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Rational Design and Development of a Lit-active Photoswitchable Inhibitor Targeting CENP-E

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Abstract

In the emerging field of photopharmacology, synthetic photoswitches based on reversible photochemical reactions are fused to bioactive molecules. Azobenzene derivatives, which can undergo *trans-cis* photoisomerization, are typical photoswitches. Most azobenzene-based photochemical tools are active in the thermodynamically stable *trans*, but not *cis*, form. *Cis*-active photochemical tools would be ideal because they can be "initially inactive and active after light illumination" in a reversible mode only by light illumination. However, only a few rational strategies for constructing such "lit-active" photopharmacological tools has been developed. Herein, we report a rationally designed lit-active photoswitchable inhibitor targeting centromere-associated protein E (CENP-E). Using the lit-active inhibitor, we were able to photoregulate CENP-E-dependent mitotic chromosome movement in cells. This study provides a framework to facilitate further progress in the development of phototpharmacological tools.

Main text

Photopharmacology is a promising approach that uses non-invasive light stimuli to precisely control biological systems with high spatiotemporal resolution.[1] In this emerging field, photochemical tools that enable both the irreversible and reversible regulation of biological processes have been elaborately developed. For ideal applications of photopharmacology, initially inactive molecules should be instantly and dramatically activated by photoillumination to exert their biological effects. In this context, the

photocaging strategy is a well-established technique in which bioactive molecules, such as ions, ligands, and inhibitors, are masked with photodegradative scaffolds, such as 2-nitrobenzyl and coumarinylmethyl groups.[2] Using the photocaging techniques, bioactive compounds are released upon the instantaneous removal of the photolabile units through photoillumination.

Since these photochemical reactions are based on one-way uncaging reactions, biological effects can be irreversibly switched from OFF to ON or from ON to OFF. Thus, the reversible regulation is intrinsically difficult to accomplish with the photocaging strategy. As an alternative and potent approach, photopharmacological tools have been elaborately developed. Reversibly photoresponsive chromophores,[3] including azobenzenes[3e], diarylethenes[3f], fulgides[3g], and hemithioindigos[3h] are synthetically fused into bioactive units. In particular, azobenzenes are the most common photoswitch because of their small size and drastic changes in angular geometry of the two aromatic rings connected to an -N=N- bond and the dipole moment. Azobenzenes can display two different geometric isomers that are thermodynamically stable *trans* and metastable *cis* isomers. These two isomers can be reversibly converted with illumination at the appropriate wavelengths (typically ultraviolet light for *trans*-to-*cis* isomerization and visible light for *cis*-to-*trans* isomerization). In practical usages of the photochemical property, azobenzenes are introduced into or substituted for a part of the core structures of target biomolecules for the development of photopharmacological tools. A representative example of such azobenzene-based photopharmacological tools is Photostatins, which works as an inhibitor of microtubule

dynamics in cells in a photoswitchable manner using the *trans-cis* photoisomerization of the azobenzene unit.[4]

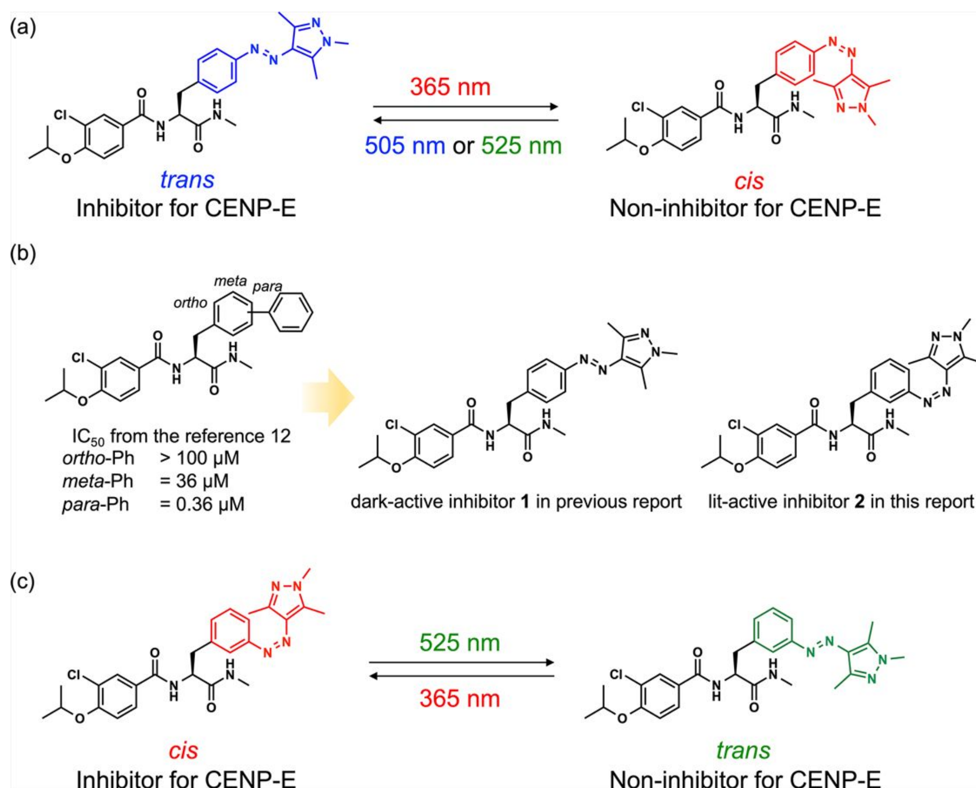


Figure 1. Design of the lit-active centromere-associated protein E (CENP-E) inhibitor. (a) Reversible photoswitching of the dark-active photoswitchalbe CENP-E inhibitor **1**.[6] (b) Molecular design of lit-active photoswtichable CENP-E inhibitor **2**. (c) Reversible photoswitching of lit-active CENP-E inhibitor **2**.

We also reported a photoswitchable centromere-associated protein E (CENP-E) inhibitor **1**[6] based on an arylazopyrazole photoswitch[7] (Figure 1a). CENP-E is a motor protein that can deliver chromosomes from mitotic poles to the metaphase plate along spindle microtubules in mitotic cells.[5] Our

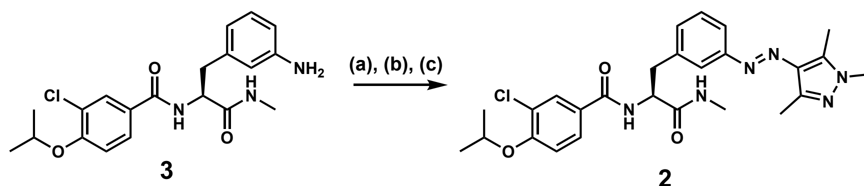
photoswitchable inhibitor **1** exhibited potent CENP-E inhibition with a thermodynamically stable *trans* state but did not with the metastable *cis* state. Using this "dark-active" **1**, we could modulate mitotic chromosome movement and the progression of mitosis, which enabled us to reveal an essential function of mitosis that CENP-E activity is not required for the maintenance of chromosome alignment during mitosis. These results highlighted the utility of **1** for studying CENP-E function. But lit-active, not dark-active nature must be generally more preferred for practical applications. With such lit-active tools showing no bioactivity before light illumination, the action can be instantly triggered and deactivated by illumination with the appropriate wavelength of light. In this context, some lit-active tools, including photostatins[4] and optojasps[8] (photoswitchable actin inhibitors) have been developed to date.

Recently, another strategy for potentially lit-active photoswitches using bridged azobenzenes (diazocines),[9] which show metastable *trans* and thermodynamically stable *cis* isomers, has also emerged. In this strategy, biological effects can be photochemically switched with the "initially inactive and active after light illumination" mode using diazocine derivatives based on dark-active compounds. Thus, diazocine-based photopharmacological tools have been considered highly promising as lit-active ones.[10] However, it is still difficult to rationally design azobenzene-based tools with the dark- or lit-active properties partially because of the difficulty of the synthesis. Furthermore, there are only a few strategies for changing dark-active compounds into lit-active ones through rational design.[9,11]

Herein, we report the rational design of lit-active (*cis*-active) photoswitchable CENP-E inhibitor **2** with

reference to the properties of dark-active (*trans*-active) inhibitor **1** (Figure 1b,c). The *trans* isomer of **2** exhibits no inhibitory effect on CENP-E, whereas the *cis* isomer shows moderate inhibition of CENP-E through experiments in test tubes and in cells. To develop a lit-active photoswitchable CENP-E inhibitor, we focused on a previous report of a structure–activity relationship study on an authentic CENP-E inhibitor (**GSK923295)**.[12] The CENP-E inhibitory properties of phenyl-substituted phenylalanine (biphenylalanine) derivatives depended significantly on the position of the phenyl group substitution (Figure 1b). The trends for CENP-E inhibitory abilities were *ortho*- (IC_{50} > 100 μM) < *meta*- (IC_{50} = 36 μM) < *para*- (IC_{50} = 0.36 μM) substitutions[12], respectively, which suggests that CENP-E strictly recognizes the steric environment surrounding the phenyl group and that the *para*-substituted biphenylalanine is more favorable for the interaction with CENP-E than the *meta*- or *ortho*-substituted ones. From these data, we presumed that the *trans* form of the *para*-substituted azo-inhibitor **1** occupies the key environment for inhibition, whereas the *cis* form does not. Therefore, we designed **2** as the photoswitchable lit-active inhibitor targeting CENP-E, where the azopyrazole photoswitch was introduced at the *meta*-position on the corresponding phenylalanine derivative. We considered that the pyrazole unit in *trans*-**2** sterically hindered the binding with CENP-E and that the pyrazole unit in the bent *cis*-**2** faced and located the *para*-position on the phenylalanine derivative, which could relieve the steric hindrance. Such an effect of stronger interaction at *cis*-state than *trans*-state of a meta-substituted azobenzene derivative as a guest molecule has been reported for photoresponsive chiral dopants in liquid crystal host

media.[13]



Scheme 1. Synthesis of **2**. Conditions: (a) $NaNO_2$, AcOH, HCl, H_2O. (b) Acetylacetone, NaOAc, EtOH, H_2O. (c) Methylhydrazine, EtOH.

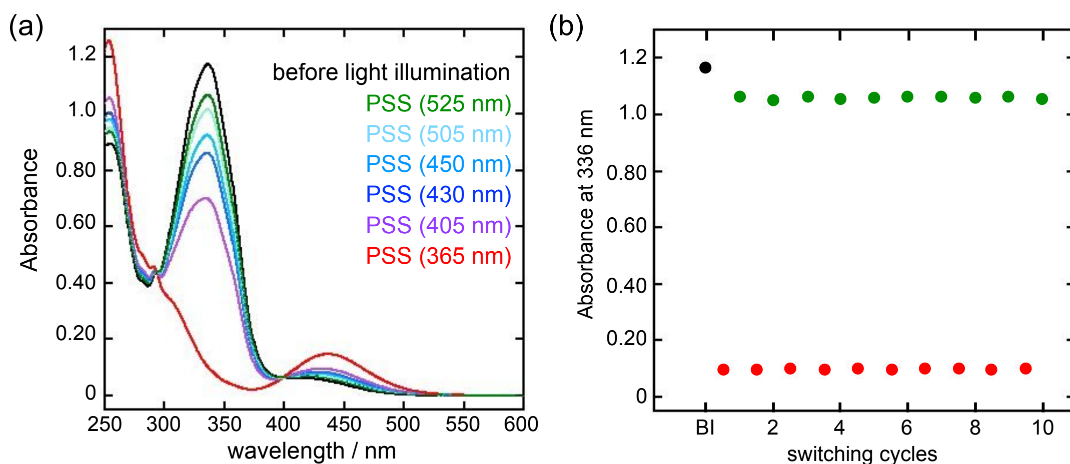


Figure 2. Photophysical properties of **2**. (a) Absorption spectral changes of **2** (50 μM) in aqueous solution after light illumination at 365, 405, 430, 450, 505, and 525 nm. (b) Switching property of the reversible photoisomerization reaction of **2**. BI, before light illumination (black dot). 365 nm light, 1800 mJ/cm^2 (red dots). 525 nm light, 1500 mJ/cm^2 (green dots).

To validate this design, **2** was chemically synthesized (for characterization, see Figures S1 and S2) from the corresponding amine derivative **3**[6] (scheme 1). The photophysical and photochemical properties of **2** were studied through absorbance measurements with illuminating light. Before light illumination, **2** exhibited the well-separated π-π* and n-π* transition bands typical for arylazopyrazoles[7] (Figure 2a), which exists as 100% *trans* isomers in aqueous solution (acetonitrile:BRB80 buffer = 1:1) as proved by HPLC analysis (Figure S3). Changes in absorption spectra were recorded with various wavelengths of light. In particular, light illumination at 365 nm dramatically altered the absorption spectrum to reach a photostationary state (PSS) containing a 95% *cis* isomer composition (Figures 2a and S3). At $PSS_{525\ nm}$ including a 93% *trans* isomer composition with light illumination at 525 nm, the absorption spectrum exhibited almost the same state as that before light illumination. This almost quantitative photoswitching was reversibly permitted for many cycles without fatigue (Figure 2b). *Cis*-**2** also underwent spontaneous and relatively slow thermal relaxation with a lifetime > 72 h at 37 °C (Figure S4).

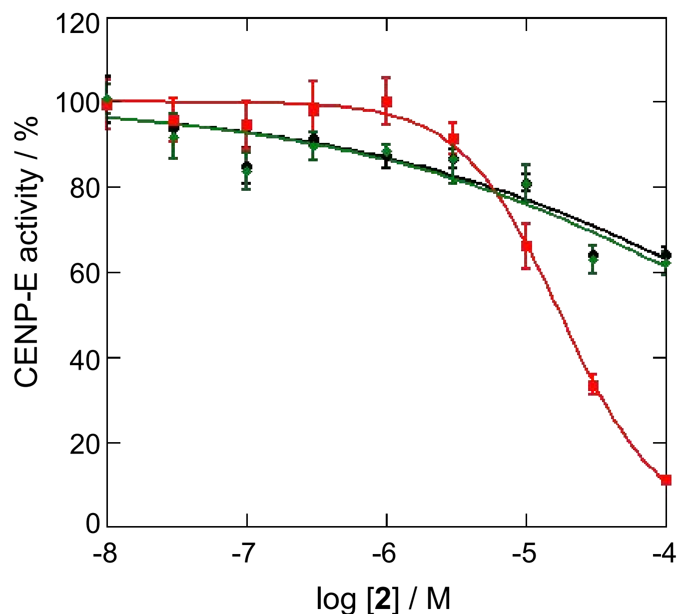


Figure 3. *In vitro* ATPase assay with CENP-E of **2** with/without 365 nm light and 525 nm light illumination. Black circles: without light illumination, green circles: light illumination at 525 nm, red squares: light illumination at 365 nm. Error bars show the standard errors from triplicate experiments.

Next, an *in vitro* ATPase assay was carried out using a chemiluminescence-based ADP-Glo system (Promega, Madison, WI, USA). Under the *trans*-rich condition of **2** before light illumination and with light illumination at 525 nm, no inhibition of CENP-E ATPase activity was observed (IC_{50} > 100 μM), although the mild inhibition of CENP-E activity was achieved under the *cis*-rich state of **2** with light illumination at 365 nm (IC_{50} = 18 μM). These results clearly demonstrate that *cis*-**2** is an active CENP-E inhibitor, whereas *trans*-**2** is not.

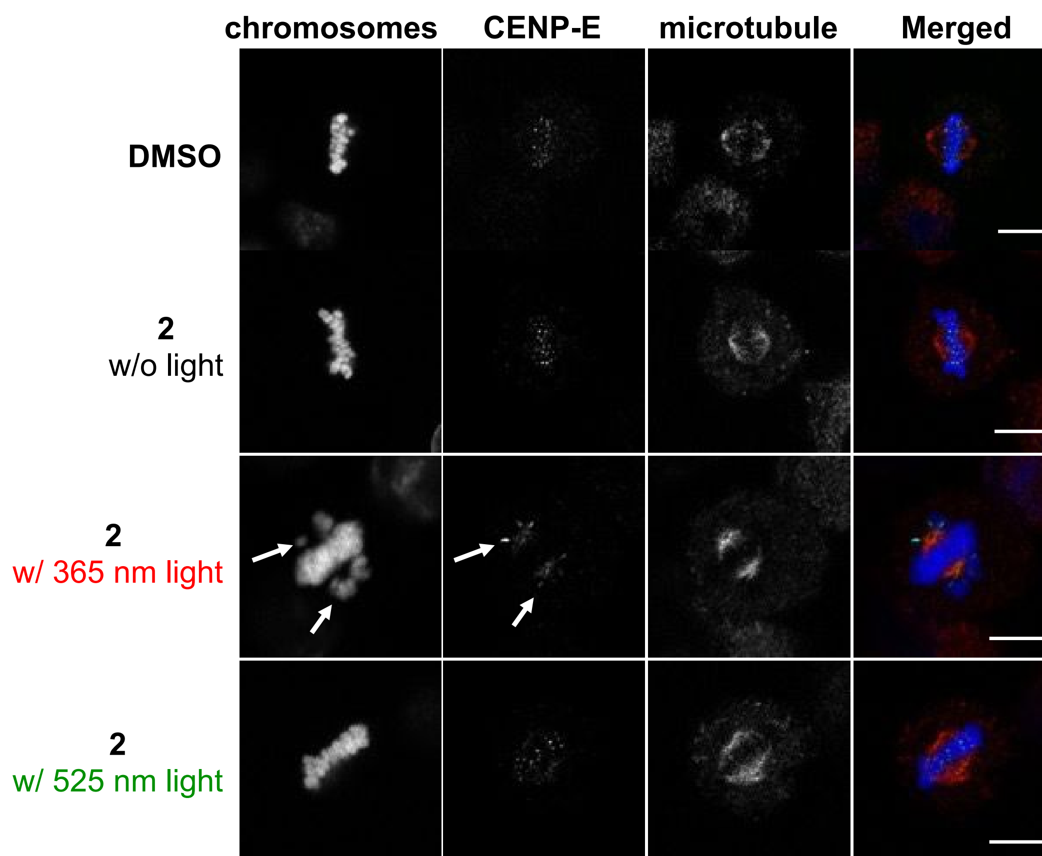


Figure 4. Immunofluorescence images of chromosomes, CENP-E, and α-tubulin in HeLa cells treated with dimethyl sulfoxide (DMSO) or **2** (100 μM) with or without light illumination at 365 nm or 525 nm in the presence of MG-132 (20 μM) for 2 h. White arrows show the misaligned chromosomes and CENP-E on their kinetochores. All scale bars are 5 μm. Representative images from triplicate experiments are shown (> 20 cells in total for each condition). w/o: without. w/: with.

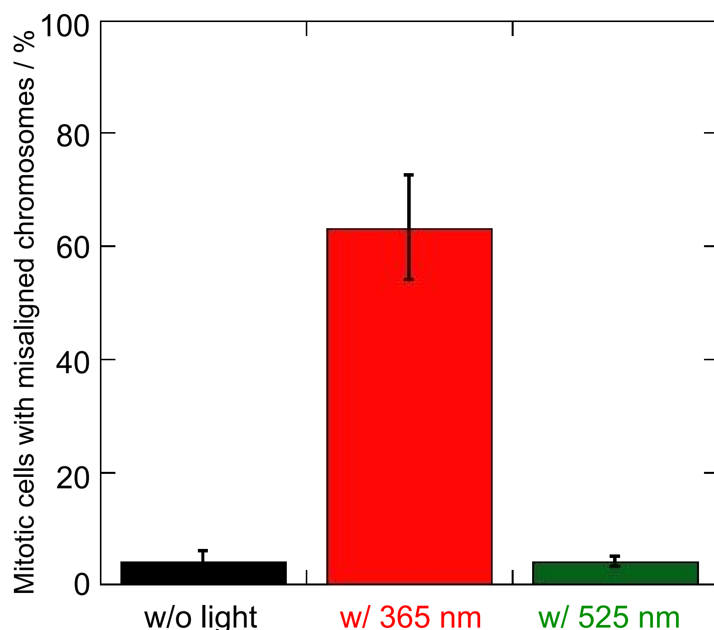


Figure 5. Frequency of misaligned chromosomes in **2**-treated cells with/without light illumination at 365 nm or 525 nm. Error bars show the standard errors from triplicate experiments (>150 cells in total). w/o: without. w/: with.

To determine the availability of **2** in cells, we conducted immunofluorescence experiments with HeLa-Kyoto cells to visualize the localization of CENP-E and chromosomes. According to our previous report on **1**[6], the proteasome inhibitor MG-132[14] was co-incubated under all conditions to block the onset of anaphase. All chromosomes in mitotic cells were completely aligned at the cell equatorial plane (metaphase plate) with co-localization of CENP-E at their kinetochores in the presence of **2** (100 μM) in 93% or 100% *trans* state with or without 525 nm light illumination, respectively (Figure 4). These results were in accordance with those of dimethyl sulfoxide (DMSO)-treated cells for the control experiment. In contrast, with light illumination at 365 nm providing 95% *cis* and 5% *trans* of **2**, many chromosomes and

CENP-E signals were accumulated around the spindle poles because of CENP-E inhibition mediated by *cis*-**2** (100 μM). Under each condition, the populations of cells containing aligned/misaligned chromosomes were quantified, which revealed that > 90% of cells contained completely aligned chromosomes under the *trans*-rich conditions, whereas > 60% of cells contained misaligned chromosomes in the *cis*-rich condition. Thus, lit-active **2** could regulate the chromosome alignment and CENP-E localization in a photoswitchable manner, but a high concentration (100 μM) of **2** was necessary because of the low affinity with CENP-E. Using **2**, we tried to investigate the applicability to live cell experiments. However, the light stimuli at 365 nm and 525 nm used for the *in situ* photoswitching from *trans* to *cis* and *vice versa* significantly induced cytotoxic effects. Thus, we abandoned further live cell experiments with **2**. Follow-up studies should focus on improving the affinity of the lit-active photoswitchable CENP-E inhibitor.

In conclusion, through this study, we successfully demonstrated a lit-active photoswitchable inhibitor **2** targeting CENP-E. The previous systematic study of the structure-activity relationship on CENP-E inhibitors[12] inspired us to rationally design the lit-active CENP-E inhibitor **2** by changing the substituted position of the pyrazole unit from *para*- into *meta*-position. Using **2** with almost quantitative photoswitchability via light illumination at 365 nm and 525 nm, CENP-E activity was regulated in test tubes and in cells. However, due to the low affinity of CENP-E for **2**, the dynamic control of CENP-E-driven chromosome movements is extremely difficult. We are currently further exploring structure–

activity relationship studies to discover lit-active CENP-E inhibitors with higher affinities.

Conflicts of interest

There are no conflicts to declare.

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