Electronic Supplementary Material (ESI) for ChemCommn.

Supporting Information

Simple and fast screening for structure-selective G-quadruplex ligands

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Table Contents

Experimental Procedures

Materials

All oligonucleotides were purchased from Sigma-Aldrich Co. LLC (St. Louis, Missouri, U.S.A.). ThT was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan) and used without further purification. In-house library compounds were purchased from Wako Pure Chemical Industries Ltd. and Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). 22AG is an oligonucleotide derived from the human telomeric repeat: [5'-A(GGGTTA)3GGG-3'] (ε_{260} = 228,500 M^{-1} cm^{-1}). Extinction coefficients for single-strand DNA were calculated from mono and dinucleotide data using the nearest-neighbor approximation model[1]. The stock solutions of oligonucleotides were stored at -20 ˚C. Single-strand concentrations of the oligonucleotides were determined by measuring the absorbance at 260 nm at 90 ˚C.

The stock solution of ThT (100 μM in Milli-Q) was stored at 4 ˚C in the dark. ThT concentration was determined by measuring the absorbance at 412 nm (extinction coefficient ε = 32,000 M^{-1} cm^{-1})[2]. Before being used, the oligonucleotides in a buffer consisting of 50 mM 2-(*N*-morpholino) ethanesulfonate (MES)-LiOH (pH 7.0) and 100 mM KCl were heated at 90 ˚C for 5 min, gently cooled at -0.5 ˚C min^{-1}, and incubated at 25 ˚C.

Fluorescence spectroscopy

Fluorescence spectra of ThT were measured using a Varioskan LUX (Thermo Fisher Scientific K. K., Waltham, Massachusetts, U.S.A.) with a 96-well plate (Sumitomo Bakelite Co., Ltd., Tokyo, Japan). The fluorescence spectra were measured from 470 nm to 600 nm. All experiments were performed in buffer consisting of 100 mM KCl and 50 mM MES-LiOH (pH 7.0) at 25 ˚C. The excitation wavelength was 450 nm. The oligonucleotides were titrated into 20 μM ThT. The values of fluorescence intensity at 485 nm (F_{485}) were plotted against the concentration of the oligonucleotide, and were fitted with the following equation using KaleidaGraph (Synergy Software, U.S.A.) to evaluate the dissociation constants of ThT ($K_{\text{d-ThT}}$) for 22AG:

$$F_{485} = a\frac{([\text{DNA}] + [\text{ThT}] + K_{\text{d-ThT}}) - \sqrt{([\text{DNA}] + [\text{ThT}] + K_{\text{d-ThT}})^2 - 4[\text{DNA}][\text{ThT}]}}{2[\text{ThT}]} + b$$

where a is the scaling factor and b is the initial fluorescence intensity at 485 nm.

ThT-displacement assay

Each experiment was performed in a 96-well plate in a buffer consisting of 50 mM LiOH-MES and 100 mM KCl (pH 7.0). The total volume was 60 μL. The ThT displacement assay was conducted as follows: 5 μM 22AG was mixed with 2 mM (as nucleotide concentration) CT-DNA and 20 μM ThT. The addition of each 20 μM ligand was followed by a 24-hour equilibration period, after which the fluorescence spectrum of ThT was recorded. The K_i values of the ligands were calculated with the following equation:

$$\text{I}_{\text{Affinity}} = 1 - \frac{[\text{Ligand}]}{[\text{Ligand}] + K_i\left(1 + \frac{[\text{ThT}]}{K_{\text{d-ThT}}}\right)}$$

where $K_{\text{d-ThT}}$ is the dissociation constant between 22AG and ThT.

T7 RNA polymerase stop assay

Each transcription reaction solution contained 2 μM DNA template, 1 mM KCl, 40 mM Tris-HCl (pH 7.2), and 8 mM $MgCl_2$. Note that this KCl concentration was used to reduce the thermal stability of G4. The samples were heated to 93 ˚C for 5 min, then cooled -0.5 ˚C min^{-1}. After annealing, NTP and DTT were added to a final concentration of 1 mM and 5 mM, respectively. T7 RNA polymerase (100 units, Takara Bio, Inc., Shiga, Japan) was added to the reaction buffer to start the reaction. The final reaction buffer contained 1 mM KCl, 40 mM Tris-HCl (pH 7.2), 8 mM $MgCl_2$, and 5 mM DTT. Each mixture was incubated at 37 ˚C for 120 min for the reaction. Reactions were quenched by incubation

with 10 units of DNase I (Takara Bio, Inc.) for 20 min, then a 10-fold excess volume of transcription stop solution (80 wt% formamide, 10 mM Na_2EDTA, and 0.01% blue dextran) was added. Each sample was cooled rapidly after heating to 93 ˚C for 5 min. The samples were loaded onto a 10% polyacrylamide and 7 M urea gel and run at 60 ˚C. After electrophoresis, the gels were stained with SYBR Gold (Thermo Fisher Scientific, Inc., Tokyo, Japan) and fluorescent bands were imaged by FLA-7000 (Fujifilm, Tokyo, Japan). Band intensities were quantified using ImageJ software distributed by the National Institutes of Health, U.S.A.

Circular dichroism spectroscopy

CD spectra of DNA oligonucleotides were measured using a J-820 spectropolarimeter (JASCO Co., Ltd., Tokyo, Japan) at 5 μM 22AG concentration in 100 mM KCl and 50 mM MES-LiOH (pH 7.0) buffer. The samples were heated to 90 ˚C, and then cooled at a rate of -0.5 ˚C min^{-1}. Before measurement, we added 50 μM ligands to the samples and incubated at 25℃ for 1 hour. The spectra at 25 ˚C were obtained using at least three scans between 200 to 350 nm in a cuvette with a path length of 0.1 cm. For CD melting experiments, samples were heated from 25 ˚C to 90 ˚C at a rate of 0.5 ˚C min^{-1}. Thermal denaturing behavior was traced at 295 nm.

Cell culture

Human cervical cancer HeLa cells obtained from the American Type Culture Collection were cultured in Dulbecco's modified Eagle's medium (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. All culture incubations were performed in a humidified 5% CO_2 incubator at 37 ˚C.

Cytotoxicity assay

HeLa cells on 100 mm plastic culture dish (~80% confluence) were trypsinized using 0.1 % (w/v) trypsin (Wako Pure Chemical Co., Ltd.) and then seeded into a 12-well plate (5×10^4 cells in 1 ml). After incubation for overnight, the cells were treated with 1, 3, 10, 30, or 100 μM of PDS, TMPyP4, MV, CV, or EV for 48 hours. The number of viable cells was determined by trypan blue exclusion-based cell staining using trypan blue (Sigma-Aldrich Co. LLC.) with a LUNA cell counter (Shoshin EM Corp, Okazaki, Japan). The number of the treated cells was normalized to that of nontreated cells. From plots of cell viability versus various ligand concentration, IC_{50} values were calculated with the following equation:

$$\text{Cell viability} = 1 - \frac{[\text{Ligand}]}{IC_{50} + [\text{Ligand}]}$$

Table S1. K_i values of compounds with 22AG in the absence or presence of excess DNA duplex at 25 ˚C.

	K_i (μM)		$I_{Selectivity}$[a]
	w/o duplex	w/ duplex	
Hemin	2.2 ± 0.2	4.0 ± 0.3	0.55 ± 0.03
Congo red	3.6 ± 0.2	4.3 ± 0.1	0.83 ± 0.05
Ethyl violet	3.0 ± 0.1	5.3 ± 0.2	0.56 ± 0.01
Crystal violet	1.8 ± 0.1	5.6 ± 0.1	0.32 ± 0.01
Methyl violet	1.0 ± 0.1	7.8 ± 0.2	0.13 ± 0.03

[a] $I_{Selectivity}$ = (K_i with excess DNA duplex) / (K_i without excess DNA duplex)

123 Table S2. DNA sequences used in T7 RNA polymerase stop assay.

Abbreviation	Sequences (from 5’ to 3’)
22AG template[a]	GCCGTTTCGTAGTAT**AGGGTTAGGGTTAGGGTTAGGG**CAGA GAGAGCACCGAGCCTAGTTCGTGTCATCTCCTATAGTGAGTCG TATTAGTGATC
mut22AG template[a]	GCCGTTTCGTAGTAT**AGTGTGAGTGTGGAGTGTGGAG**CAGA GAGAGCACCGAGCCTAGTTCGTGTCATCTCCTATAGTGAGTCG TATTAGTGATC
dsDNA	GCAATATTGC

124 [a] Bold letters indicate G4 forming region in 22AG template and the corresponding mutated region for mut22AG template.

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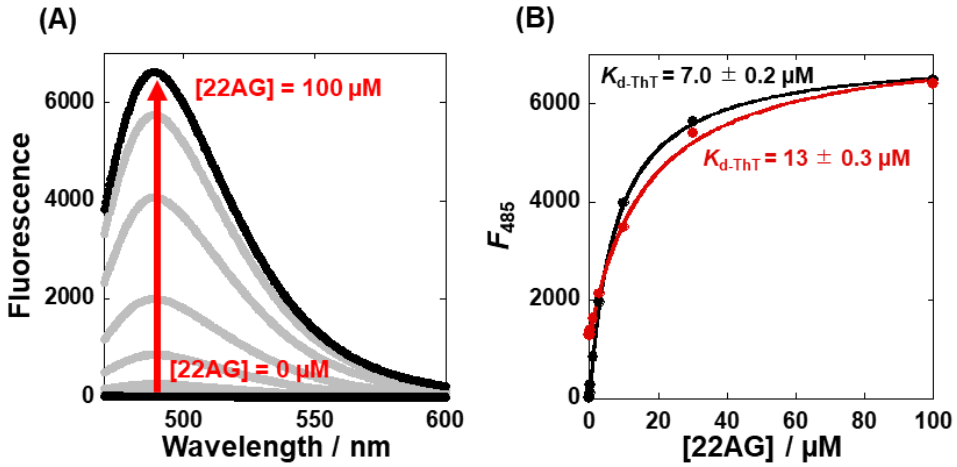


Figure S1. (A) Fluorescence spectra of 1 µM ThT with 0 to 100 µM of 22AG in the KCl buffer at 25 ˚C. Ex: 450 nm. (B) Plots of fluorescence intensity at 485 nm of 1 µM ThT (F_{485}) as a function of [22AG] in the absence (black) or presence (red) of 2 mM CT-DNA.

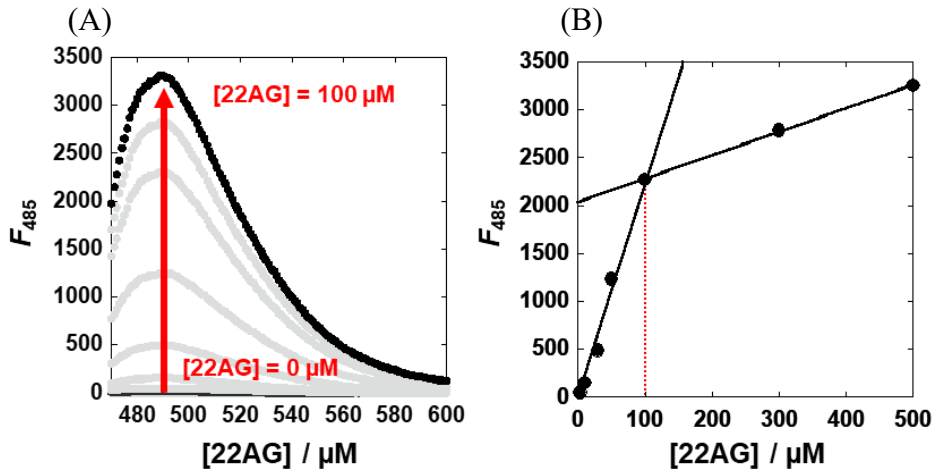
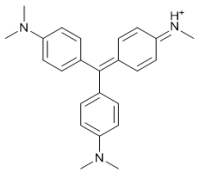
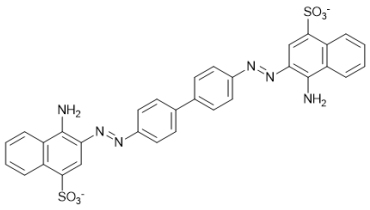


Figure S2. (A) Fluorescence spectra of 100 µM ThT with 0 to 500 µM of 22AG in the KCl buffer at 25 ˚C. Ex: 450 nm. (B) Plots of fluorescence intensity at 485 nm of 100 µM ThT (F_{485}) as a function of [22AG], showing a clear bending point at 100 µM 22AG.

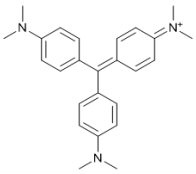
1. Methyl violet



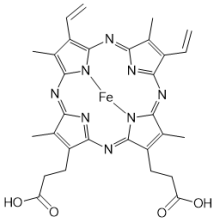
2. Congo red



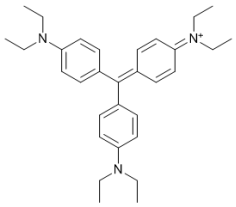
3. Crystal violet



4. Hemin



5. Ethyl violet



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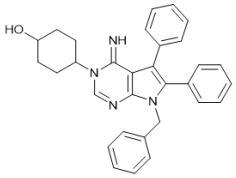
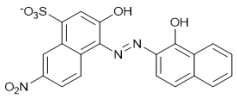


7. Eriochrome black T



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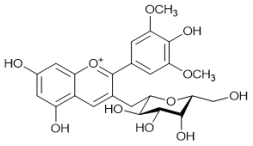
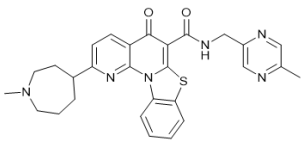
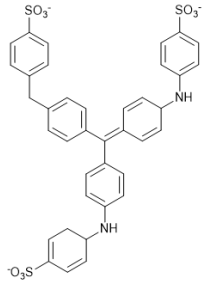


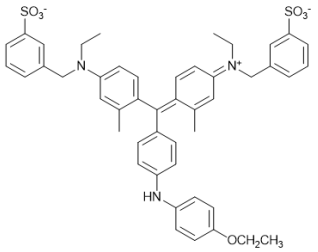
9. CX-5461



10. Methyl blue



11. Coomassie brilliant blue G-250



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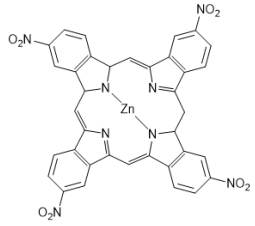
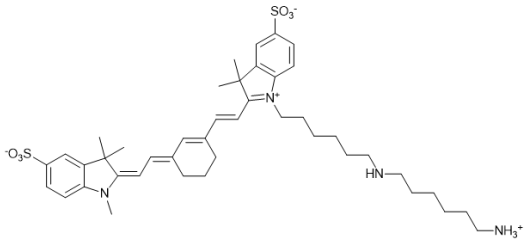
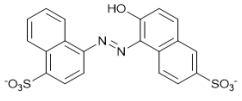


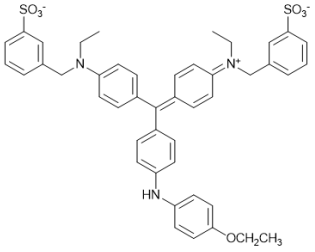
13. Sulfo-Cy7-amine



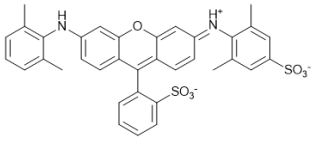
14. Acid red 13



15. Coomassie brilliant blue R-250



16. Acid red 289



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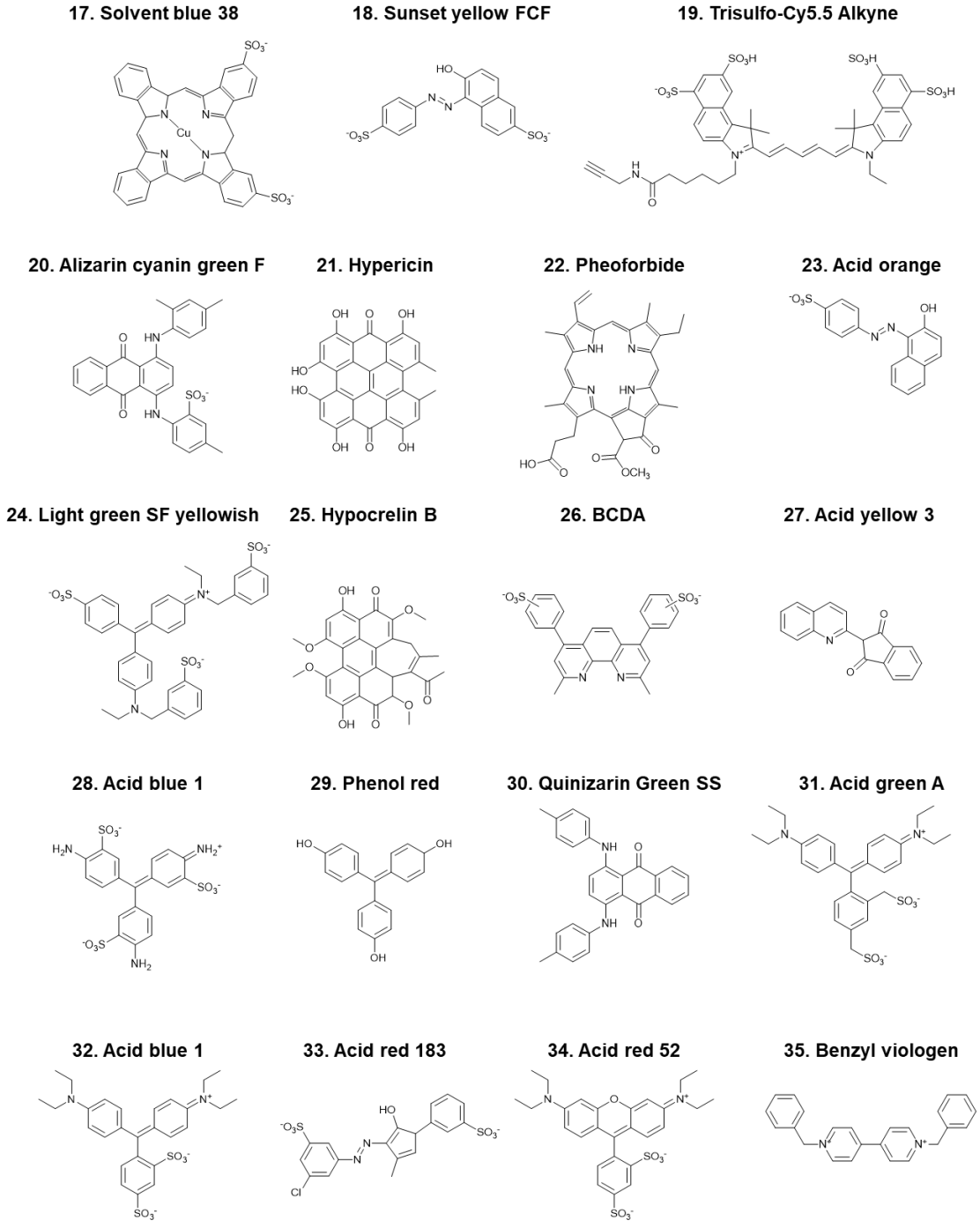
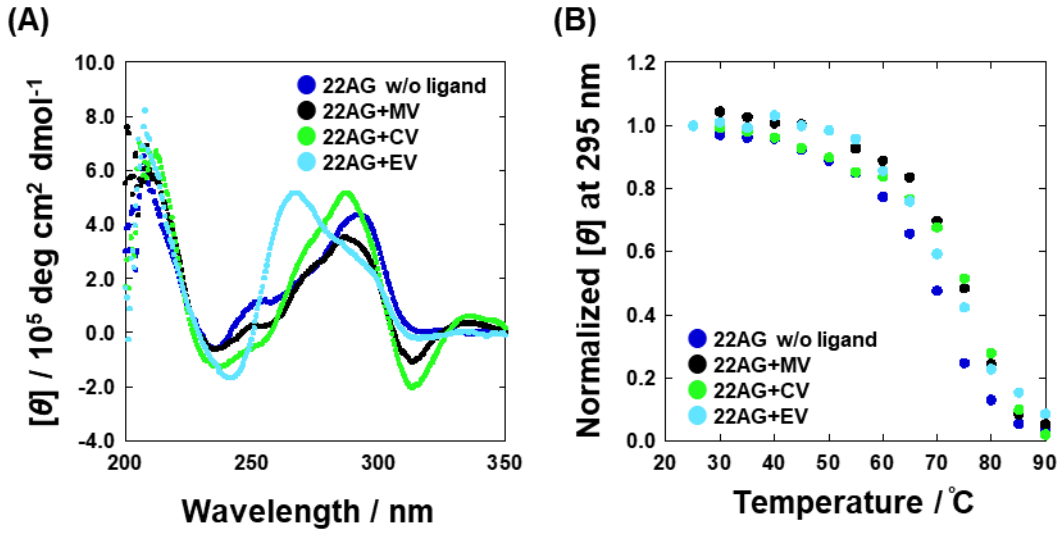


Figure S3. The chemical structure of the compounds in the in-house library. Compounds are listed in the order of the $I_{Affinity}$ value in the absence of excess DNA duplex.



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145 Figure S4. (A) CD spectra of 5 μM 22AG in the absence of G4 ligand and in the presence of 50 μM MV, CV, or EV at 25 ℃ in the
146 KCl buffer. All CD spectra show two positive peaks around 260 and 295 nm, indicating a mixed G4 in the experimental conditions,
147 although a local conformation is altered by the G4 ligands. (B) Normalized CD intensity at 295 nm as a function of temperature for 5
148 μM 22AG in the presence of MV, CV, or EV in the KCl buffer. The melting temperatures were 68, 74, 73, and 72 ℃ in the absence
149 or presence of MV, CV, and EV, respectively.

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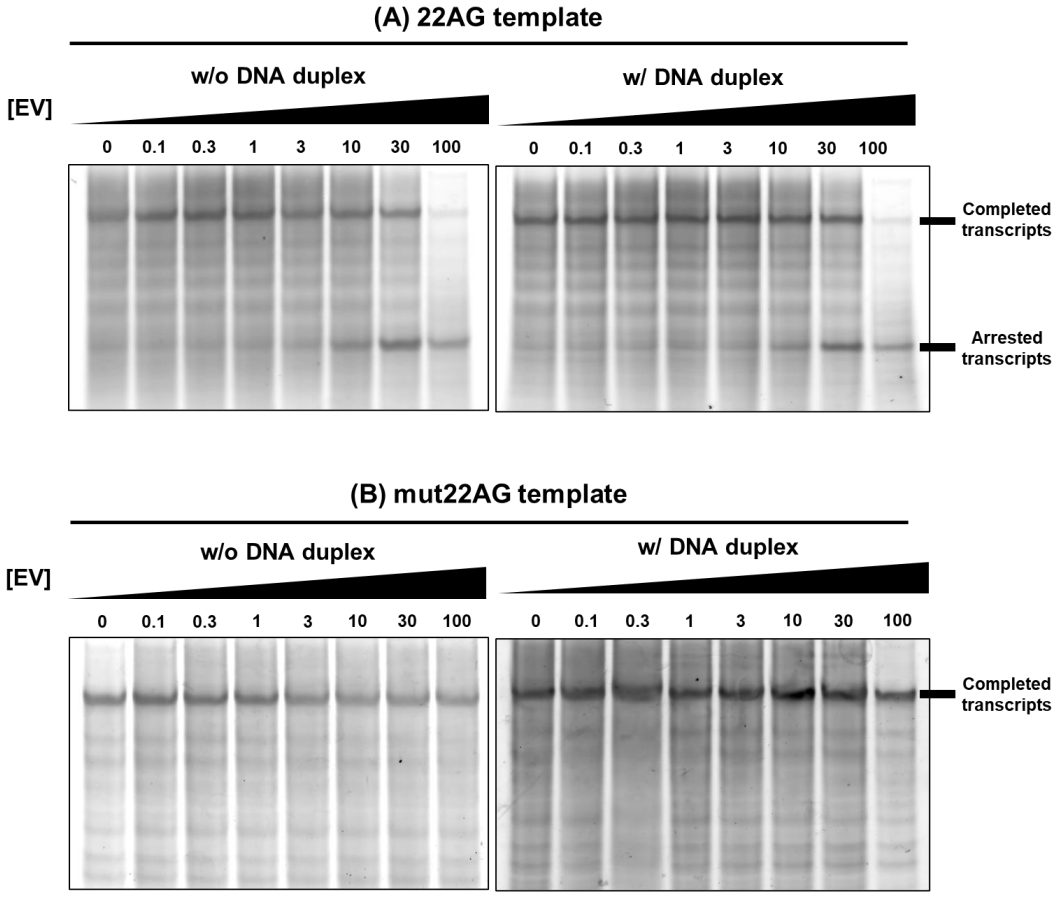

Figure S5. Denatured 10% polyacrylamide gel electrophoresis of the transcripts from 22AG template (A) or mut22AG template (B) with 0, 0.1, 0.3, 1, 3, 10, 30, or 100 μM EV in the absence (left) or presence (right) of 100 μM dsDNA. The transcription reactions were carried out in buffer consisting of 1 mM KCl, 40 mM Tris-HCl (pH 7.2) for 2 hours.

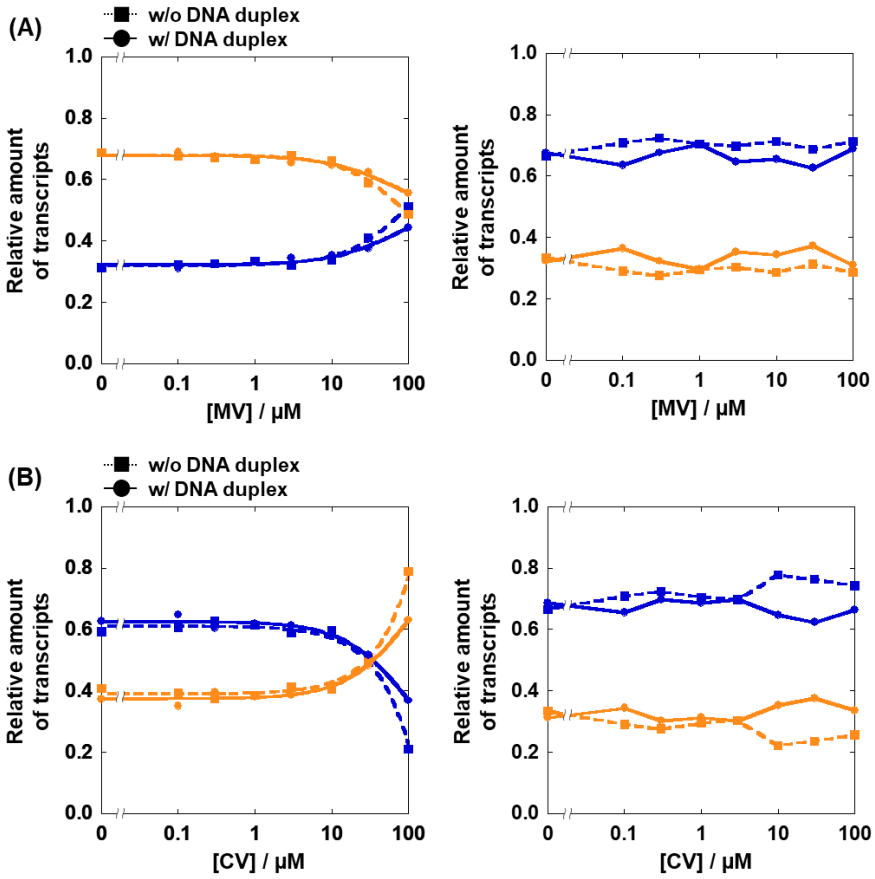

Figure S6. Relative amounts of the completed transcript (blue) and arrested transcript (orange) from the assay versus concentration of EV (A) and CV (B) with the 22AG template (left) or the mut22AG template (right) in the absence (squares with dotted line) or presence (circles with continuous line) of excess DNA duplex. Transcription reactions were carried out in buffer consisting of 1 mM KCl, 40 mM Tris-HCl (pH 7.2), 8 mM $MgCl_2$.

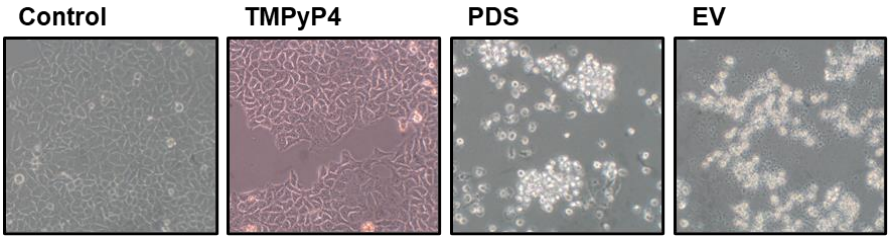

Figure S7. Images of HeLa cells cultured in medium containing 0 or 100 μM TMPyP4, PDS, or EV.

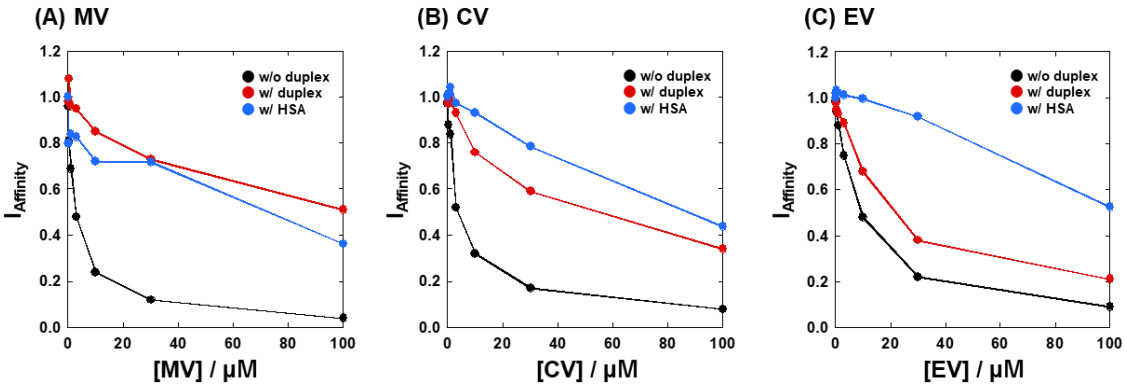

Figure S8. Plots of $I_{affinity}$ values versus the concentrations of MV, CV, and EV in the absence or presence of 2 mM CT-DNA or 50 μM HSA in the KCl buffer at 25℃

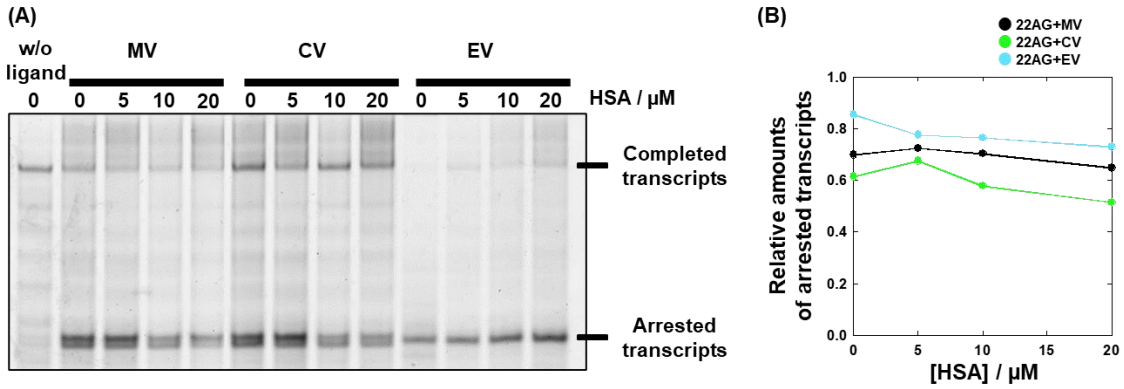

Figure S9. Denatured 10% polyacrylamide gel electrophoresis of the transcripts from 22AG template with 0, 5, 10, or 20 μM HSA in the presence of 100 μM MV, CV, or EV. The transcription reactions were carried out in buffer consisting of 1 mM KCl, 40 mM Tris-HCl (pH 7.2) for 2 hours. (B) Relative amounts of arrested transcripts versus concentration of HSA with the 22AG template in the presence of 100 μM MV (black), CV (green), or EV (blue).

References

1. N. Sugimoto, M. Nakano and S. Nakano, *Biochemistry*, 2000, **39**, 11270-11281.
2. V. Gabelica, R. Maeda, T. Fujimoto, H. Yaku, T. Murashima, N. Sugimoto and D. Miyoshi, *Biochemistry*, 2013, **52**, 5620-5628.