Electronic Supplementary Information

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

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1. General Methods

All commercially available reagents and solvents were used without further purification. Purification of the synthesized compound was carried out by reverse phase high performance liquid chromatography (RP-HPLC) system (Shimadzu). Compound was characterized with ^{1}H-nuclear magnetic resonance (NMR) and ^{13}C-NMR, and mass spectroscopy. ^{1}H NMR spectrum was recorded on a JEOL JNM-ECX 400 spectrometer and all chemical shifts are cited on the δ-scale in ppm relative to the signal of solvent (DMSO-d_6) and coupling constants (*J*) are reported in Hz. Proton-decoupled ^{13}C NMR spectrum was also recorded on a JEOL JNM-ECX 400 spectrometer and all chemical shifts are reported in ppm using solvent as the internal standard (DMSO-d_6). High resolution mass spectrum (HR-MS) was recorded by electrospray ionization (ESI) method using Thermo Scientific Exactive mass spectrometer.

2. Experimental Methods

Photophysical experiments

UV-Vis absorption spectra were measured on a Shimadzu UV-2600 spectrophotometer. The sample in the mixture of acetonitrile and BRB80 buffer (50% : 50%) was prepared (BRB80 : 80 mM PIPES, 1 mM EGTA, 1 mM $MgCl_2$, pH 6.9.). The *cis-trans* photoisomerization of **2** was induced by light illumination at 365 nm (1800 mJ/cm^2), 405 nm (3000 mJ/cm^2), 430 nm (3000 mJ/cm^2), 450 nm (3000 mW/cm^2), 505 nm (3000 mJ/cm^2), 525 nm (1500 mJ/cm^2) using light sources (CL-1503 with CL-H1-365-9-1, CL-H1-405-9-1, CL-H1-430-9-1, CL-H1-450-9-1, CL-H1-505-9-1 and CL-H1-525-7-1, ASAHI SPECTRA).

In vitro ATPase Assay of CENP-E

CENP-E (62.5 ng/mL, Cytoskeleton, Inc.) was added to the assay buffer (2 μM microtubules, 10 μM taxol, 1 mM DTT, 0.2% BSA, 0.01% Brij-35 in BRB80 buffer) in the presence of inhibitor **2** with or without light illumination at 365 nm (1800 mJ/cm^2) and 525 nm (1500 mJ/cm^2) before the ATPase reaction. ATP hydrolysis reactions in 384 well plates

started by the addition of ATP (1.25 μM). The samples were incubated for 60 min at 25 ºC. The chemiluminescent signals in each wells were detected using the plate reader (Infinite 200 Pro M Plex, Tecan).

Immunofluorescence imaging experiments

According to our previously reported method[S1], compound **2** (100 μM) was illuminated with or without light at 365 nm (1800 mJ/cm^2) and 525 nm (1500 mJ/cm^2) before the addition to cells. HeLa-Kyoto cells (1 x 10^5 cells) on a 35 mm glass bottom dish (IWAKI) were incubated in the presence of MG-132 (20 μM) for 2 h. The samples were fixed with the paraformaldehyde method. The chromosomes were stained by 1.0 μg/mL 4′,6-diamidino-2-phenylindole. Microtubules and CENP-E were detected with the anti–α-tubulin antibody (YOL1/34; Abcam) with Alexa Fluor 568-conjugated secondary antibody and the anti-CENP-E antibody (1H12, ab5093, Abcam) with Alexa Fluor 488 secondary antibody. Images were captured with a Ti2 microscope (Nikon) equipped with a Plan Apo VC ×60 objective lens (NA 1.40, Nikon).

Quantification of cells containing aligned/misaligned chromosomes by imaging

Chromosome-stained cells were prepared by the same methods as "**Immunofluorescence imaging experiments**". Fluorescence images were captured with a Ti2 microscope (Nikon) equipped with a Plan Apo VC x20 objective lens (NA 0.75, Nikon).

3. Synthetic procedures

Synthesis of 2

Sodium nitrite (70 mg, 1.0 mmol) in water (1.0 mL) was added to the solution of **3**[S1] (280 mg, 0.72 mmol) in AcOH (3 mL) and 12N HCl (1 mL) over 1 hour at 0 °C. To this solution, acetylacetone (200 μL, 1.9 mmol) in EtOH (2 mL) and sodium acetate (500 mg, 6.1 mmol) was added. The reaction mixture was stirred for 2 h at room temperature. AcOEt and brine was poured into this solution. The organic phase was extracted, washed with sat. $NaHCO_3$aq and brine, dried over $MgSO_4$, and filtered. The resulting solution was dried *in vacuo*. The crude product was used for the next reaction without further purification.

The crude mixture was added to methylhydrazine (106 μL, 2.0 mmol) in EtOH (5 mL). After the reflux for 4 h, the solution was evaporated *in vacuo*. It was purified with reverse

phase HPLC to afford compound **2** (127 mg, 37% over two steps) as a yellow solid.

^{1}H-NMR (400 MHz, DMSO-d_6): 8.65 (d, J = 8.4 Hz, 1H), 8.06 (q, J = 4.4 Hz, 1H), 7.92 (d, J = 2.4 Hz, 1H), 7.75 (dd, J = 8.4, 2.4 Hz, 1H), 7.72-7.71 (m, 1H), 7.53-7.50 (m, 1H), 7.37-7.34 (m, 2H), 7.17 (d, J = 8.8 Hz, 1H), 4.74 (septet, J = 6.4 Hz, 1H), 4.67-4.63 (m, 1H), 3.73 (s, 3H), 3.190-3.02 (m, 2H), 2.62 (d, J = 3.2 Hz, 3H), 2.53 (s, 3H), 2.34 (s, 3H).

^{13}C-NMR (100 MHz, DMSO-d_6):δ 172.11, 165.13, 155.72, 153.41, 140.71, 140.29, 140.09, 134.87, 130.86, 129.83, 129.31, 128.52, 127.31, 122.90, 122.21, 119.85, 114.72, 71.73, 55.42, 37.65, 36.47, 26.19, 22.15, 14.28, 9.94.

HR-MS (ESI) for $[M + H]^+$ calcd. 511.2219, found 511.2215.

4. Results

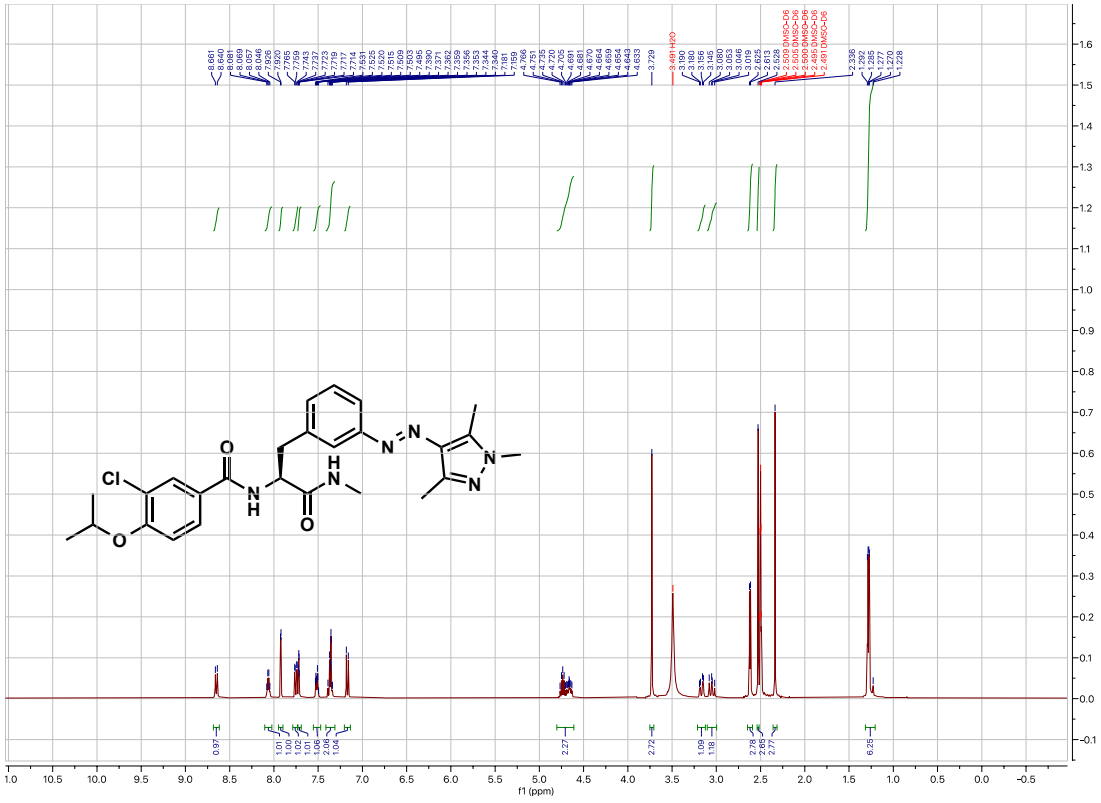

Figure S1. ^{1}H-NMR of **2**.

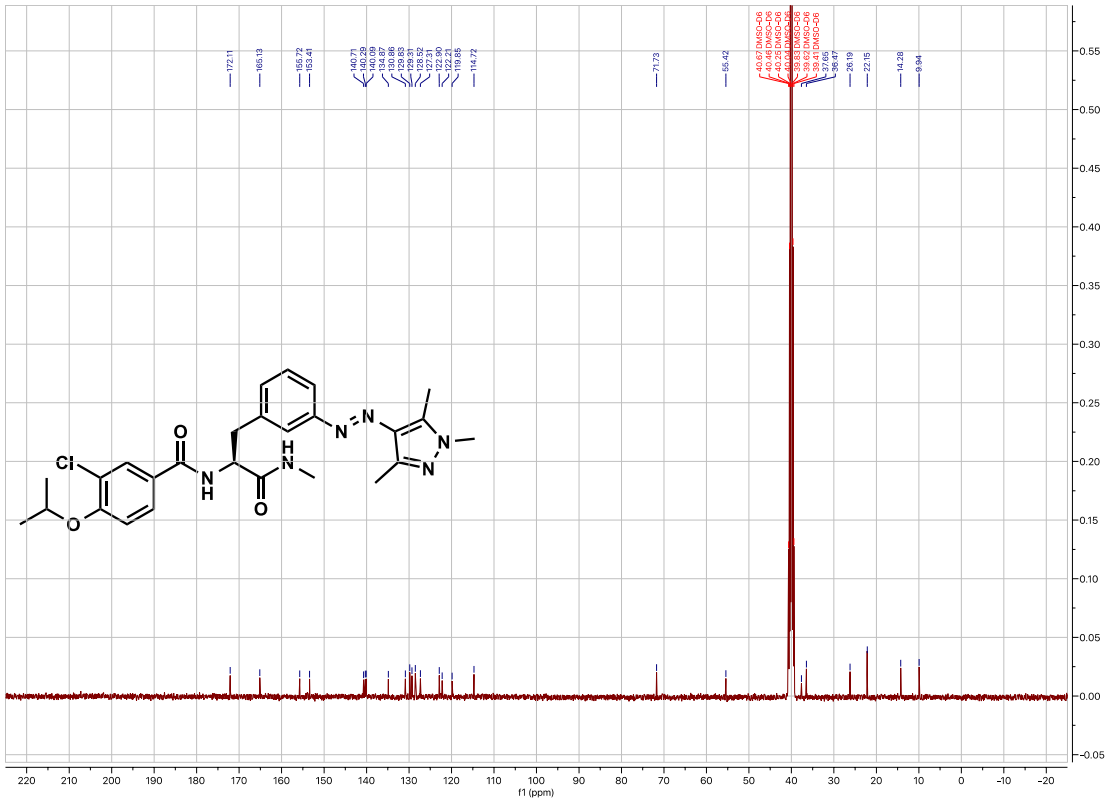

Figure S2. ^{13}C-NMR of **2**.

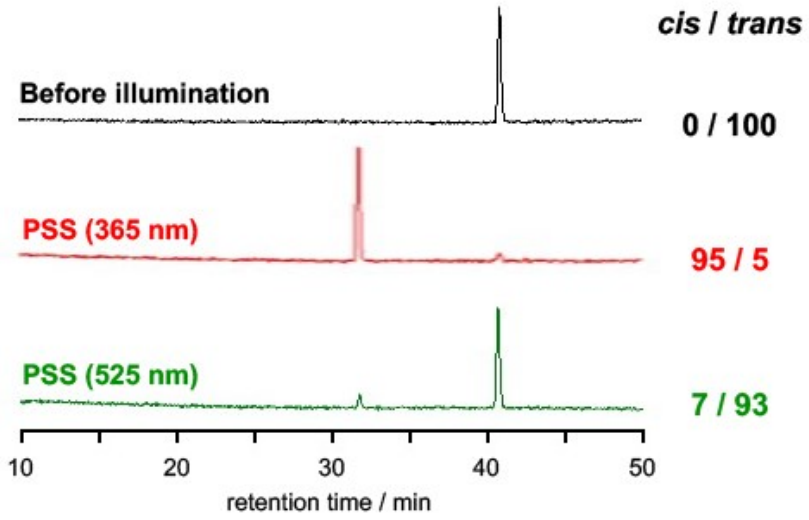

Figure S3. HPLC analyses of *trans/cis* ratios of **2** (20 μM) in BRB80 buffer with/without light stimuli. Retention time : 32 min for *cis* isomer and 41 min for *trans* isomer. HPLC condition : CH_3CN / water = 20 / 80 to 70 / 30 for 50 min. Light condition : 1800 mJ/cm^2 of 365 nm light and 1500 mJ/cm^2 of 525 nm light. Detection at 293 nm of an isosbestic point.

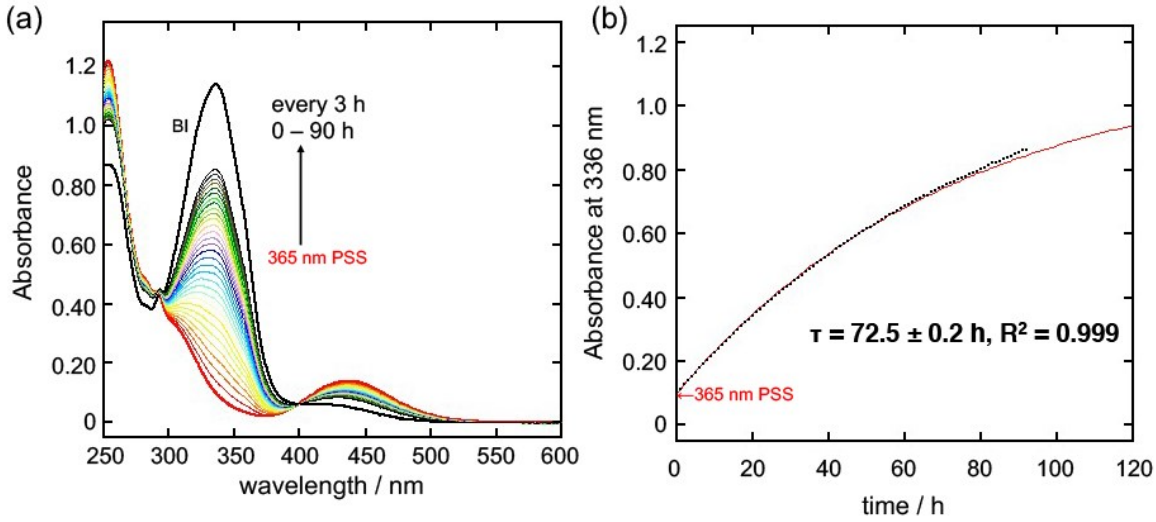

Figure S4. Thermal back reaction of *cis*-**2** (50 μM) in aqueous solution (acetonitrile : BRB80 buffer = 1 : 1) at 37 ºC. (a) Time-dependent changes of absorption spectra with interval of 3 hours for 90 h from the PSS_{365nm}. (b) The plots of absorbance at 336 nm.

The fitting equation was $Abs_{BI} - (Abs_{BI} - Abs_{PSS365\ nm}) \times \exp(-t/\tau)$, where Abs_{BI} is the absorbance before light illumination, $Abs_{PSS365\ nm}$ is the absorbance at photostationary state with 365 nm light, τ is the lifetime of *cis* isomer and t is time (hour).

5. Reference

S1. Mafy, N. N.; Matsuo, K.; Hiruma, S.; Uehara, R.; Tamaoki, N. Photoswitchable CENP-E Inhibitor Enabling the Dynamic Control of Chromosome Movement and Mitotic Progression. *J. Am. Chem. Soc.* **2020**, *142*, 1763– 1767.