Target identification of a macrocyclic hexaoxazole G-quadruplex ligand using a post-target-binding visualization

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Materials

1a, **1b**, and CO-1 were synthesized as previously reported.[1] All dual-labeled oligonucleotides were purchased from Sigma-Aldrich (HPLC purification grade) and used without further purification. Turbo™ DNase (AM2239) and ribonuclease (RNase) A (R6513) were purchased from Thermo Fischer Scientific and Sigma-Aldrich, respectively. Phen-DC3 was purchased from Polysciences (26000-5). Thiazole Orange was purchased from Sigma-Aldrich (390062). All other reagents were obtained in the molecular biology grades from Sigma-Aldrich. Fluorescent images were acquired on an IN Cell Analyzer 6000 (Cytiva). Image analysis was performed using ImageJ 1.47c (National Institutes of Health, USA).

Table S1. Sequences of Oligonucleotides used in this paper

Oligonucleotide	Sequence
Flu-ss-telo21	5'-FAM-d[GGG TTA GGG TTA GGG TTA GGG]-TAMRA-3'
Flu-ss-TERRA	5'-FAM-[GGG UUA GGG UUA GGG UUA GGG]-TAMRA-3'
Flu-ss-dsDNA	5'-FAM-d[TAT AGC TAT ATT TTT TTA TAG CTA TA]-TAMRA-3'
telo24	5'-d[TTA GGG TTA GGG TTA GGG TTA GGG]-3'
stem26	5'-d[TAT AGC TAT ATT TTT TTA TAG CTA TA]-3'

Experimental procedure.

1. FRET melting analysis

Fluorescence resonance energy transfer (FRET) melting assay was carried out according to reported methods.[2] It was performed with an excitation wavelength of 470-505 nm and a detection wavelength of 523-543 nm and use of the Thermal Cycler Dice® Real Time System III (Takara-Bio). The dual fluorescently labeled oligonucleotides (Table S1) were used in this protocol. The donor fluorophore was 6-carboxyfluorescein (FAM) and the acceptor fluorophore was 6-carbpxytetramethylrhodamine (TAMRA). All purified nucleotides were dissolved as stock solution (100 mM) in MilliQ water to be used without further purification. Further dilutions of the oligonucleotides were performed with potassium cacodylate buffer (pH 7.4, 60 mM), and FRET experiments were carried out with oligonucleotides solutions (400 nM). Dual-labeled DNA or RNA was annealed by heating 99 °C for 5 min and was allowed to cool to room temperature. Original compounds were prepared as DMSO stock solutions (10 mM) and diluted to 200 μM with DMSO.

The rest of the dilutions were performed with the potassium cacodylate buffer. FRET experiments were carried out with a 2.0 μM starting solution of compound. Next, the annealed DNA (20 μL) and the compound solution (20 μL) were aliquoted into 8 strips PCR tubes, with a total reaction volume of 40 μL, with labeled oligonucleotide (200 nM) and the compound (1.0 μM) in the presence or absence of double-stranded DNA (stem26) (20 μM). The plates were incubated at 25 °C for overnight. Subsequent experiments used the following temperature procedure in real-time PCR, finishing as follows: 25 °C for 30 min, then a stepwise increase of 1.0 °C every minute from 25 °C until 99 °C. During the procedures, the FAM fluorescence was recorded after each step. The change in melting temperature at 1.0 μM compound concentration – ΔT_m – was calculated from at least three experiments by subtraction of the blank from the averaged melting temperature of each compound (Table 1).

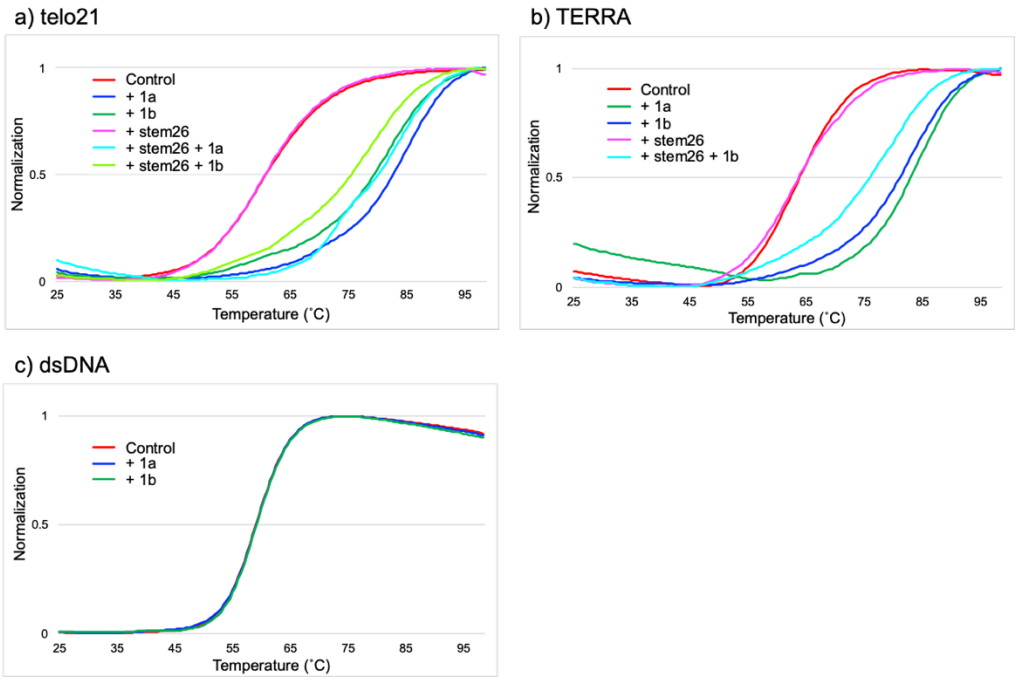

Figure S1. FRET melting assay. FAM intensities of dual-labelled (a) telo21, (b) TERRA, and (c) dsDNA in the presence or absence of **1a**, **1b**, and stem26 were recorded and normalized, respectively.

2. G4 displacement analysis

G4 displacement analyses were carried out according to reported methods with a) Thiazole orange[3a,b] and b) Thioflavin T[3c]. Prefolded telo24 and thiazole orange were mixed in 160 µL sodium cacodylate buffer (10 mM, pH 7.3, 100 mM KCl), before being incubated with G4 ligand for 3 min at room temperature. Subsequently, the fluorescence spectrum was recorded (excitation: 485 nm, fluorescence area: 501 nm). Thioflavin T (ThT)-displacement (G4-FID) analysis was carried out. Prefolded telo24 and Thioflavin T were mixed in 160 µL Tris-HCl buffer (50 mM, pH 7.4, 100 mM KCl), before being incubated with G4 ligand for 3 min at room temperature. Subsequently, the fluorescence spectrum was recorded (excitation: 425 nm, fluorescence area: 490 nm).
Fluorescent intensities were read by a Microplate Reader (SH-9000Lab, CORONA ELECTRIC Co., Ltd.).

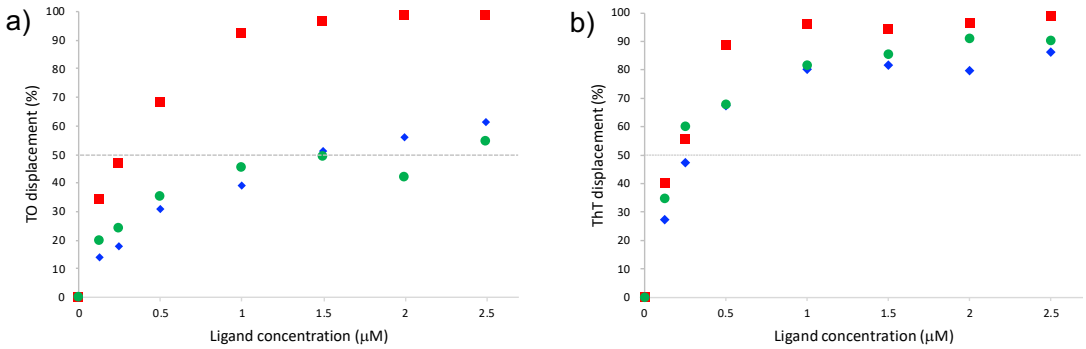

Figure S2. G4 displacement assay. a) Thiazole orange on telo24 and b) ThT displacement on TERRA ratio was plotted in the presence of 0–2.5 µM of Phen-DC3 (red), **1a** (blue), and **1b** (green).

3. HPLC analysis

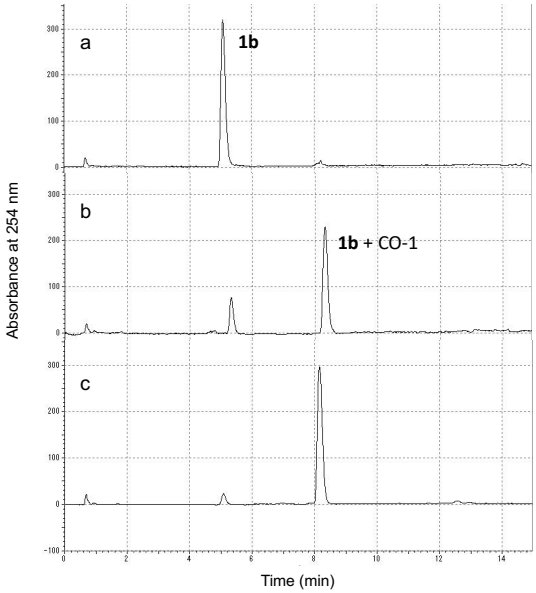

Figure S3. Reverse phase HPLC analysis of the click reaction between **1b** and CO-1. **1b** (100 μM) and CO-1 (500 μM) was mixed in MeCN at 25 ºC and the reaction was monitored at 254 nm absorbance at each time point (a) 0 min, (b) 2 hours (80%), (c) 4 hours (93%) to measure the conversion yield. The peak of **1b** and CO-1 conjugation was characterized by HRMS (ESI, M+Na) calculated for $C_{61}H_{69}BF_2N_{16}O_{11}Na$ 1273.5291, found 1273.5296). The mobile phase condition is 30–60% MeCN, 0.1% TFA for 10 min (50×4.6 mm YMC-Triart C18). CO-1 was not eluted by this method due to its hydrophobicity.

4. Growth-inhibitory activities of 6OTDs toward cancer cells

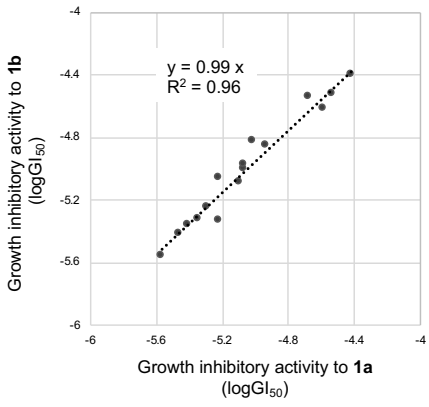

Figure S4. Correlation between the growth-inhibitory activities of **1a** and **1b** and 16 cancer cell lines (shown in Figure 2).[4] The black dashed line shows the linear regression (y = 0.99 x) for the 16 data points.

5. *Cell culture*

For all reported experiments, human osteosarcoma U2OS cells were cultured at 37 °C, 5% CO_2 in DMEM (nacalai tesque) containing 4.5 g/L glucose, 10% heat-inactivated fetal bovine serum (FBS) (Thermo Fischer Scientific), 0.01% kanamycin (Meiji Seika). Cells were grown to up to 80% confluency and passaged every 2 to 4 days using Trypsin-EDTA solution (nacalai tesque).

6. *Evaluation of cytotoxic effects of 6OTDs and Phen-DC3 on U2OS cells*

Cells (200 in 80 μ L of culture medium) were seeded into each well of a 96-well microplate and incubated for 16 h. These cells were treated with 0.1-10 μ M of **1a**, **1b**, Phen-DC3 and etoposide for 96 hours. The number of cells in each well was quantitated by using a CellTiter-Glo Luminescent Cell Viability Assay reagent (Promega) with a GENios microplate reader (Tecan).

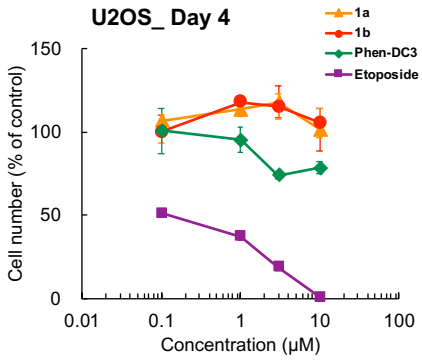

Figure S5. Effects of **1a**, **1b** and Phen-DC3 on U2OS cell growth. Cells were incubated in culture medium with various concentrations of compounds, **1a**, **1b** and Phen-DC3 for 96 hours. Then, the relative cell number was quantitated by a CellTiter-Glo Luminescent Cell Viability Assay reagent. Cells receiving only DMSO were used to define 100%. Etoposide was used as a positive control.

7. *In situ click staining with 1b and CO-1*

G4 imaging in fixed U2OS cells. Cells (0.6×10^5/ well) in 10% FBS medium were grown on coverslips in a 6-well plate and fixed with ice-cold MeOH for 10 min at -20 °C. Coverslips were washed with (-)-phosphate-buffered saline (PBS) 3 times, and incubated with 0.2 U/μL Turbo^{TM} DNase or 1 mg/mL RNase A for 2 h at 37 °C. Nuclease-digested cells were washed with Tris-EDTA buffer (3 times) and then treated with **1b** (10 μM) for 1 h at room temperature. After labelling with **1b**, cells were treated with 1 mM iodoacetaminde and labelled with 1 μM CO-1 at room temperature under shade conditions. DNA was stained with 4′,6-diamidino-2-phenylindole (DAPI). Microscopic analyses were performed

with the IN Cell Analyzer 6000.

G4 imaging in living U2OS cells. Cells (4.0×10^3/80 μL) in 10% FBS medium were seeded in 96-well microplates, cultured overnight and then treated with 10 μM **1b**, in culture media for 24 h at 37 °C. After incubation, cells were washed and added with 3.0 μM CO-1 probe. Labelling was allowed to proceed for 1 h at 37 °C in the incubator chamber. Following incubation, cells were treated with Hoechst 33342 (5 μg/mL) for nuclear staining. Microscopic analyses were performed with the IN Cell Analyzer 6000.

8. Phen-DC3 treatment before in situ click staining of the fixed U2OS cells

Cells (1.0×10^5/ well) in 10% FBS medium were grown on coverslips in a 6-well plate and fixed with ice-cold MeOH for 10 min at -20 °C. Coverslips were washed with PBS(-) (3 times), and incubated with Phen-DC3 (0, 10, 20 and 100 μM) for 1 h at room temperature. Following Phen-DC3 binding, coverslips were washed with Tris-EDTA buffer (3 times), and cells were incubated with 10 μM **1b** for 1 h at room temperature. Then, cells were treated with 1 mM iodoacetaminde and labelled with 1 μM CO-1 at room temperature under shade conditions. DNA was stained with DAPI. Microscopic analyses were performed with the IN Cell Analyzer 6000.

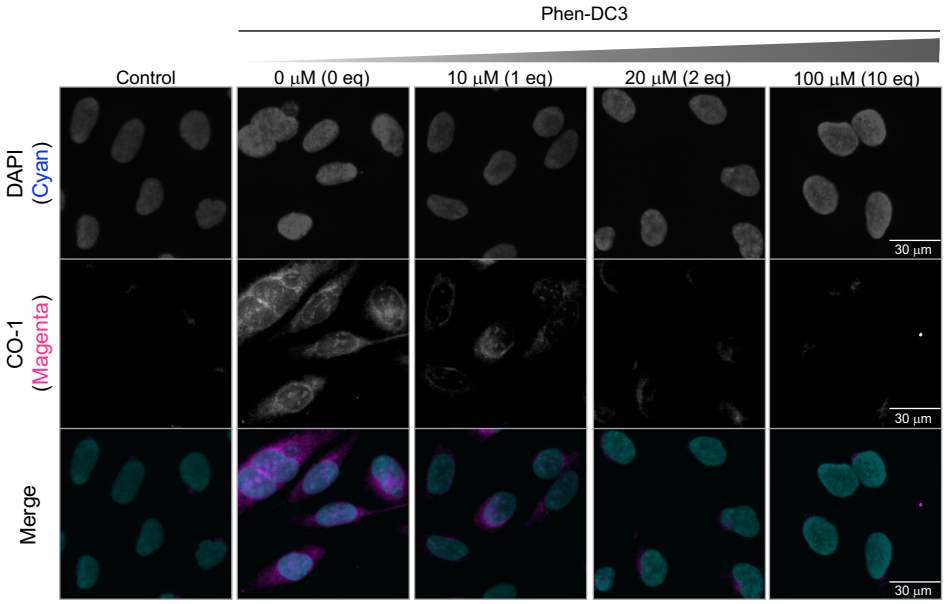

Figure S6. Phen-DC3 treatment (0–100 μM) before *in situ* click staining by **1b** and CO-1 in the fixed U2OS cells.

9. Phen-DC3 treatment after in situ click staining of the fixed U2OS cells

Cells (1.0×10^5/ well) in 10% FBS medium were grown on coverslips for 12 h and fixed with ice-cold MeOH for 10 min at -20 °C. Coverslips were washed with PBS(-) (3 times), and incubated with 10 μM **1b** for 1 h at room temperature. Following **1b** binding, coverslips were washed with Tris-EDTA buffer (3 times), and cells were treated with 1 mM iodoacetaminde and labelled with 1 μM CO-1 at room temperature under shade conditions. Then, the cells were incubated with Phen-DC3 (0, 10 and 50 μM) for 1 h at room temperature, and nuclei were stained with DAPI. Microscopic analyses were performed with the IN Cell Analyzer 6000.

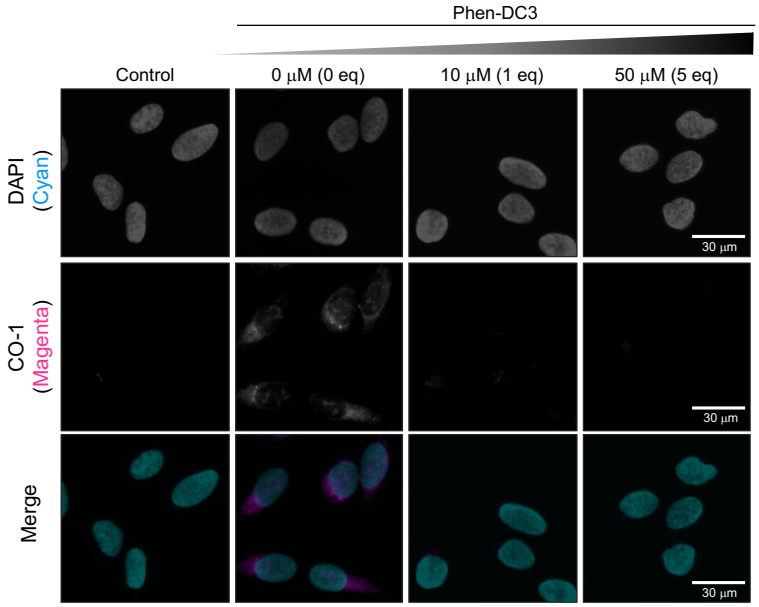

Figure S7. Phen-DC3 treatment (0–50 μM) after *in situ* click staining by **1b** and CO-1 in the fixed U2OS cells.

10. Phen-DC3 treatment before in situ click staining in the living U2OS cells

Cells (1.6×10^4/ well) in 10% FBS-DMEM (Kanamycin 0.1 mg/mL) medium were grown on 35 mm PLL-coated glass bottom dishes for 48 hours and then treated with 10 μM **1b**, in culture media for 3.5 h at 37 °C. Cells were washed with 10% FBS-DMEM (twice), and treated with Phen-DC3 (0, 10, or 100 μM) for 3.5 h at 37 °C. Following Phen-DC3 binding, cells were washed with 10% FBS-DMEM (twice), incubated with 600 nM CO-1. Labelling was allowed to proceed for 1 h at 37 °C in the incubator chamber. Following incubation, cells were treated with Hoechst 33342 (5 μg/mL) for nuclear staining. Cells were rinsed with 3 mL medium and 1 mL 1×Opti-Klear, and added to 1 mL 1×Opti-Klear. Microscopic analyses were performed with the fluorescence microscope (OLYMPUS).

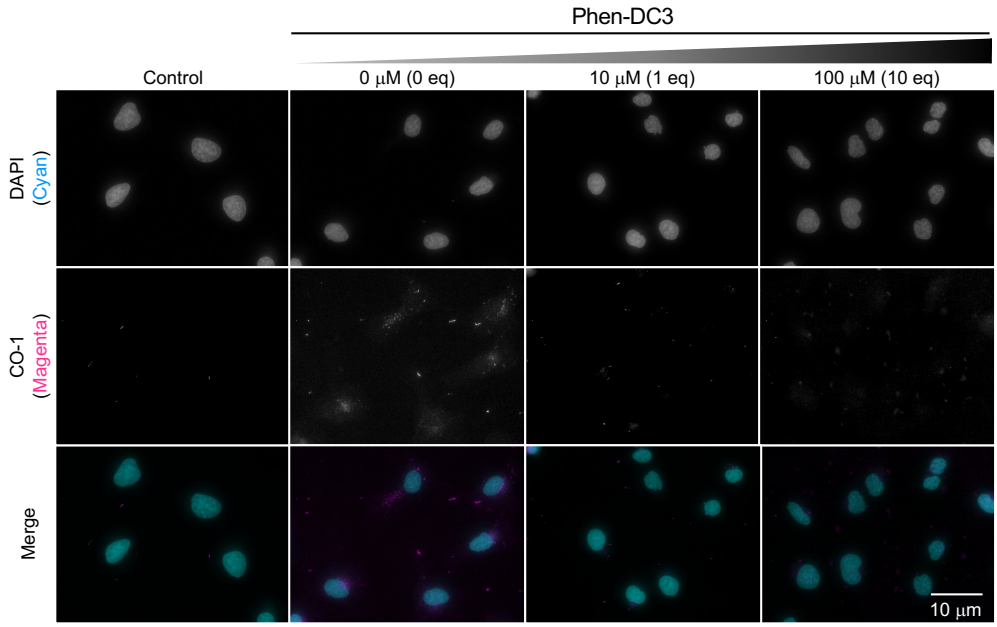

Figure S8. Phen-DC3 treatment (0–100 μM) before *in situ* click staining by **1b** and CO-1 in the living U2OS cells.

11. Supplementary References

1. J. A. Punnoose, Y. Ma, M. E. Hoque, Y. X. Cui, S. Sasaki, A. H. Guo, K. Nagasawa and H. B. Mao, *Biochemistry*, 2018, **57**, 6946-6955.
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3. (a) E. Largy, F. Hamon and M. P. Teulade-Fichou, *Anal. Bioanal. Chem.*, 2011, **400**, 3419-3427; (b) D. Monchaud, C. Allain and M. P. Teulade-Fichou, *Bioorg. Med. Chem. Lett.*, 2006, **16**, 4842-4845; (c) Shujuan Xu, Qian Li, Junfeng Xiang, Qianfan Yang, Hongxia Sun, Aijiao Guan, Lixia Wang, Yan Liu, Lijia Yu, Yunhua Shi, Hongbo Chen & Yalin Tang, *Scientific Reports*, 2016, **6**, 24793.
4. T. Yamori, *Cancer Chemother. Pharmacol.*, 2003, **52**, S74-S79.