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Supporting Information

NRAS DNA G-quadruplex-targeting molecules for sequence-selective enzyme inhibition

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1. Experimental Procedures

1.1 Materials

All oligonucleotides were purchased from Sigma-Aldrich Co. LLC (St. Louis, Missouri, U.S.A.). ZnAPC [zinc (II) phthalocyanine 4,4',4'',4'''-tetrasulfonic acid, tetrasodium salt] was purchased from Frontier Scientific and used without further purification. Thioflavin T (ThT) were purchased from Wako Pure Chemical Industries Ltd. Library compounds were purchased from Wako Pure Chemical Industries Ltd., Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), and TargetMol Chemicals Inc. (Boston, Massachusetts, USA). NRAS and TELO are the oligonucleotide derived from human *NRAS* and human telomere (Table S1). Extinction coefficients for single-strand NRAS (ε_{260} = 186,500 M^{-1} cm^{-1}) and TELO (ε_{260} = 228,500 M^{-1} cm^{-1}) were calculated from mono and dinucleotide data using the nearest-neighbor approximation model[1]. The stock solutions of DNA were stored at -30 °C. Single-strand concentrations of the DNA were determined by measuring the absorbance at 260 nm at 90 °C. The stock solution of ZnAPC (100 μM in Milli-Q) and of ligands (1 mM in dimethyl sulfoxide) was stored at -30 °C in the dark. Before measurement, the DNA in a buffer containing 100 mM KCl 50 mM 2-(*N*-morpholino) ethanesulfonate (MES)-LiOH (pH 7.0) were heated at 93 °C for 5 min, gently cooled at -0.5 °C min^{-1}, and incubated at 25 °C.

1.2 Identification of NRAS and TELO structure

Structure of NRAS and TELO was studied using UV melting and circular dichroism (CD) measurements. 10 μM DNA solution was prepared in a buffer containing 100 mM KCl and 50 mM MES-LiOH (pH 7.0). UV melting curves were recorded on a spectrophotometer (UV-1800; Shimadzu, Kyoto, Japan). The melting curves were traced at 295 nm with 1 cm path length quartz cuvette and samples were heated at a rate of 0.5 °C/min. from 10 to 90 °C to trace thermal denaturation. The hypochromic transition at 295 nm shows a G4 formation[2] (Fig. S1B). CD spectra were measured using a J-820 spectropolarimeter (JASCO Co., Ltd., Tokyo, Japan) with 0.1 cm path length quartz cuvette at 25 °C. CD spectra of NRAS and TELO showed a parallel G4 and a hybrid G4, respectively[3].

1.3 Absorption spectroscopy

Visible absorption spectra for 2 μM ZnAPC were recorded on a spectrophotometer (UV-1800; Shimadzu, Kyoto, Japan) with a quartz cuvette with 1 cm path length. All the measurements were carried out in the buffer containing 100 mM KCl and 50 mM MES-LiOH (pH 7.0) at 25 °C. ΔAbs_{680}, (abs. at 680 nm with DNA) - (abs. at 680 nm without DNA), 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 (K_d) of ZnAPC for NRAS:

$$\Delta Abs_{680} = a\frac{([DNA] + [ZnAPC] + K_d) - \sqrt{([DNA] + [ZnAPC] + K_d)^2 - 4[DNA][ZnAPC]}}{2[ZnAPC]} \quad (1)$$

where a is the scaling factor.

1.4 Fluorescence spectroscopy

Fluorescence spectra of ZnAPC from 650 nm to 750 nm were measured using a FP-8200 spectrofluorometer (JASCO, Tokyo, Japan) with 0.3 cm path length quartz cuvette. The fluorescence spectra were measured with excitation wavelength of 620 nm. All experiments were performed in the buffer containing 100 mM KCl and 50 mM MES-LiOH (pH 7.0) at 25 °C.

1.5 Fluorescent indicator displacement (FID) assay with ZnAPC

Fluorescent indicator displacement (FID) assay was carried out in the buffer containing 100 mM KCl and 50 mM LiOH-MES (pH 7.0) 25 °C. The total reaction volume was 60 μL, which contains 5 μM NRAS, 20 μM ZnAPC, and 20 μM ligand in the absence or presence of 50 μM TELO. First, NRAS was mixed with TELO and ZnAPC and the mixture were heated at 93 °C for 5 min, gently cooled at -0.5 °C

min^{-1}. After the annealing, fluorescence spectra of 20 μM ZnAPC in the absence of the ligand were measured at 25 °C from 650 mm to 750 nm with excitation wavelength of 620 nm 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 mixture of NRAS, ZnAPC and the ligand was incubated under the dark at 25 °C for 24 hours after the addition of the ligand. After the incubation, fluorescence spectra of ZnAPC were measured in the presence of the ligand under the same condition. $I_{Affinity}$ for FID assay with ZnAPC was calculated from the F_{690} (fluorescence intensity of ZnAPC at 690 nm) as the following: (F_{690} with the ligand) / (F_{690} without the ligand).

1.6 ThT displacement (TD) assay

ThT displacement (TD) assay[4] was carried out in the buffer containing 100 mM KCl and 50 mM LiOH-MES (pH 7.0) at 25 °C. The total reaction volume was 60 μL, which contains 1 μM TELO and 20 μM ThT. First, TELO was mixed with ThT and the mixture were heated at 93 °C for 5 min, gently cooled at -0.5 °C min^{-1}. After the annealing, fluorescence spectra of 20 μM ThT in the absence of the ligand were measured at 25 °C from 470 mm to 600 nm with excitation wavelength of 450 nm 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 mixture of TELO, ThT, and the ligand was incubated under the dark at 25 °C for 24 hours after the addition of the ligand. After the incubation, fluorescence spectra of ThT were measured in the presence of the ligand under the same condition. $I_{Affinity}$ for TD assay was calculated from the F_{485} (fluorescence intensity of ThT at 485 nm) as the following: (F_{485} with the ligand) / (F_{485} without the ligand).

1.7 Binding analysis

The observed K_d values (K_i) of the ligands were calculated with the following equation:

$$I_{Affinity} = 1 - \frac{[Ligand]}{[Ligand] + K_i\left(1 + \frac{[FI]}{K_d}\right)} \qquad (2)$$

Where $I_{Affinity}$ is the affinity index of ligands, [FI] is concentration of ZnAPC and ThT, and K_d is dissociation constants of ZnAPC and ThT for NRAS and TELO, respectively.

1.8 Thermal analysis

The values of melting temperature (T_m) of NRAS were evaluated by CD melting curves traced at 265 nm. The CD melting curves of NRAS were measured in a cuvette with a path length of 0.1 cm using a J-820 spectropolarimeter (JASCO Co., Ltd., Tokyo, Japan) in a buffer containing 1 mM KCl and 50 mM MES-LiOH (pH 7.0). Before measurement, a mixture of 50 μM ligand and 10 μM NRAS was prepared and incubated at 25 °C for 1 hour. For CD melting experiments, samples were heated from 20 °C to 80 °C at a rate of 0.5 °C min^{-1}.

1.9 T7 RNA polymerase stop assay

Each transcription reaction solution contained 1 μM DNA template, 1 mM KCl, 99 mM LiCl, 8 mM $MgCl_2$, and 40 mM Tris-HCl (pH 7.2). Note that this condition was used to reduce the arrested transcripts in the absence of a G4 ligand. The samples were heated to 93 °C for 5 min, then cooled to 37 °C at a rate 0.5 °C min^{-1}. After annealing, NTP and DTT were added to be a final concentration of 1 mM and 5 mM, respectively. T7 polymerase (100 units, Takara Bio, Inc., Shiga, Japan) was added to the reaction buffer to start the reaction. The final volume of the reaction mixture was 20 μL. Each mixture was incubated at 37 °C for 2 hours for the reaction. Reactions were quenched by incubation with 10 units of DNase I (Takara Bio, Inc.) for 1 hour, then a 25-fold excess volume of the transcription stop solution (80wt% formamide, 10 mM Na_2EDTA, and 0.2% 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 gel containing 7 M urea and run at 70 °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. The IC_{50} values of the ligands for transcription reaction were calculated with the following equation:

$$Relative\ amounts\ of\ arrested\ transcripts = \frac{[Ligand]}{[Ligand] + IC_{50}} \qquad (3)$$

Relative amounts of arrested transcripts were evaluated as the following: (band intensity of arrested transcript) / [(total band intensity of the arrested transcript) + (band intensity of the completed transcript)].

1.10 Two-step TRAP assay

We previously reported two-step telomeric repeat amplification protocol (tsTRAP) assay[5], which was improved on the basis of manufacture's protocol the TRAPEZE telomerase detection kit (Sigma-Aldrich Co. LLC, St. Louis, Missouri, U.S.A.). Prior to conducting the tsTRAP assay, telomerase was extracted from HeLa cells obtained from the American Type Culture Collection. The HeLa cells (10^5 cells) were lysed in 1 mL of CHAPS lysis buffer containing 10 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 1 mM EGTA, 0.1 mM benzamidine, 5 mM β-mercaptoethanol, 0.5% (3-[(3-Cholamidopropyl)-dimethyl-ammonio]-1-propane sulfonate), and 10% glycerol and then incubated on ice for 30 min. Subsequently, the cell lysate was centrifuged at 4 °C, and 800 µL of the supernatant was collected as the telomerase solution. The telomerase solution was stored at -80 °C. The detailed methodology and quantification procedures for the tsTRAP assay are described in our previous publication[5].

Table S1. DNA sequences used in this study

Abbreviation	Sequence (from 5’ to 3’)
NRAS	GGGAGGGGCGGGTCTGGG
TELO	AGGGTTAGGGTTAGGGTTAGGG
NRAS template[a]	GCCGTTTCGTAGTAT**GGGCGGGGAGGGTCTGGG**CAGAGAGAGCACCG AGCCTAGTTCGTGTCATCTCCTATAGTGAGTCGTATTAGTGATC
mutNRAS template[a]	GCCGTTTCGTAGTAT**GAGCGAGGAGGGTCTGAG**CAGAGAGAGCACCG AGCCTAGTTCGTGTCATCTCCTATAGTGAGTCGTATTAGTGATC

[a] Bold letters indicate the G4 forming region in NRAS template and the corresponding mutated region in mutNRAS template.

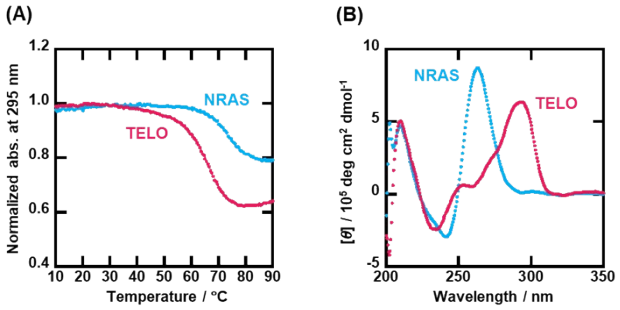
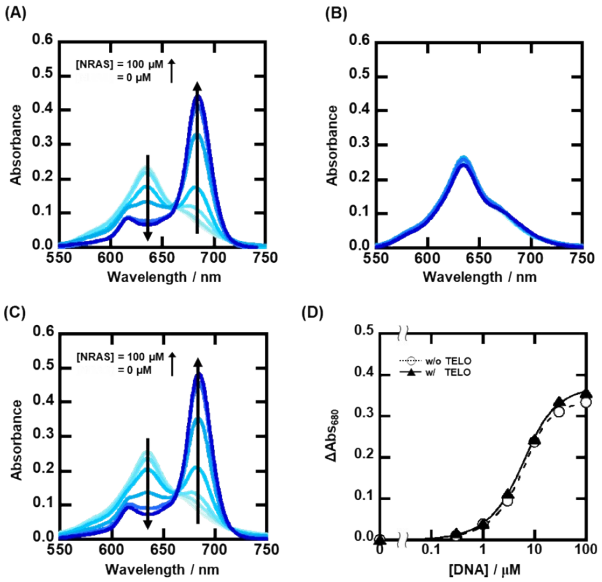


Figure S1 (A) Normalized UV melting curves traced at 295 nm for 10 μM NRAS (blue) and TELO (red). UV melting measurements were carried out from 10 °C to 90 °C. (B) CD spectra of 10 μM NRAS (blue) and TELO (red) in the buffer containing 100 mM KCl and 50 mM MES-LiOH (pH 7.0) at 25 °C.



(E)

DNA	K_d (μM)
NRAS	5.8 ± 0.7
TELO	N.D.
NRAS w/ 50 μM TELO	6.0 ± 0.6

Figure S2 Visible absorbance spectra of 2 μM ZnAPC with 0, 0.3, 1, 3, 10, 30, and 100 μM NRAS (A) and TELO (B). (C) Visible absorbance spectra of 2 μM ZnAPC with various concentrations of NRAS in the presence of 50 μM TELO. All measurements were performed in the buffer containing 100 mM KCl and 50 mM MES-LiOH (pH 7.0) at 25 °C. (D) Plots of Δabs_{680} *versus* concentration of NRAS in the absence (circles with dotted line) or presence (triangles with continuous line) of 50 μM TELO. (E) The K_d values of ZnAPC for NRAS and TELO at 25 °C.

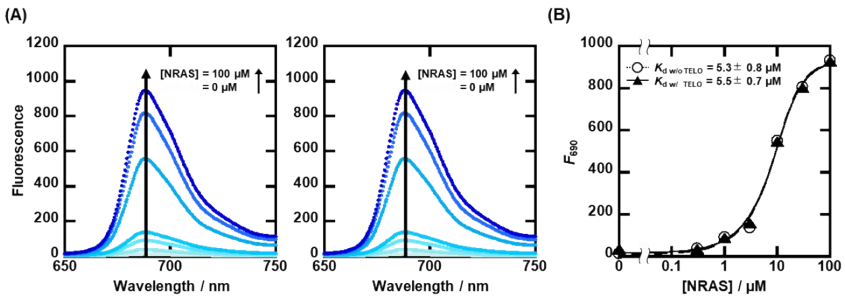


Figure S3. (A) Fluorescence spectra of 2 μM ZnAPC with 0, 0.3, 1, 3, 10, 30, and 100 μM NRAS in the absence (left) or presence (right) of 50 μM TELO in the buffer containing 100 mM KCl and 50 mM MES-LiOH (pH 7.0) at 25 °C. (B) Plots of F_{690} *versus* concentration of NRAS in the absence (circles with dotted line) or presence (triangles with continuous line) of 50 μM TELO. The K_d values of ZnAPC for NRAS in the absence and presence of 50 μM TELO at 25 °C were shown in the panel.

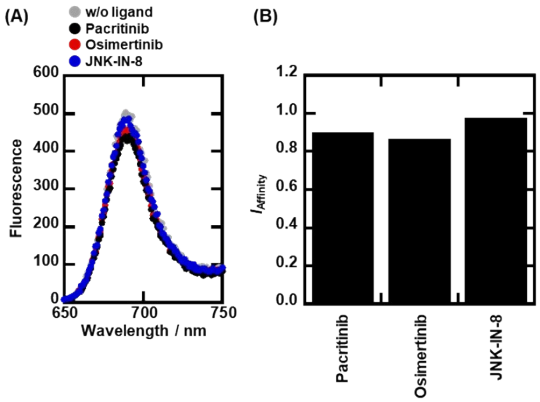
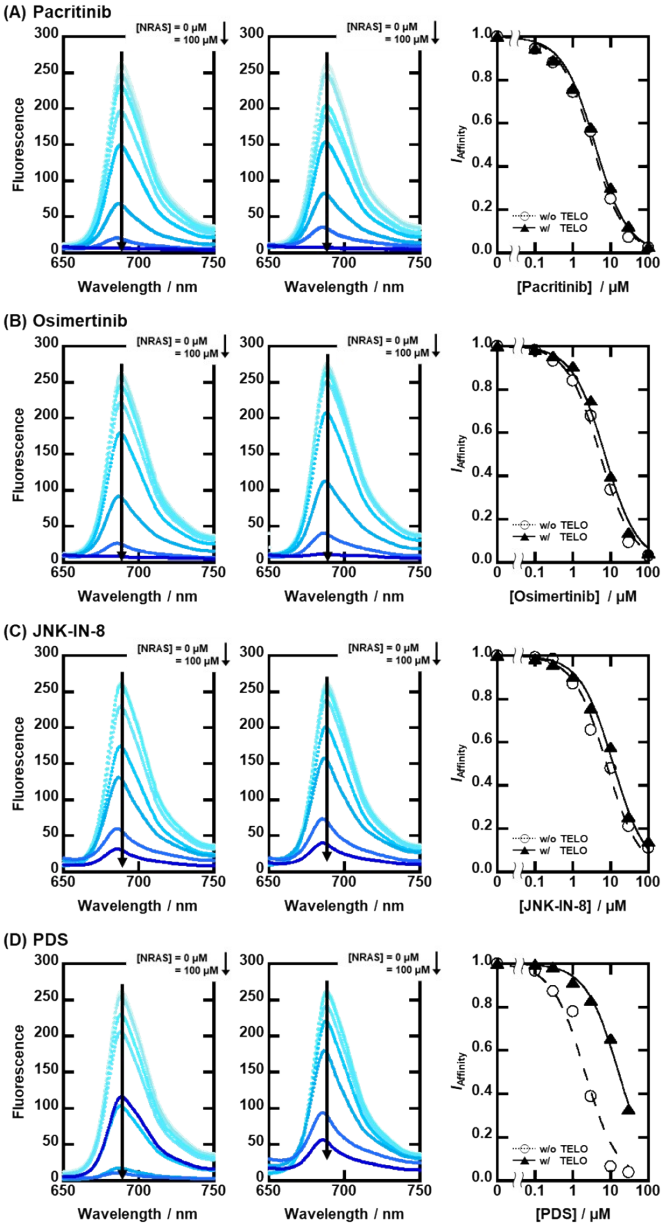


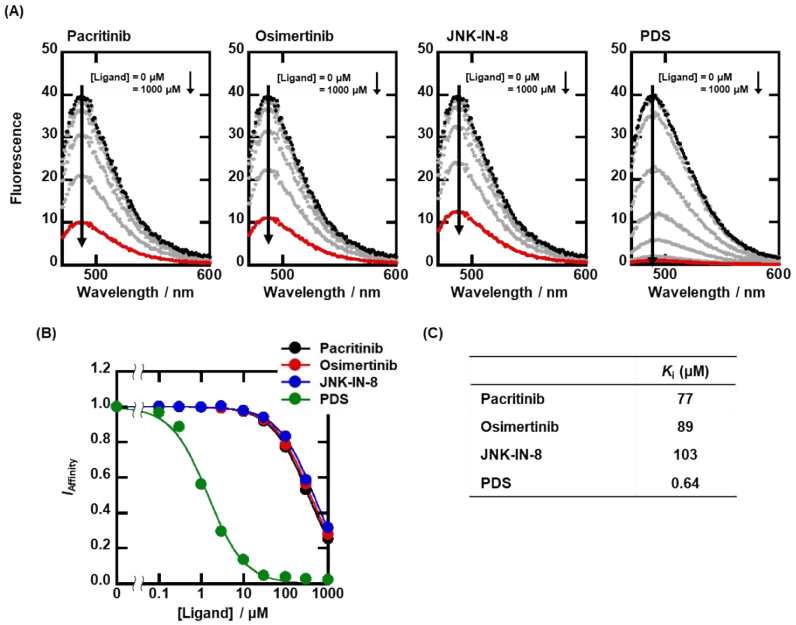
Figure S4. (A) Fluorescence spectra of 20 µM ZnAPC with 20 µM pacritinib, osimertinib, and JNK-IN-8 in the buffer containing 100 mM KCl, 50 mM MES-LiOH, and 80 v/v% DMF. (B) $I_{Affinity}$ of 20 µM pacritinib, osimertinib, and JNK-IN-8.



(E)

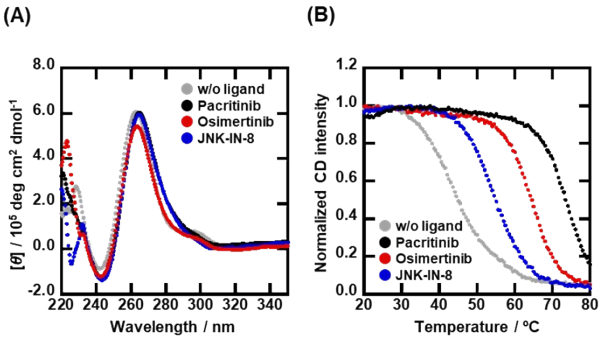
	K_i (μM)	
	w/o TELO	w/ TELO
Pacritinib	0.74 ± 0.11	0.88 ± 0.09
Osimertinib	0.85 ± 0.08	0.96 ± 0.10
JNK-IN-8	1.7 ± 0.1	2.6 ± 0.2
PDS	0.47 ± 0.08	4.1 ± 0.1

Figure S5. Fluorescence spectra of 20 μM ZnAPC and 1 μM NRAS with 0, 0.1, 0.3, 1, 3, 10, 30, and 100 μM pacritinib (A), osimertinib (B), JNK-IN-8 (C), or PDS (D) in the absence (left row) or presence (center row) of 50 μM TELO. All measurements were carried out in the buffer containing 100 mM KCl, 50 mM MES-LiOH (pH 7.0) at 25 °C. Right row: plots of $I_{Affinity}$ of pacritinib (A), osimertinib (B), JNK-IN-8 (C), and PDS (D) *versus* concentration of the ligands in the absence (circles with dotted line) or presence (triangles with continuous line) of 50 μM TELO. (E) The K_i values of pacritinib, osimertinib, JNK-IN-8, and PDS for NRAS in the absence and presence of 50 μM TELO at 25 °C.



	K_i (μM)
Pacritinib	77
Osimertinib	89
JNK-IN-8	103
PDS	0.64

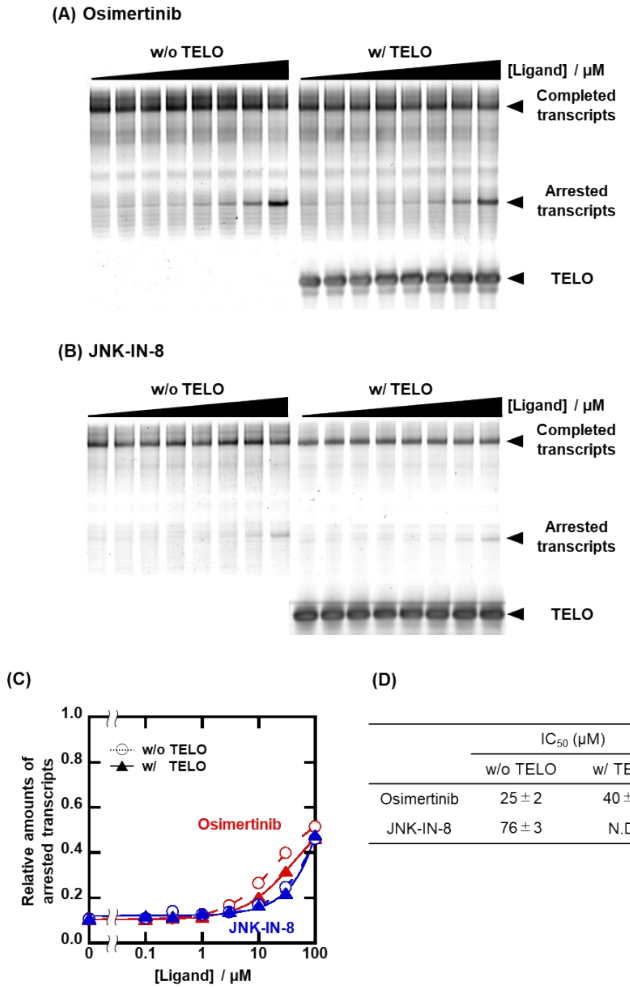
Figure S6. (A) Fluorescence spectra of 20 μM ThT with 1 μM TELO in the presence of 1 μM TELO with 0, 0.1, 0.3, 1, 3, 10, 30, and 100 μM pacritinib, osimertinib, JNK-IN-8, or PDS. All measurements were carried out in the buffer containing 100 mM KCl and 50 mM MES-LiOH (pH 7.0) at 25 °C. (B) Plots of $I_{Affinity}$ of pacritinib, osimertinib, JNK-IN-8, and PDS *versus* concentration of the ligands. (C) The K_i values of pacritinib, osimertinib, JNK-IN-8, and PDS for TELO at 25 °C.



(C)

	T_m (°C)	ΔT_m (°C)
w/o ligand	41.0 ± 0.9	
Pacritinib	72.7 ± 0.6	31.7
Osimertinib	63.9 ± 0.6	22.9
JNK-IN-8	54.2 ± 0.3	13.2

Figure S7. (A) CD spectra of 10 µM NRAS in the absence (gray) or presence of 50 µM pacritinib (black), osimertinib (red), and JNK-IN-8 (blue) in the buffer containing 1 mM KCl and 50 mM MES-LiOH (pH 7.0) at 25 °C. (B) CD melting curves traced at 265 nm for 10 µM NRAS in the absence (gray) or presence of 50 µM pacritinib (black), osimertinib (red), and JNK-IN-8 (blue). (C) The T_m values of 10 µM NRAS in the absence and presence of 50 µM pacritinib, osimertinib, and JNK-IN-8.



	IC_{50} (μM)	
	w/o TELO	w/ TELO
Osimertinib	25 ± 2	40 ± 4
JNK-IN-8	76 ± 3	N.D.

Figure S8. Transcripts from NRAS template with 0, 0.1, 0.3, 1, 3, 10, 30, and 100 osimertinib (A) or JNK-IN-8 (B) in the absence (left) or presence (right) of 50 μM TELO. The transcription reactions were carried out in the buffer containing 1 mM KCl, 99 mM LiCl, 8 mM $MgCl_2$, and 40 mM Tris-HCl (pH 7.2) for 2 hours at 37 °C. (C) Plots of relative amounts of the arrested transcripts with NRAS template *versus* concentration of osimertinib (red) or JNK-IN-8 (blue) in the absence (circles with dotted line) or presence (triangles with continuous line) of 50 μM TELO. (D) The IC_{50} values of osimertinib and JNK-IN-8 in the absence and presence of 50 μM TELO at 37 °C.

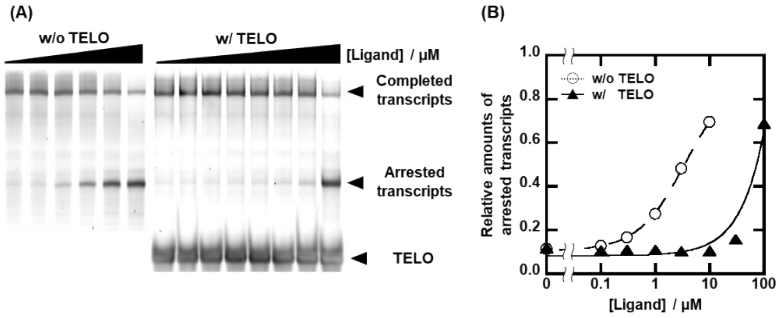


Figure S9. Transcripts from NRAS template with 0, 0.1, 0.3, 1, 3, and 10 μM PDS in the absence (left) or presence (right) of 50 μM TELO. The transcription reactions were carried out in the buffer containing 1 mM KCl, 99 mM LiCl, 8 mM $MgCl_2$, and 40 mM Tris-HCl (pH 7.2) for 2 hours at 37 °C. (D) Plots of relative amounts of the arrested transcripts with NRAS template *versus* concentration of PDS in the absence (circles with dotted line) or presence (triangles with continuous line) of 50 μM TELO.

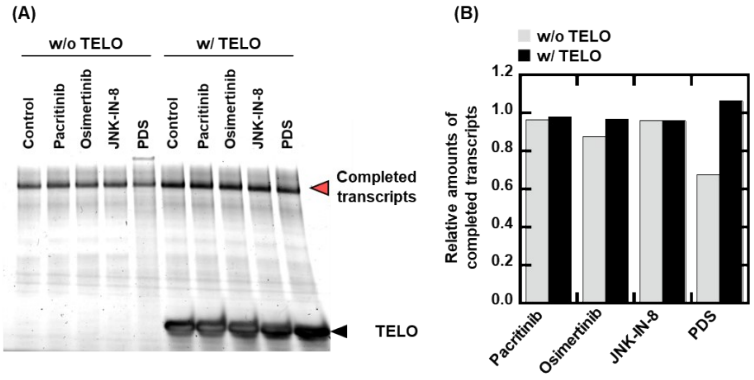


Figure S10. (A) Transcripts from mutNRAS template with 100 μM pacritinib, osimertinib, JNK-IN-8, or PDS in the absence or presence of 50 μM TELO. The transcription reactions were carried out in the buffer containing 1 mM KCl, 99 mM LiCl, 8 mM $MgCl_2$, and 40 mM Tris-HCl (pH 7.2) for 2 hours at 37 °C. (B) Relative amounts of the competed transcripts of pacritinib, osimertinib, JNK-IN-8, and PDS in the absence (gray) and presence (black) of 50 μM TELO.

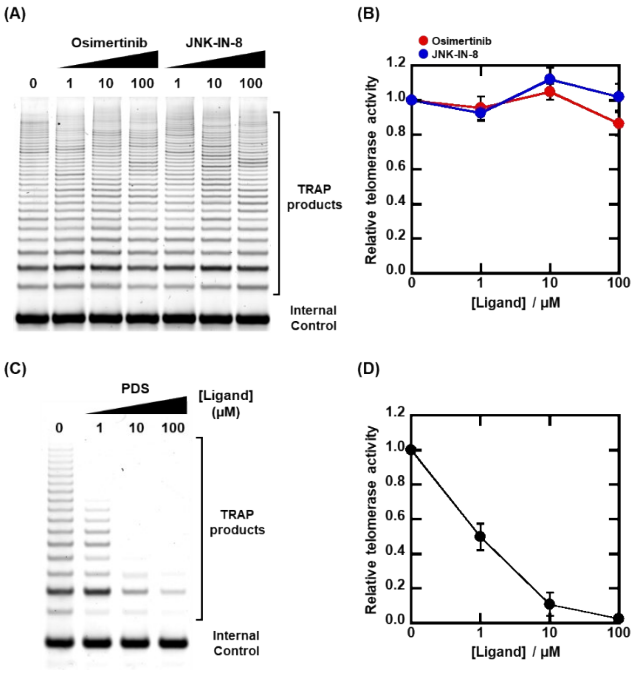


Figure S11. (A, C) Results of the tsTRAP assay with 0, 1, 10, and 100 μM osimertinib (A), JNK-IN-8 (A), or PDS (C) in the buffer containing 200 mM Tris-HCl (pH 8.3), 15 mM $MgCl_2$, 630 mM KCl, 0.5% Tween 20, and 10 mM EGTA for 30 min. at 30 °C. (B, D) Plots of relative telomerase activity *versus* concentrations of osimertinib (B), JNK-IN-8 (B), and PDS (D) at 30 °C.

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