Supporting Information

Trigeminal star-like platinum complexes induce cancer cell senescence through quadruplex-mediated telomere dysfunction

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Experimental Procedures

General Methods. ^{195}Pt NMR (500 MHz) spectra were recorded on a Varian INOVA-500 spectrometer, and K_2PtCl_4 was used as internal references (δ=0). ^{1}H and/or ^{13}C NMR (300 MHz) spectra were recorded on a Mercury-Plus 300 spectrometer. Elemental analysis was recorded on a Vario EL elemental analyzer. Fluorescence resonance energy transfer (FRET) thermal melting curves were recorded on a Roche Light Cycler II real-time PCR machine. Surface plasmon resonance (SPR) experiments were performed on ProteOn XPR36 Protein Interaction Array system (Bio-Rad Laboratories, Hercules, CA) using a Neutravidin-coated GLM sensor chip. Circular dichroism (CD) spectra were recorded on a JASCO J-810 spectropolarimeter. Isothermal titration calorimetry (ITC) experiments were performed on a high-sensitivity isothermal titration calorimeter (VP-ITC, MicroCal, Inc., Northampton, MA, U. S. A.). Oligonucleotides purified by reversed-phase HPLC were purchased from Sangon (China). Unless otherwise noted, materials obtained from commercial suppliers were used without further purification.

Synthesis of {[Pt(dien)]$_3$(tpt)}·(NO$_3$)$_6$ (1): (dien = diethylenetriamine, tpt = 2,4,6-tri(pyridin-4-yl)-1,3,5-triazine): [Pt(dien)Cl]Cl·HCl (202.81mg, 0.50mmol), synthesized according to a literature reported procedure,[1] and $AgNO_3$ (254.81mg, 1.50mmol) were stirred in water (9mL) in a stoppered flask with the exclusion of light for 36h at 45 ^{o}C and then filtered from AgCl. The clear filtrate was then transferred to another stoppered flask where tpt (49.97mg, 0.16mmol) was added and heated at 90^{o}C for 3 days under N_2. The solution was then concentrated to about 2mL, and addition of ethanol (30mL) produced the precipitate of the desired complex. This was collected by filtration, washed with ethanol and ether, and dried under vacuum. Yield: 96%. ^{1}H NMR (300MHz, D_2O, 25 ^{o}C): δ = 8.93 (d, 6 H), 8.64 (d, 6 H), 3.25-2.80 (m, 24 H); ^{13}C NMR (300MHz, D_2O, 25 ^{o}C): δ = 170.27, 153.45, 145.22, 127.70, 54.15, 50.45; ^{195}Pt NMR (500MHz, D_2O): -1222.20 ppm. Elemental analysis (calcd, found for $C_{30}H_{51}N_{21}O_{18}Pt_3·6H_2O$): C (21.36, 21.20), H (3.76, 3.76), N (17.43, 17.52).

Synthesis of {[Pt(dpa)]$_3$(tpt)}·(NO$_3$)$_6$ (2): (dpa = 2,2′-Dipicolylamine, tpt = 2,4,6-tri(pyridin-4-yl)-1,3,5-triazine): [Pt(dpa)Cl]Cl (232.62mg, 0.50mmol), synthesized according to a literature reported procedure,[1] and $AgNO_3$ (169.87mg, 1.00mmol) were stirred in water (10mL) in a stoppered flask with the exclusion of light for 46h at 60 ^oC and then filtered from AgCl. The clear filtrate was then transferred to another stoppered flask where tpt (49.97mg, 0.16mmol) was added and heated at 90 ^oC for 3 days under N_2. The solution was then concentrated to about 2mL, and addition of ethanol (30mL) produced the precipitate of the desired complex. This was collected by filtration, washed with ethanol and ether, and dried under vacuum. Yield: 92%. ^1H NMR (300MHz, D_2O, 25 ^oC): δ = 8.09 (m, 9 H), 8.70 (s, 2 H), 7.61 (m, 18 H), 7.32 (m, 9 H), 4.7-4.9 (m, 12 H); ^{13}C NMR (300MHz, D_2O, 25 ^oC): δ = 170.33, 165.91, 154.00, 148.42, 146.49, 141.70, 127.30, 125.44, 123.11, 59.59; ^{195}Pt NMR (500MHz, D_2O): -798.80 ppm. Elemental analysis (calcd, found for $C_{54}H_{51}N_{21}O_{18}Pt_3 \cdot 12H_2O$): C (31.13, 31.27), H (3.63, 3.58), N (14.12, 14.07).

Fluorescence resonance energy transfer thermal (FRET) melting assay: Fluorescence melting curves were made on a Roche Light Cycler II real-time PCR machine with excitation at 470nm and detection at 530nm. Fluorescence readings were taken at intervals of 1 ^oC over the range 37-99 ^oC, with a constant temperature being maintained for 30s prior to each reading to ensure a stable value. Final analysis of the data was carried out using Origin 7.0 (OriginLab Corp.). The DNA with the following sequences was used (where FAM is 6-carboxyfluorescein and TAMRA: 6-carboxy-tetramethylrhodamine):

hTel G-quadruplex, 5′-FAM-GGGTTAGGGTTAGGGTTAGGG-TAMRA-3′,

c-*kit1* , 5′-FAM-AGGGAGGGCGCTGGGAGGAGGG-TAMRA-3′

c-myc, 5'-FAM-TGGGGAGGGTGGGGAGGGTGGGGAAGG-TAMRA-3'

bcl2, 5'-FAM-AGGGGCGGGCGCGGGAGGAAGGGGGCGGGAGCGGGGCTG-TAMRA-3'

dsDNA, 5′-FAM-TATAGCTATA-HEG-TATAGCTATA-TAMRA-3′, HEG linker is [(-CH_2-CH_2-O-)$_6$])

FRET Competition assays: were performed in a similar manner. Compound dilutions were prepared in concentrations four times the final ones and added as 25μL aliquots. To hTel G4 DNA (50μL), complex (25μL, 4μM), and the appropriate competitor calf thymus DNA (Sigma-Aldrich, UK),

synthesized in-house, were added at the following concentration ratios of 1:1, 1:10, 1:50, 1:100, 1:200. The percentage of retained hTel G4 melting temperatures (ΔT_m) upon addition of increasing concentrations of competitor was averaged from three experiments, normalized with standard deviation to the ΔT_m (0.4μM) for that ligand in the absence of competitor.

Surface Plasmon Resonance (SPR): SPR measurements were performed on a ProteOn XPR36 Protein Interaction Array system (Bio-Rad Laboratories, Hercules, CA) using a Neutravidin-coated GLM sensor chip. Six concentrations were injected simultaneously at a flow rate of 40μL/min for 3~6 min of association phase, followed by 3 min of disassociation phase at 25 ^{o}C. The GLM sensor chip was regenerated with short injection of 1M NaCl between consecutive measurements. The final graphs were obtained by subtracting blank sensorgrams from the duplex DNA or hTel G4 sensorgrams. Data are analyzed with ProteOn manager software, using the Equilibrium and the two states model for fitting kinetic data. The DNA with the following sequences was used in SPR experiments:

hTel G4, 5'-biotin-d[AGGGTTAGGGTTAGGGTTAGGG]-3'

duplex DNA, 5'-biotin-d[CGAATTCGTCTCCGAATTCG]-3'

Isothermal titration calorimetry (ITC): Calorimetric experiments were performed using a high-sensitivity isothermal titration calorimeter (VP-ITC, MicroCal, Inc., Nor-thamplatinumon, MA, U. S. A.). For a typical titration, serial 10 μL of complex (the concentrations of **1** and **2** are 1.2mM and 2.9mM, respectively) were injected into a sample cell of the DNA solution at 240s intervals with a stirring speed of 372 r.p.m at 25 ^{o}C. The heat output per injection was obtained through integration, and it was corrected by subtracting the dilution heat, which was determined in parallel experiments using an injection of the same concentrations into the buffer solution. The corrected binding isotherms were fitted to obtain the K_b value, the number of binding sites (n), enthalpy change (ΔH) and entropy change (ΔS) using Origin 7.0.

Circular dichroism (CD) studies: A CD study was conducted to observe the effect of **1** and/or **2** on the formation of hTel G4. CD studies were performed on a JASCO J-810 spectropolarimeter at room temperature using a cell length of 1cm, and over a wavelength range of 220-360nm, with a scan speed of 200nm/min with 5 acquisitions. The oligomer 22AG (5′-$AG_3(T_2AG_3)_3$-3′) at a final concentration

of 3μM was resuspended in Tris-HCl buffer (10mM, pH 7.4) containing 100mM K^+ or no metal ions and heated to 90 °C for 5 minutes and then gradually cooled to room temperature and incubated at 4 °C overnight. CD spectra were baseline-corrected for signal contributions due to the buffer. Then CD titration was performed at a fixed 22AG concentration (3μM) with various concentrations of the complexes (3mM in water). After each addition of complex, the reaction was stirred and allowed to equilibrate for at least 10 min (until no elliptic changes were observed) and a CD spectrum was collected. Final analysis of the data were carried out using Origin 7.0 (OriginLab Corp.).

Cell lines and culture conditions: HeLa, A549, HTC75, U2OS, SAOS2 and VA13 cells were obtained from Cell Resource Center of Peking Union Medical College and were cultured at 37°C under 5% CO_2 in DMEM (Sigma) supplemented with 10% fetal calf serum (PPA) and 100 U/mL penicillin and streptomycin (HyClone).

The telomeric repeat amplification protocol (TRAP) assay: TRAP assay was performed according the previous reports[2-5]. Firstly, telomerase extract (0.75 ng/μl, 1μl) was prepared from HeLa cells and used for every reaction in a final volume of 20μl consisting of 1×TRAP buffer (20mM Tris-HCl, pH 8.3, 1.5mM $MgCl_2$, 63mM KCl, 0.05% Tween-20 and 1mM EGTA in DEPC water), bovine serum albumin (BSA, 0.05μg $sample^{-1}$, 1.0μL), 0.125mM dNTP, 0.25μM TS primer, 0.25μM ACX primer, 0.25μM NT primer, $2*10^{-22}$mol TSNT template, 1U hot-shart Taq ploymerase and different concentrations (1, 3, 6μM) of the platinum(II) complex (5μl). Then the mixtures were transferred to a thermal cycler (Bio-Rad S1000, USA) for the initial elongation step: 30 °C for 30 min, followed by 94 °C for 10 min and a final maintain at 20 °C. Secondly, the QIA quick nucleotide purification kit (Qiagen, 28304) was used to purify the elongated product and remove the extra Pt(II) complexes. Finally, the purified extended samples were then subjected to PCR amplification again: 94 °C for 90s, followed by 35 cycles of PCR reaction: 95 °C for 30s, 50 °C for 30s and 72 °C for 60s. DNA products were resolved on 8% polyacrylamide gel and visualize under UV illumination.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays: The cells were harvested during the exponential phase and seeded equivalently into 96-well plates. The Pt(II) complexes were added to the wells to achieve final concentrations. Twenty microliters of a stock MTT dye solution (5mg/mL) was added to each well after a 44h incubation. DMSO (100μL) was added to solubilize the MTT formazan after an additional incubation period of 4h. The percentage of cellular viability was calculated using the following equation: (mean OD of treated cells/mean OD of control cells) × 100%. Cells treated with vehicle (1% DMSO) were used as controls. Data are presented as the means of three independent experiments ± standard deviations.

Long-term proliferation studies: Long-term proliferation experiments were carried out using the HeLa cell line. 5.0×10^5 Cells were seeded to $10cm^2$ dish, after incubating for 6h, the complex was added to a final concentration of 1.0, 3.0 or 6.0μM. The medium was changed every three days until cell proliferate arrest was reached.

Senescence-associated beta-galactosidase (SA-β gal) activity: SA-β gal activity was quantified with the beta-galactosidase (SA-β gal) staining Kit from Sigma.

Telomere Restriction Fragment (TRF) Assay: To measure the telomere length, genomic DNA was obtained by lysissing the drug-treated cells according to the protocol afforded by "AxyPrep blood genomic DNA MiniPrep kits", and then the genomic DNA was digested with Hinf1/Rsal restriction enzymes and Rnase A enzymes. The digested DNA fragments were separated on 0.7% agarose gel, and the telomere DNA fixed on the gel by baking the gel at vacuum for 1h. Gel was hybridized with a G-proof probe for telomeric repeats and TRF was performed by chemiluminescence detection.

Fluorescence in situ hybridization (FISH) and immunofluorescence (IF): FISH and IF were performed as previously described[2]. Briefly, cells were fixed with 4% paraformaldehyde, permeablized in 0.5% Triton X-100 (in 1×PBS). Cells were incubated overnight at 4 °C with primary antibodies against G-quadruplex (BG4) and 53BP1(Novus) washed three times, and incubated with secondary antibodies, DyLight 488-conjugated anti-mouse. Cells were then incubated with FITC/Cy3-labeled $(CCCTAA)_3$ PNA probe (Panagene) at 37°C for 2 hours, stained with DAPI. Fluorescence was detected and imaged using Zeiss Microscope. 200 cells were used to the statistical analysis.

Telomere fluorescent in situ hybridization (FISH): FISH was performed as previously described.[6, 7] Cy3-labeled (CCCTAA)3 PNA probe (Panagene, Korea) was used. Fluorescence of telomeres was digitally imaged on a Zeiss microscope with Cy3/DAPI filters. The frequency of telomere-free ends was analyzed by statistical analysis.

ICP-MS: For Pt measurement, HeLa cells are firstly co-culture with **1** and/or **2** for 48h, and then harvested. Followed by treating with 60% HNO_3 and stood for over 24h at room temperature to ensure complete digestion. Each sample was then diluted with twice-distilled water to achieve a final volume of 10mL containing 2% HNO_3. In addition, a standard curve was made for the quantitative determination. The concentration of Pt was determined by an inductively coupled plasma mass spectrometer (ICAP Qc, Thermo Fisher, USA), associating with cell number to afford the absolute Pt content per 1000 cells. Values are the average ± SD of three independent experiments.

Statistical analysis: The student's 2-tailed unpaired t-test was used to determine statistical significance and the resulting P-values are indicated in the figures (* $P<0.05$; ** $P<0.01$; *** $P<0.001$).

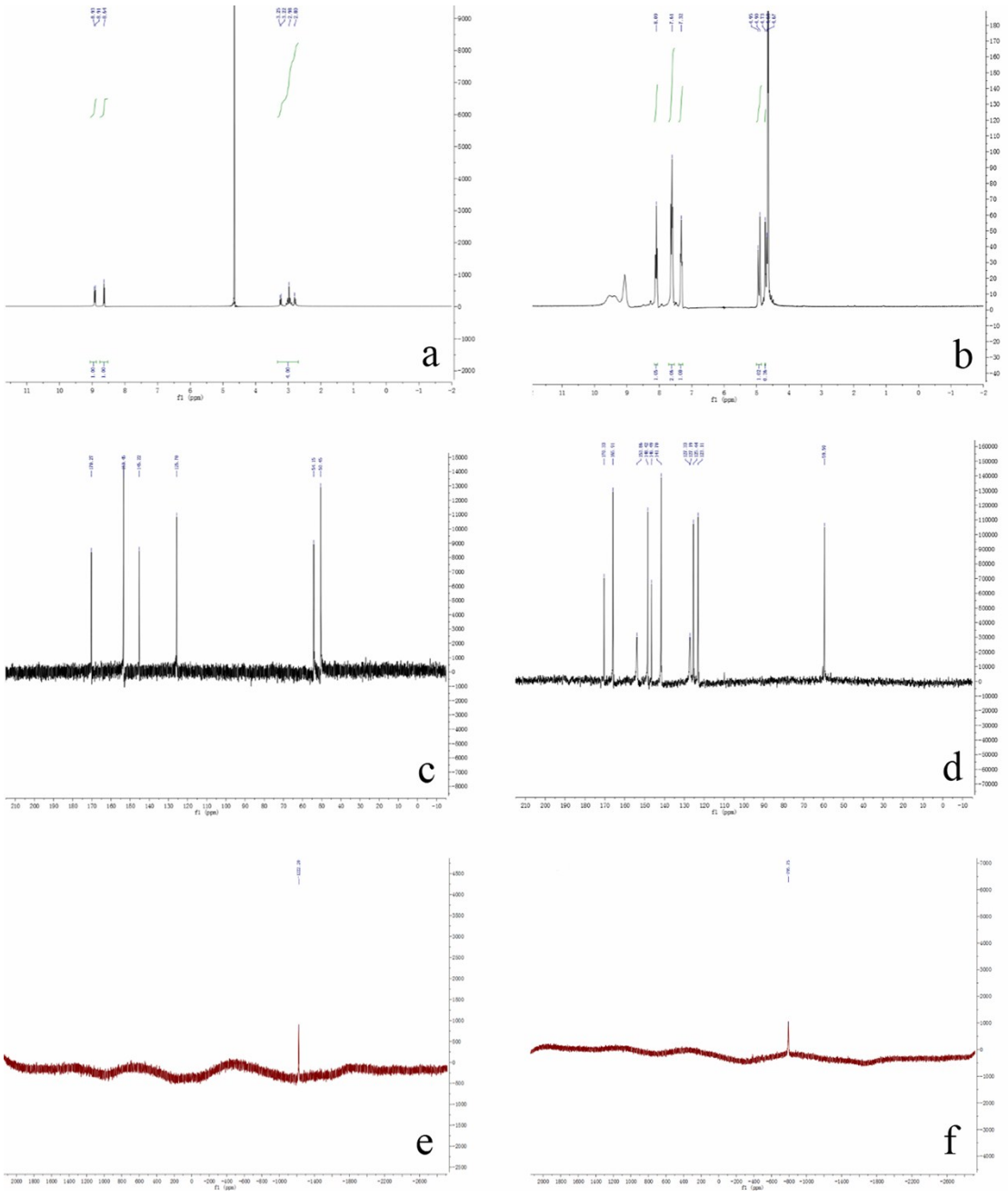

Figure S1. ^{1}H NMR spectra of complexes **1** (a) and **2** (b) at 25 ^{o}C; ^{13}C NMR spectra of complexes **1** (c) and **2** (d) at 25 ^{o}C; ^{195}Pt NMR spectra of complexes **1** (e) and **2** (f) at 25 ^{o}C..

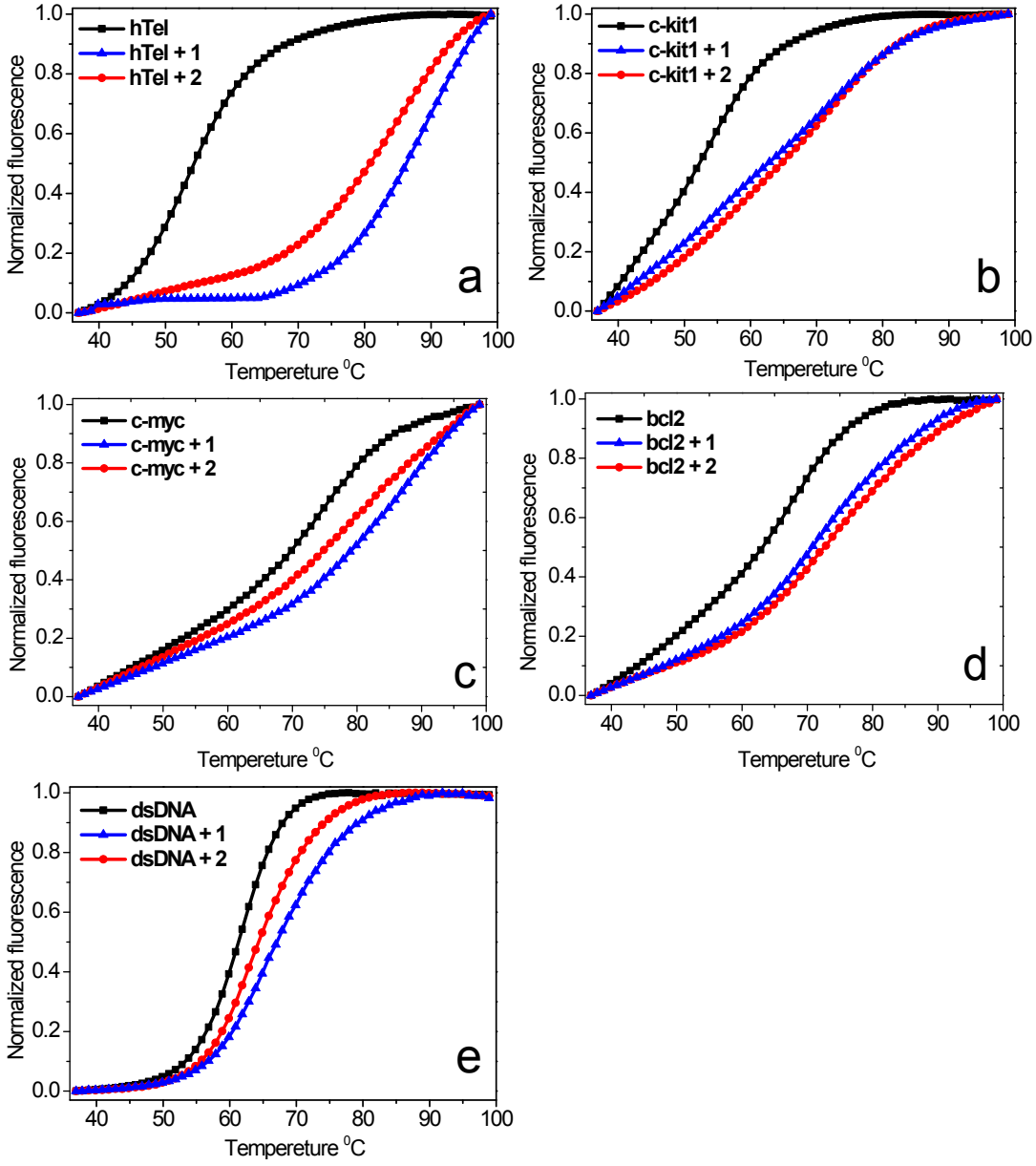

Figure S2. FRET stabilization curves of **1** and **2** (0.4 μM) with human telomere G-quadruplex (hTel G4) (a), *c-kit1* (b), c-myc (c), bcl2 (d) and dsDNA (e). The concentration of quadruplex DNA were 400 nM in 60 mM potassium cacodylate buffer (pH 7.4).

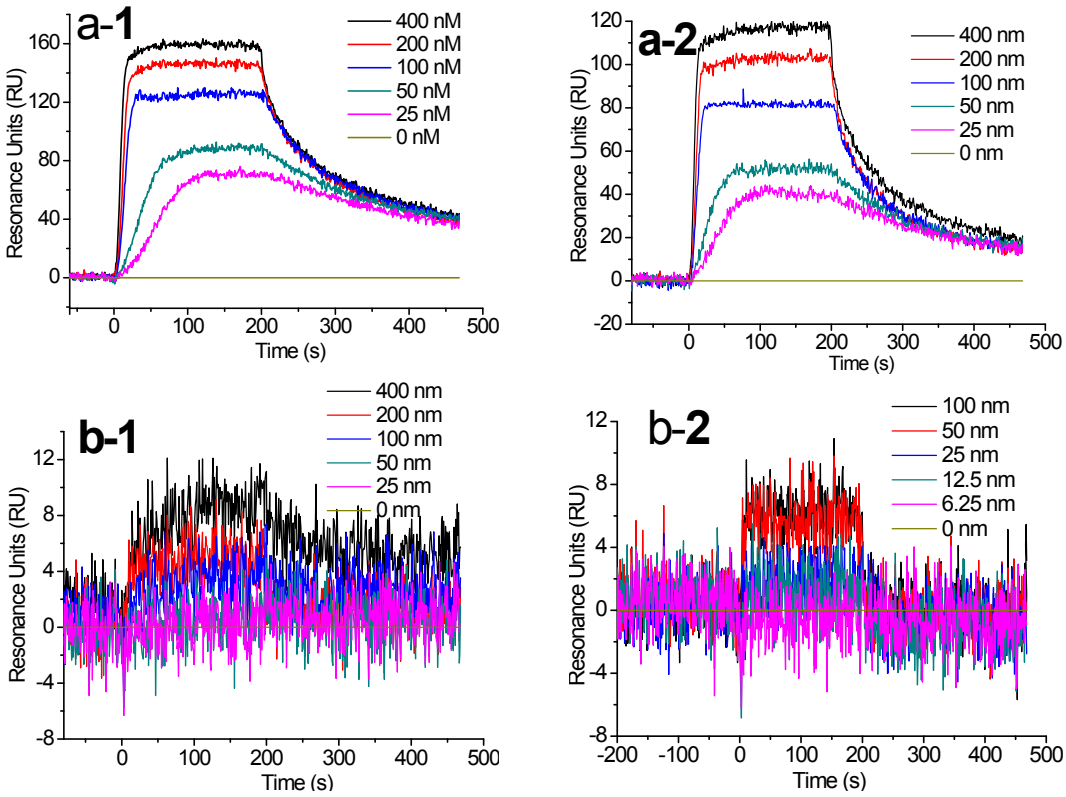

Figure S3. SPR sensorgram overlay for binding of **1** (a-**1**, b-**1**) and **2** (a-**2**, b-**2**) to the immobilized DNA in the presence of 50mM Tris-HCl, pH 7.4, 100mM KCl, 0.005% Tween-20 at 25 °C. **a,** the human telomere G-quadruplex (hTel G4); **b,** the duplex DNA (dsDNA).

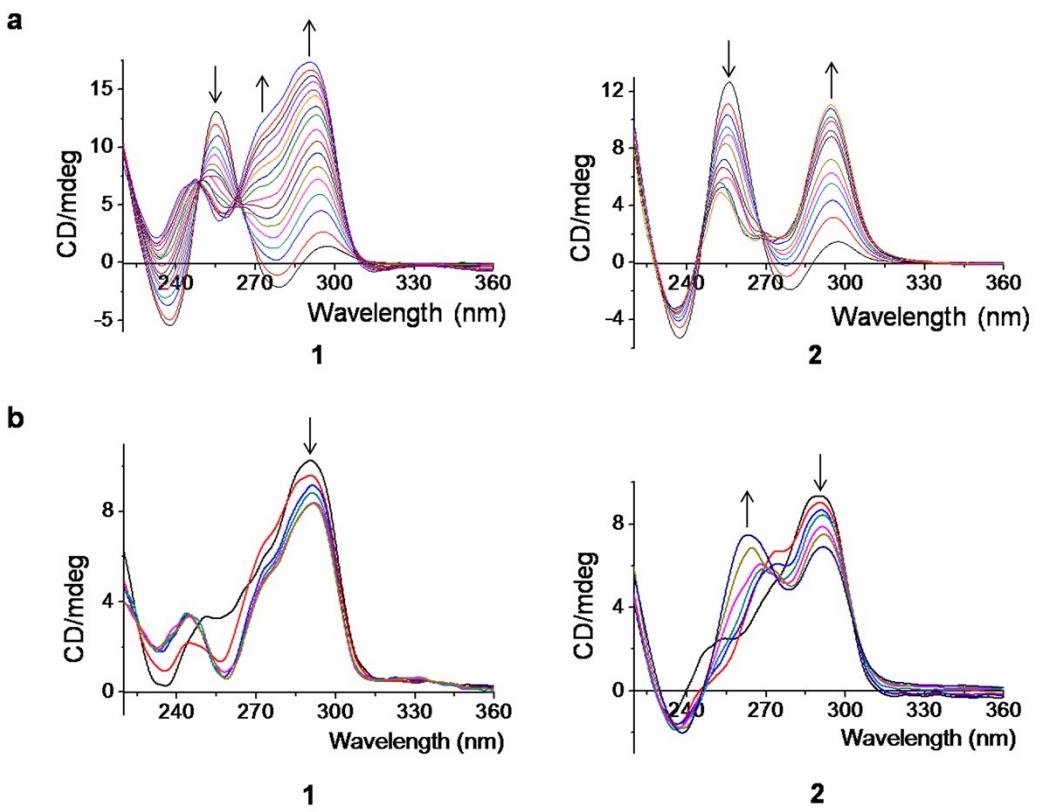

Figure S4. CD spectra of **1** - **2** with a 3μM solution of G-quadruplex in 10mM Tris-HCl, pH 7.4, at room temperature, (r = C_{ML}/C_{DNA}). (a) absence of metal ion, **1**: r =0.2 ~ 3.2, **2**: r =0.2 ~ 2.2. (b) in the presence of 100mM K^+, **1**: r =1.0 ~ 5.0, **2**: r = 0.5 ~ 3.0.

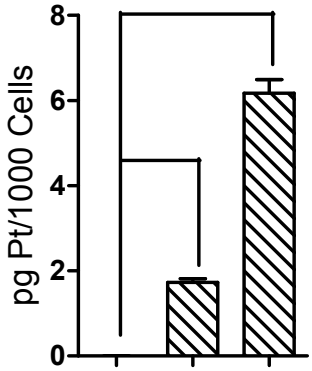

Figure S5. Cellular platinum concentration in HeLa cells after 24 h exposure to **1** or **2** (6.0 μM).

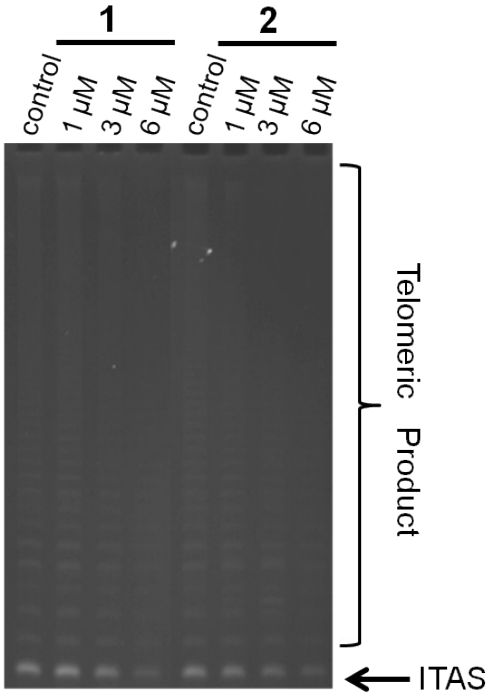

Figure S6. Inhibition effect of **1** and 2 on the telomerase activity by TRAP assay.

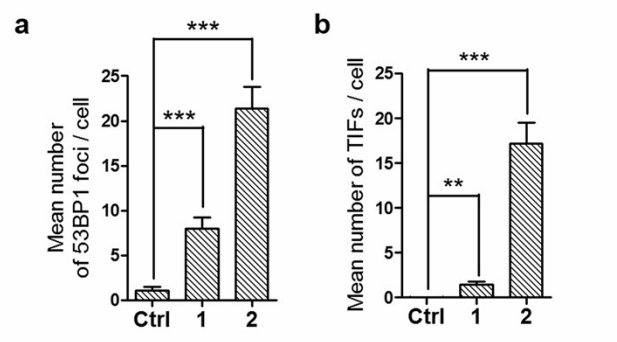

Figure S7. (a) Quantification of figure 3b. The mean number of 53BP1 foci per cell was determined. (b) Quantification of figure 3b. The mean number of TIF foci per cell was determined. For each group, ≥200 cells were examined and values are the average ± SD of three independent experiments.

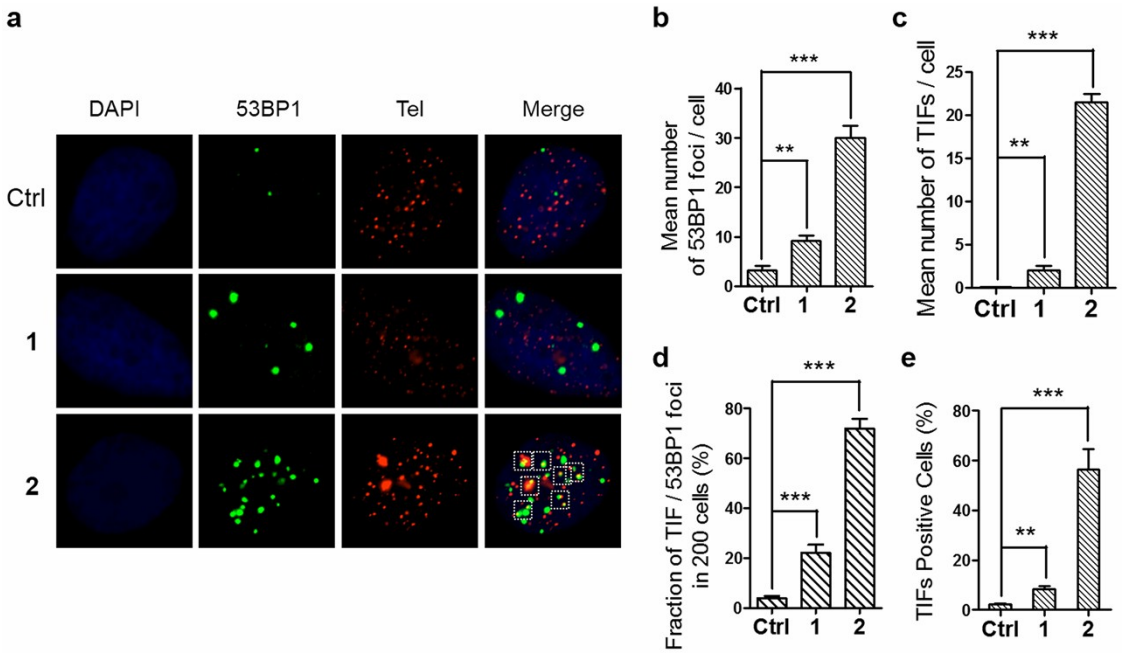

Figure S8. (a) IF-FISH assay for evaluating the telomeric DNA damage. SAOS2 cells were treated with 0.01% DMSO (Ctrl) or **1** and/or **2** (6.0 μM) for 20 days. DAPI, 53BP1 and Telo-Cy3 was the nucleus dye (blue), DNA damage marker (green) and telomere probe (red), respectively. In the bottom panel, higher-magnification view showed the co-localization (yellow) of 53BP1 foci and Telo-Cy3 foci in nucleus termed as Telomere dysfunctional-foci (TIF). (b) Quantification of a. The mean number of 53BP1 foci per cell was determined. (c) Quantification of a. The mean number of TIF foci per cell was determined. (d) Histograms show the fraction of TIFs/53BP1 foci among 200 untreated and Pt(II)-treated cells, respectively. (e) Histograms show the percentage of TIFs-positive cells among 200 untreated and Pt(II)-treated cells, respectively. Cells with three or more 53BP1/Telo-Cy3 foci were scored as TIF positive. For each group, ≥200 cells were examined and values are the average ± SD of three independent experiments.

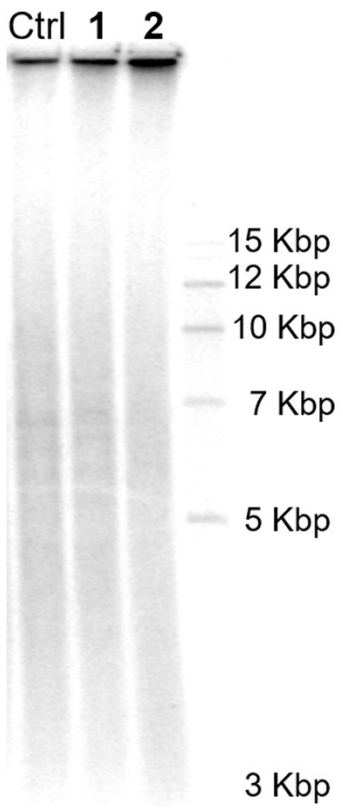

Figure S9. TRF lengths measured in untreated (0.01% DMSO as Ctrl) and **1**- or **2**-treated (6.0 μM over 20 days) SAOS2 cells.

Reference

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