ($10 \text{ mW}/\text{cm}^2$, lower) for 1 h. The representative images are shown from triplicate experiments (> 10 cells for each condition). w/o: without. w/: with. White arrows indicated the misaligned chromosomes in the column of chromosomes and the CENP-E accumulation around mitotic poles in the column of CENP-E.

at 470 nm (Fig. 5). Without light illumination, over 80% of the

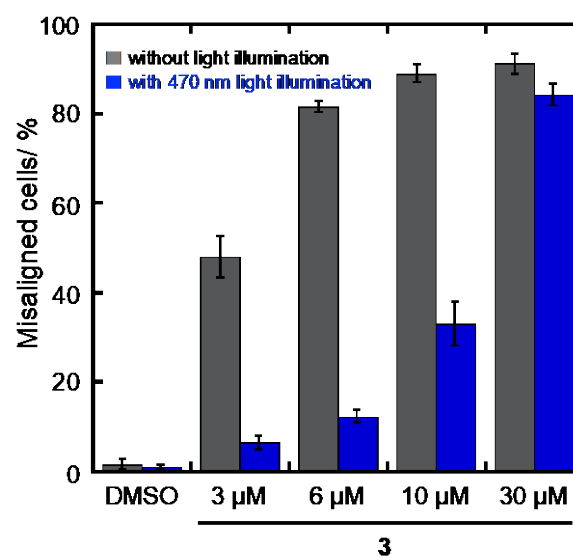


Fig. 5. Cell population percentage of misaligned chromosomes due to the CENP-E inhibition by **3** with DMSO (< 0.5%) as a cosolvent. Error bars represent the standard errors from triplicate experiments (> 50 cells for each experiment).

chromosomes were misaligned due to CENP-E inhibition at a concentration higher than $6.0 \mu\text{M}$ of **3**, where half maximal effective concentration (EC_{50}) of exhibiting the cells with misaligned chromosomes was calculated to be $2.9 \mu\text{M}$. In

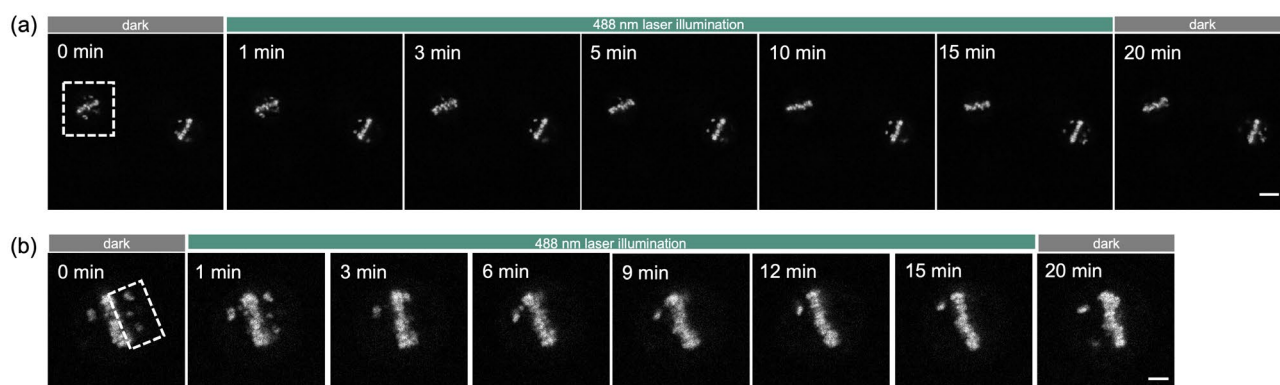


Fig. 6. Live cell experiments for the photocontrol of mitotic chromosomes by **3** (6.0 μM) with DMSO (0.1%) as a co-solvent in the setup of confocal laser scanning microscopy. (a) Time lapse images of the photocontrol of the mitotic chromosomes in one cell out of the two lined up mitotic cells. White square at 0 min indicates the region of laser illumination at 488 nm. Scale bar: 10 μm . (b) Time lapse images showing the precise photocontrol of chromosome positioning and their asymmetric distribution at the subcellular level. White dotted square at 0 min indicates the region illuminated with 488 nm laser. Scale bar: 5 μm . All images are representative of triplicate experiments.

contrast, with the continuous light illumination at 470 nm, the population of cells with misaligned cells was comparatively lower, with an EC_{50} value of 13 μM . Interestingly, this 4.5-fold changes in EC_{50} values were in complete accordance with the changes in IC_{50} values in ATPase assay *in vitro*. Therefore, **3** worked as the photoswitchable CENP-E inhibitor that is controllable using a visible light at 470 nm both in test tubes and in cells.

We further verified the applicability and spatiotemporal controllability of **3** through the live cell experiments. Chromosomes in mitotic cells were stained using the far-red fluorescent probe for nuclei, SiR-DNA¹⁹ which can be excited by laser illumination at 632 nm without inducing photoisomerization of **3**. After incubation with **3** (6.0 μM) for an hour, cells were subjected to the live imaging using the standard setup of confocal laser scanning microscopy (CLSM) with the continuous laser illumination at 488 nm for the induction of *trans* to *cis* photoisomerization of the azo unit. In the experiment where one out of two different mitotic cells was targeted and illuminated with 488 nm laser, throughout the duration of the laser illumination, the misaligned chromosomes in the targeted cell moved from the mitotic poles towards the metaphase plate and eventually, fused to the plate within 15 min (Fig. 6a). By contrast, the misaligned chromosomes in another cell without laser illumination were never restored to the metaphase plate. For a more local photocontrol of chromosome movements and positioning in a single mitotic cell, the 488 nm laser was continuously illuminated to the misaligned chromosomes locating around the right-half poles (white dotted square, Fig. 6b). The polar chromosomes in the designated area for laser illumination moved and reached to the metaphase plate, while misaligned chromosomes around another mitotic pole remained misaligned. Eventually, the asymmetric distribution of mitotic chromosomes in single cell were induced by the pin-point laser illumination.

Conclusions

In this research, we developed a photoswitchable CENP-E inhibitor **3**, which afforded the 4.3-fold affinity changes with and without light illumination. This drastic change in CENP-E inhibition was controllable with blue-green light, which has lesser phototoxic effects than violet (405 nm) light. Using this less toxic light stimulus, the precise and remote manipulation of mitotic chromosomes was demonstrated at subcellular resolutions. The techniques developed in the current study will enable the construction of novel photochemical system for the alteration of cell fates through the engineering of mitotic chromosomes.

Author contributions

K.M. and N.T. conceptualized the project, and wrote the manuscript. K.M. synthesized and analysed the compounds and carried out biochemical and cell experiments. K.M. and T.K. carried out the flash photolysis experiments. T.W., A.K. and N.T. supported and supervised the project.

Conflicts of interest

There are no conflicts to declare.

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