Pt(II) squares as selective and effective human telomeric G-quadruplex binders and potential cancer therapeutics

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Electronic Supporting Information

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). Elemental analysis was recorded on a Vario EL elemental analyzer. X-ray data were taken on a Rigaku R-AXIS SPIDER Image Plate diffractometer with graphite-monochromated Mo Kα radiation (λ = 0.71073 Å). 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.). The flow cytometry assay were performed on a FACSCalibur (BD, USA). Oligonucleotides purified by reversed-phase HPLC were purchased from Sangon (China). Unless otherwise noted, materials obtained from commercial suppliers were used without further purification.

X-ray diffraction measurements: X-ray diffraction measurements were performed on Rigaku R-AXIS SPIDER Image Plate diffractometer with graphite-monochromated Mo Kα radiation (λ = 0.71073 Å). The structures of **4** were solved with direct methods and refined with a full-matrix least-squares technique with the SHELXL program package. Anisotropic thermal parameters were applied to all non-hydrogen atoms. All hydrogen atoms were included in calculated positions and refined with isotropic thermal parameters riding on those of the parent atoms.

FRET melting assay: The fluorescent labeled oligonucleotide F21T (5′-FAM-$G_3[T_2AG_3]_3$-TAMRA-3′, FAM: 6-carboxyfluorescein, TAMRA: 6-carboxy-tetramethylrhodamine), *bcl2* (5′-FAM-AGGGGCGGGCGCGGGAGGAAGGGGGCGGGAGCGGGGCTG-3′-TAMRA) and F10T (5′-FAM-dTATAGCTATA-HEG-TATAGCTATA-TAMRA-3′, HEG linker is $[(-CH_2-CH_2-O-)_6]$) used as the FRET probes, were diluted in 60 mM potassium cacodylate buffer (pH 7.4) and then annealed by heating to 90 ^oC for 5 min, followed by cooling slowly to room temperature overnight. Fluorescence melting curves were determined with a Roche Light Cycler II real-time PCR machine, using a total reaction volume of 20 μL, with a series of different concentrations of complexes and 0.4 μM of labeled oligonucleotide. Measurements were made on a RT-PCR with excitation at 470 nm and detection at 530 nm. Fluorescence readings were taken at intervals of 1 ^oC over the range 37-99 ^oC, with a constant temperature being maintained for 30 s prior to each reading to ensure a stable value. Final analysis of the data was carried out using Origin 7.0 (OriginLab Corp.).

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. In a typical experiment, biotinylated duplex DNA, and biotinylated G-quadruplex (see below) were folded in filtered and degassed running buffer (Tris-HCl 50 mM, pH 7.4, 100mM KCl, 0.005% Tween-20). The DNA samples were then captured (~1000 RU) in flow cells 1, 2 and 3, leaving the fourth flow cell as a blank. Complexes solutions were prepared with running buffer by serial dilutions from stock solutions. Six concentrations were injected simultaneously at a flow rate of 40 μL/min for 4~6 min of association phase, followed by 3 min of disassociation phase at 25 ^oC. 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 G-quadruplexes sensorgrams. Data are analyzed with ProteOn manager software, using the Equilibrium and two states for fitting the data.

Sequences of oligomers used in SPR experiments:

Human telomeric quadruplex, 5'-biotin-d[AGGGTTAGGGTTAGGGTTAGGG]-3'

bcl2 promoter, 5'-biotin-d[AGGGGCGGGCGCGGGAGGAAGGGGGCGGGAGCGGGGCTG]-3'

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

PCR-stop assay: The oligonucleotide HTG21 (5′-GGGTTAGGGTTAGGGTTAGGG-3′) and the corresponding complementary sequence (5′-ATCGCTTCTCGTCCCTAACC-3′, HTG21rev) were used here. The reactions were performed in 1 × PCR buffer, containing 10 pmol of each oligonucleotide, 0.2 mM dNTP, 2.5 U Taq polymerase, and different concentrations of complexes. Reaction mixtures were incubated in a thermocycler with the following cycling conditions: 94 °C for 3 min, followed by 30 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s. PCR products were then analyzed on 15% nondenaturing polyacrylamide gels in 1 × TBE and silver stained.

CD studies: A circular dichroism (CD) study was conducted to observe the effect of **1**, **2**, **3** and **4** on the structure of the htelo G-quadruplex. CD studies were performed on a JASCO J-810 spectropolarimeter at room temperature using a cell length of 1 cm, and over a wavelength range of 220-360 nm, with a scan speed of 200 nm/min with 5 acquisitions. The oligomer 22AG (5′-$AG_3(T_2AG_3)_3$-3′) at a final concentration of 3 μM was resuspended in Tris-HCl buffer (10 mM, pH 7.4) containing 100 mM K^+, 100 mM Na^+, or no metal ions and heated to 90 ^oC 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 (3 μM) with various concentrations of the complexes (3 mM 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.).

Isothermal titration calorimetry (ITC): Calorimetric experiments were performed using a high-sensitivity isothermal titration calorimeter (VP-ITC, MicroCal, Inc., Northampton, MA, U. S. A.). Calorimetric experiments were performed in a K^+-containing solution using the human telomeric G-quadruplex DNA concentration of 20 μM in the sample cell. The concentrations of **1**, **2**, **3** and **4** in the injection tube varied from 800 to 2400 μM. For a typical titration, serial 10 μL of complex were injected into a sample cell of the DNA solution at 240 s intervals with a stirring speed of 372 r.p.m at 25 ^oC. 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 of **1**, **2**, **3** and **4** 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.

Cell lines and culture conditions: HepG2 (Human hepatocellular liver carcinoma cell line), HeLa (Human cervix carcinoma cell line), MCF-7 (Human breast adenocarcinoma cell line), A549 (Human lung adenocarcinoma cell line) and A549/cisR were supplied by Center of Experimental Animal Sun Yat-sen University (Guangzhou, China). Cells were routinely maintained in DMEM (Dulbecco's modified eagle's medium, Gibco BRL) supplemented with 10% fetal bovine serum

(Hyclone), penicillin G (Sigma-Aldrich, 100 U/mL) and streptomycin (Sigma-Aldrich, 100 μg/mL) at 37.0 °C in a humidified atmosphere containing 5% CO2.

The telomeric repeat amplification protocol (TRAP) assay: Inhibition of telomerase activity was detected by a telomeric repeat amplification protocol (TRAP). Telomerase extract (0.75 ng/μl) was prepared from HeLa cells and used for every reaction in a final volume of 20 μl consisiting of TRAP buffer (20 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 63 mM KCl, 0.05% Tween-20 and 1 mM EGTA in DEPC water), 0.125 mM dNTP, 0.25 μM TS primer, 0.25μM ACX primer, 0.25 μM NT primer, $2*10^{-22}$ mol TSNT template, 1 U hot-shart Taq ploymerase and the Pt(II) square. And then program the mixture for 30 min of incubation at 30 ^{o}C, followed by 35 cycles of PCR reaction: 95 ^{o}C for 30s, 50 ^{o}C for 30s, and 72 ^{o}C for 60s. DNA products were resolved on a 8 % polyacrylamide gel and visualize under UV illumination.

MTT assay: The cells were harvested during the exponential phase and seeded equivalently into 96-well plates The Pt(II) squares were added to the wells to achieve final concentrations. Twenty microliters of a stock MTT dye solution (5 mg/mL) was added to each well after a 44 h incubation. DMSO (100 μL) was added to solubilize the MTT formazan after an additional incubation period of 4 h. 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.

The flow cytometry: A549/cisR cells were seeded in a 12-well plate at 37 ^{o}C and cultured until they reached 70% confluency. Cisplatin and Pt(II) squares solutions (60 μM) were added and incubated with the cells for 4 h. The followed experimental protocols was afford by the Alexa Fluor® 488 annexin V/Dead Cell Apoptosis Kit with Alexa® Fluor 488 annexin V and PI for Flow Cytometry.

Synthetic Details and Characterization

Synthesis of [Pt(en)(4,4'-dipyridyl)]$_4$(NO$_3$)$_8$ (1): was prepared using the same procedure applied by Sleiman[1]. Yield: 88%. Elemanal. Calcd (%) for $C_{48}H_{64}N_{24}O_{24}Pt_4 \cdot 12H_2O$ (2356.54): C, 24.45; H, 3.76; N, 14.26. Found: C, 24.32; H, 3.53; N, 14.26. ^{195}Pt NMR (500MHz, D_2O): -1080 ppm.

Synthesis of [Pt(NH$_3$)$_2$(4,4'-dipyridyl)]$_4$(NO$_3$)$_8$ (2): This was prepared using the procedure as for **4**, except that 4,4'-dipyridyl (0.173 g, 0.50 mmol), and at 95 ^{o}C for 4 days under N_2. Yield: 73%. Elemanal. Calcd (%) for $C_{40}H_{56}N_{24}O_{24}Pt_4 \cdot 11H_2O$ (2234.48): C, 21.49; H, 3.52; N, 15.04. Found: C,21.69; H, 3.29; N, 14.72. ^{195}Pt NMR (500MHz, D_2O): -877 ppm.

Synthesis of [Pt(en)(pyrazine)]$_4$(NO$_3$)$_8$ (3): Pt(en)Cl$_2$ (0.16 g, 0.50 mmol), synthesized according to a literature reported procedure[2], and $AgNO_3$ (0.17 g, 1.00 mmol) were stirred in water (5 mL) in a stoppered flask with the exclusion of light for 24 h at 60 ^{o}C and then filtered from AgCl. The clear filtrate was then transferred to another stoppered flask where pyrazine (0.04 g, 0.50 mmol) was added and heated at 100 ^{o}C for 2 hours under N_2. The solution was then concentrated to about 2 mL, and addition of ethanol (25 mL) produced the precipitate of the desired complex. This was collected by filtration, washed with ethanol and ether, and dried under vacuum. Yield: 86%. Elemanal. Calcd (%) for $C_{24}H_{48}N_{24}O_{24}Pt_4 \cdot 2H_2O$: C, 15.39; H, 2.80; N, 17.95. Found: C,15.36; H, 2.81; N, 18.10. ^{195}Pt NMR (500 MHz, D_2O): -1123 ppm.

Synthesis of [Pt(NH$_3$)$_2$(pyrazine)]$_4$(NO$_3$)$_8$ (4): was prepared using the same procedure applied by Lippert[3] and characterized by X-ray structure analysis. Yield: 76%. Elemanal. Calcd (%) for $C_{16}H_{40}N_{24}O_{24}Pt_4 \cdot 3.67H_2O$: C, 10.68; H, 2.65; N, 18.69. Found: C,10.78; H, 2.58; N, 18.61. ^{195}Pt NMR (500 MHz, D_2O): -895 ppm. Slow evaporation of a concentrated aqueous solution of **4** yielded white suitable for X-ray analysis.

Table S1. The main kinetic rate constants and the chi2 (x^2) value of SPR data

DNA	k_a ($M^{-1}S^{-1}$)[a]	k_d (S^{-1}) [a]	K_A (M^{-1}) [b]	K_D (M) [b]	Chi2 [c]
			1		
htelo	-	-	2.95×10^6	3.39×10^{-7}	8.12
bcl2	-	-	6.29×10^5	1.59×10^{-6}	4.29
dsDNA	-	-	4.44×10^5	2.55×10^{-6}	6.89
			2		
htelo	1.19×10^4	1.34×10^{-3}	8.85×10^6	1.13×10^{-7}	7.35
bcl2	3.57×10^4	1.26×10^{-1}	2.82×10^5	3.54×10^{-6}	5.61
dsDNA	7.70×10^3	3.12×10^{-2}	2.46×10^5	4.06×10^{-6}	9.58
			3		
htelo	-	-	7.58×10^5	1.32×10^{-6}	3.84
bcl2	-	-	3.27×10^5	3.06×10^{-6}	2.94
dsDNA	-	-	2.46×10^5	4.06×10^{-6}	2.86
			4		
htelo	-	-	5.75×10^5	1.74×10^{-6}	3.39
bcl2	-	-	4.85×10^4	2.06×10^{-5}	5.04
dsDNA	-	-	4.15×10^5	2.41×10^{-6}	5.68

[a] Kinetic constants (k_a and k_d are the corresponding association and dissociation rate constants) determined from ProteOn analysis by using Langmuir model fitting of 4~6 min association and 3 min disassociation. [b] K_A determined from k_a / k_d or 1/ K_D, K_D determined from k_d / k_a or the equilibrium model. [c] The chi2 (x^2) value is a standard statistical measure of the closeness of fit. x^2 is of the same order of magnitude as the noise in RU. And the fitting process is terminated automatically when a minimum value is found for x^2.

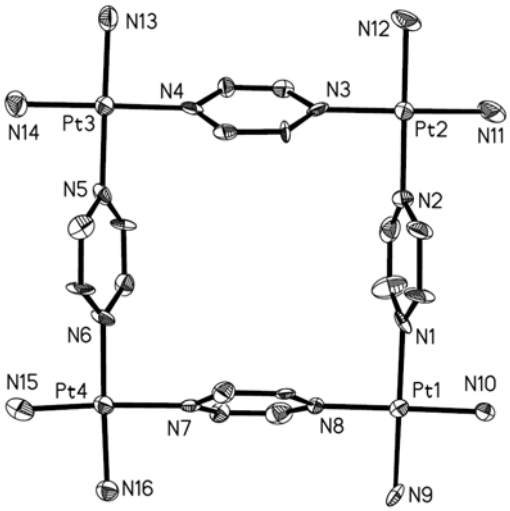

Fig. S1 X-Ray crystal structure of **4**. Hydrogen atoms are omitted for clarity. Selected bond distances (Å), the distances of Pt atoms and angles (deg): Pt1-N1 1.993(17), Pt1-N8 1.934(15), Pt1-N9 2.036(18), Pt1-N10 2.001(16), Pt1···Pt2 6.800, Pt2···Pt3 6.795, N8-Pt1-N1 90.2(6), N3-Pt2-N2 90.7(7), N4-Pt3-N5 90.2(7), N7-Pt4-N6 90.7(7).

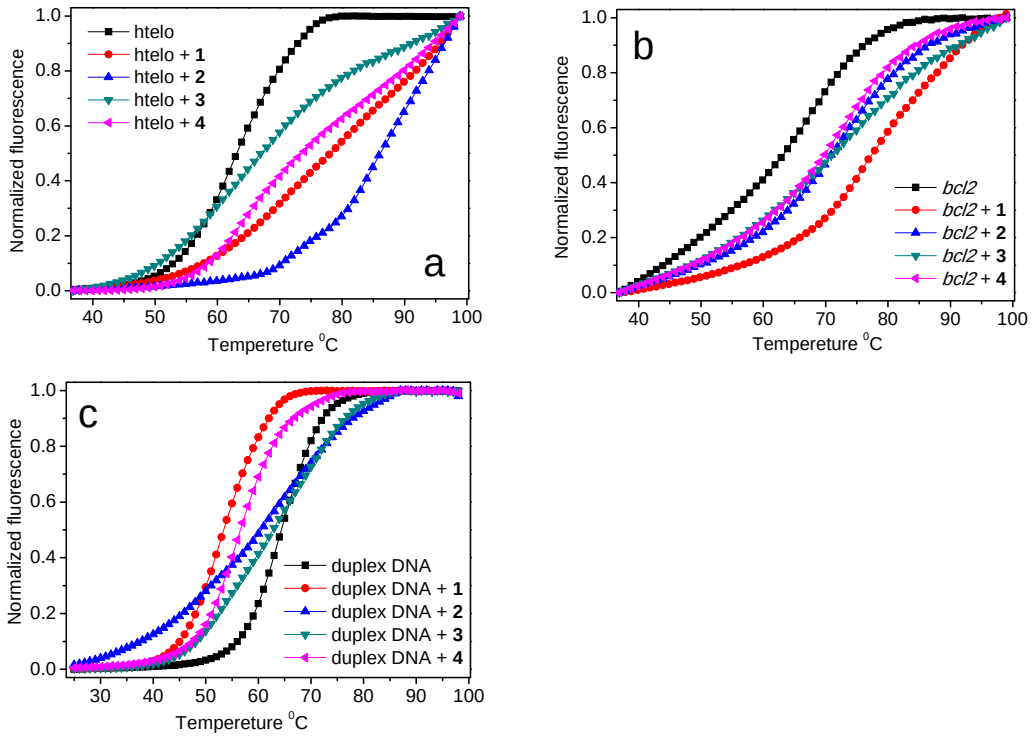

Fig. S2 The FRET stabilization curves of **1** (0.6 μM), **2** (0.6 μM), **3** (1.0 μM) and **4** (1.0 μM) with two G-quadruplex and duplex DNA. The concentrations of DNA were 400 nM in 60 mM potassium cacodylate buffer (pH 7.4). a, the human telomeric (htelo) G-quadruplex; b, *bcl2* promoter G-quadruplex; c, the duplex DNA.

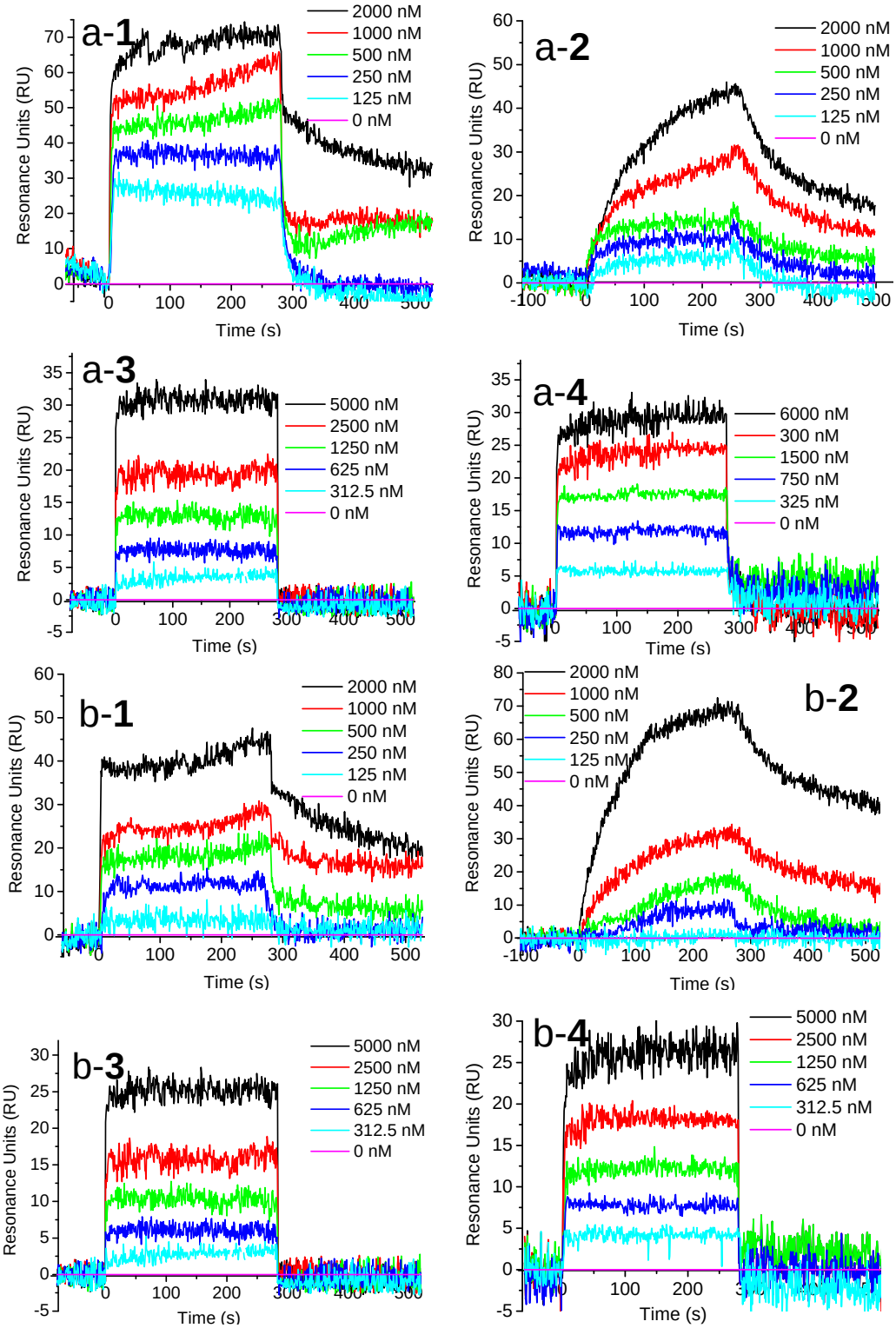

Fig. S3 SPR sensorgram overlay for binding of **1** (a-**1**, b-**1**), **2** (a-**2**, b-**2**), **3** (a-**3**, b-**3**) and **4** (a-**4**, b-**4**) to the immobilized G-quadruplex in the presence of 50 mM Tris-HCl, pH 7.4, 100mM KCl, 0.005% Tween-20 at 25 ^{o}C. **a,** *bcl2* G-quadruplex; **b,** the duplex DNA.

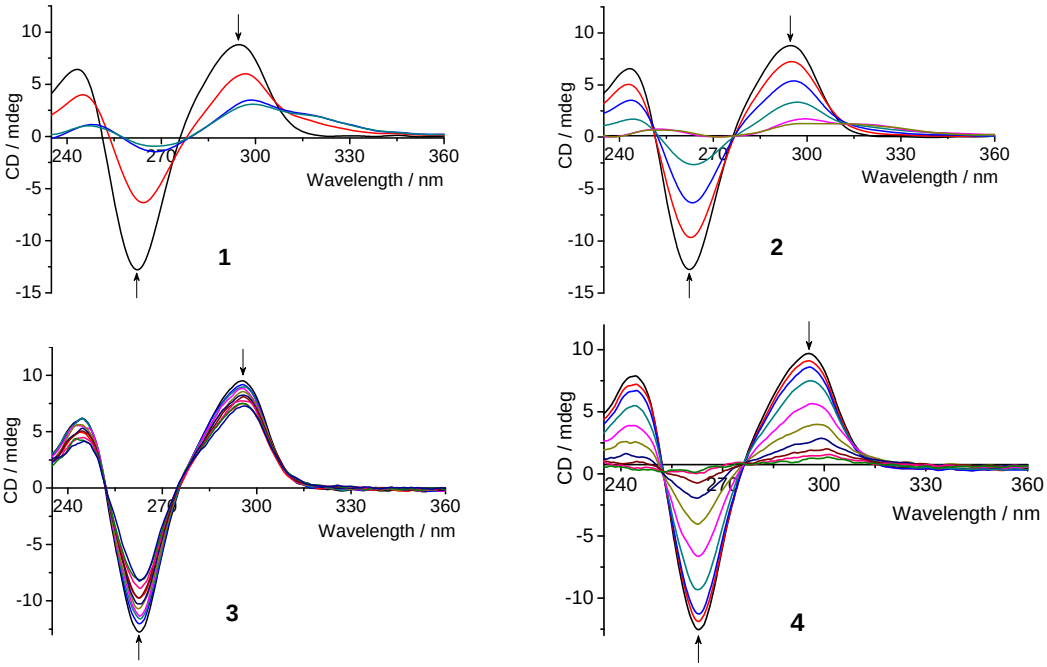

Fig. S4 CD spectra of G-quadruplex induced by **1**, **2**, **3** and **4** with a 3 μM G-quadruplex in 10 mM Tris-HCl, pH 7.4, 100 mM NaCl at room temperature. (r = C_{ML}/C_{DNA}). **1**: r =0 ~ 3; **2**: r =0 ~ 5; **3**: r =0 ~ 10; **4**: r =0 ~ 9.

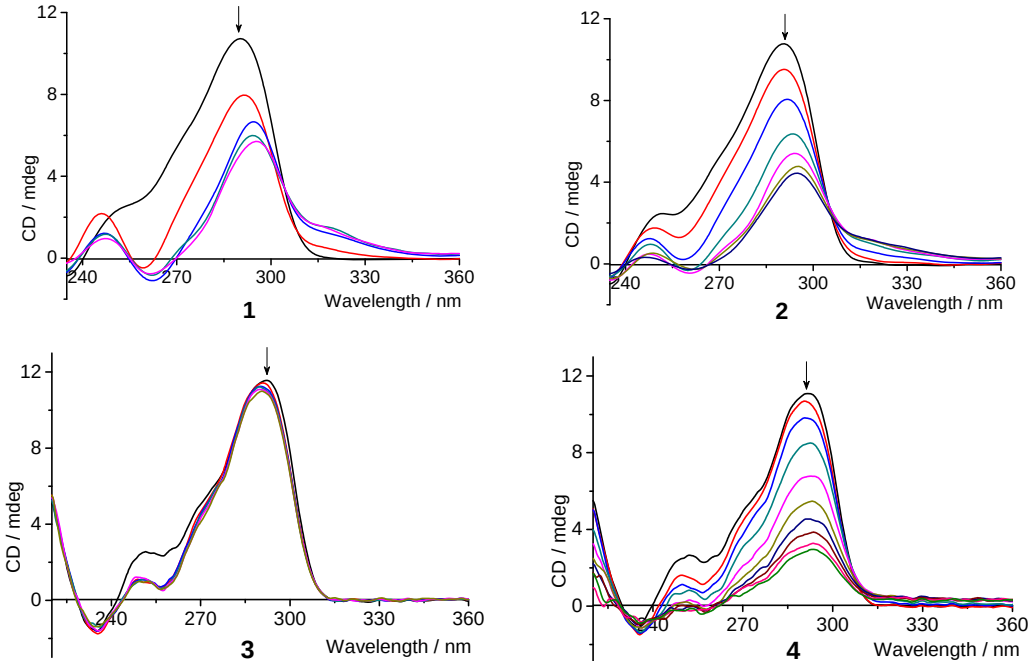

Fig. S5 CD spectra of G-quadruplex induced by **1**, **2**, **3** and **4** with a 3 μM G-quadruplex in 10 mM Tris-HCl, pH 7.4, 100 mM KCl at room temperature. (r = C_{ML}/C_{DNA}). **1**: r =0 ~ 4; **2**: r =0 ~ 6; **3**: r =0 ~ 5; **4**: r =0 ~ 9.

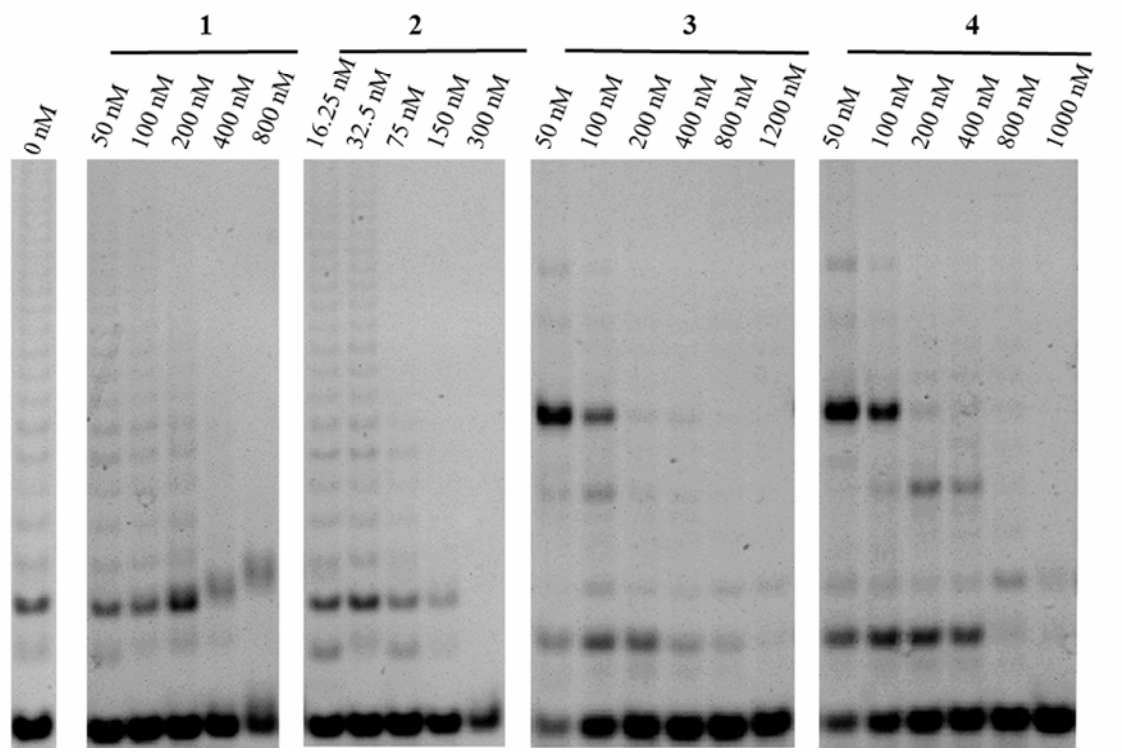

Fig. S6 The TRAP assay for **1**, **2**, **3** and **4**, showing ladders generated by the action of telomerase on a TS primer (PCR amplified).

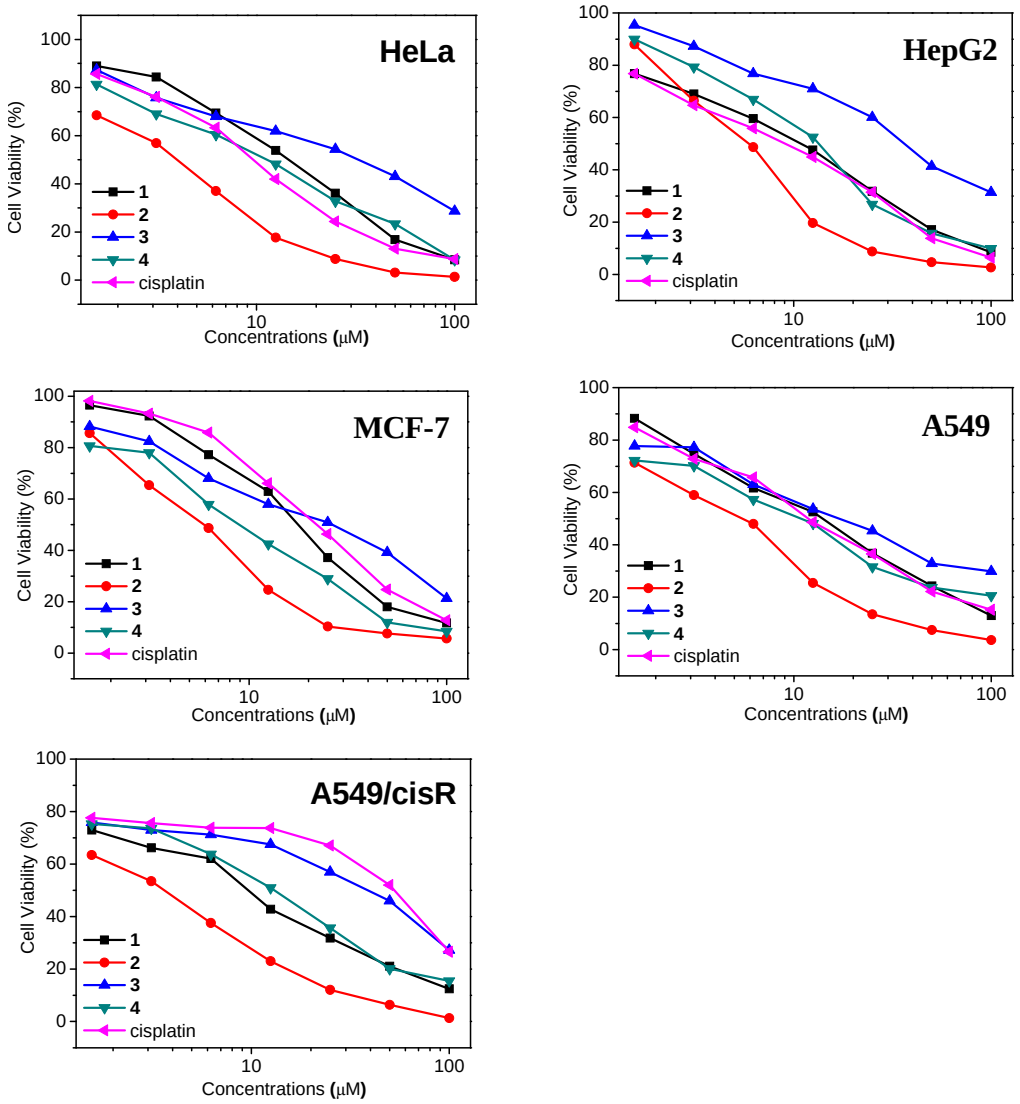

Fig. S7 Concentration-dependent cytotoxic effects of Pt(II) squares and cisplatin on indicated cell lines determined by MTT assay after 48 h treatment. Data were presented as means of three independent experiments ± standard deviations and the duration of treatment was 48 h.

References.

1. Kieltyka, R.; Englebienne, P.; Fakhoury, J.; Autexier, C.; Moitessier, N.; Sleiman, H. F., A platinum supramolecular square as an effective G-quadruplex binder and telomerase inhibitor. *J Am Chem Soc* **2008,** *130* (31), 10040-1.

2. Kemp, S.; Wheate, N. J.; Buck, D. P.; Nikac, M.; Collins, J. G.; Aldrich-Wright, J. R., The effect of ancillary ligand chirality and phenanthroline functional group substitution on the cytotoxicity of platinum(II)-based metallointercalators. *J Inorg Biochem* **2007,** *101* (7), 1049-58.

3. Willermann, M.; Mulcahy, C.; Sigel, R. K.; Cerda, M. M.; Freisinger, E.; Sanz Miguel, P. J.; Roitzsch, M.; Lippert, B., Pyrazine as a building block for molecular architectures with PtII. *Inorg Chem* **2006,** *45* (5), 2093-9.