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Platinum squares with high selectivity and affinity for human telomeric G-quadruplexes

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Electronic Supporting Information

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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). 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.). UV/Vis spectra were recorded on a Varian Cary 100 UV-Vis spectrophotometer equipped with a temperature controller. Molecular Modeling Analysis performed on a Surflex-Dock suite1 (SYBYL 7.3.5, Tripos, Inc., St. Louis, MO, U. S. A.) and the images in the manuscript were created from SYBYL 7.3 with the intercalation site. 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 **1** were solved with direct methods and refined with a full-matrix least-squares technique with the SHELXL program package[1]. 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-tetramethylrhod amine) 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, 3 and 4, leaving the fifth 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

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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 two states model for fitting kinetic data.

Sequences of oligomers used in SPR experiments:

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

c-myc promoter, 5'- biotin-d[TGGGGAGGGTGGGGAGGGTGGGGAAGG]-3'

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

duplex (ds) 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** and **2** on the structure of the 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^+, 10 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** and **2** 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** and **2** 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.

Continuous variation analysis: Continuous variation analysis was performed according to a previously reported literature procedure.[2] Stock solutions 10 μM of **1** and **2** were prepared. The human telomeric G-quadruplex solution was made to match the concentration of the stock solutions in 10 mM Tris-HCl and 100 mM KCl at pH 7.4. The total concentrations of the complex solutions were

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maintained at 10 μM throughout the titrations. Before each spectrum collected, the complex solutions were allowed to equilibrate for at least 60 min. The data were collected at the feature absorption wavelength of each complex, and the complex at the concentration without G-quadruplex being measured as the reference. The data analysis was performed in Origin 7.0.

Molecular modeling analysis: Molecular docking analyses of **1** - **2** and the crystallographic structure of parallel 22-mer telomeric G-quadruplexes (PDB ID 1KF1) were performed using the Surflex-Dock suite[3] (SYBYL 7.3.5, Tripos, Inc., St. Louis, MO, U. S. A.). Hydrogen atoms were neglected in **1-2**, and water molecules were removed from the PDB file. The Surflex-Dock used an empirical scoring function and a patented search engine to dock the complexes into telomeric G-quadruplexes to identify the most stable and favorable orientations. The images in the manuscript were created from SYBYL 7.3 with the intercalation site.

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 (cisplatin sensitive) and A549/cisR (cisplatin resistant) human lung adenocarcinoma cell line 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% CO_2.

The telomeric repeat amplification protocol (TRAP) assay: Inhibition of telomerase activity was detected by a telomeric repeat amplification protocol (TRAP).[4] 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 ^oC, followed by 30-35 cycles of PCR reaction: 95 ^oC for 30s, 50 ^oC for 30s, and 72 ^oC for 60s. DNA products were resolved on a 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) 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.

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Synthetic Details and Characterization

Synthesis of [Pt(en)(quinoxaline)]$_4$(NO$_3$)$_8$ (1): Pt(en)Cl$_2$ (0.163 g, 0.50 mmol), synthesized according to a previously reported procedure.[5] And AgNO$_3$ (0.170 g, 1.00 mmol) was stirred in water (5 mL) in a stoppered flask with the exclusion of light for 24 h at 60 ^{o}C and filtered to remove AgCl. The clear filtrate was transferred to another stoppered flask. and quinoxaline (0.065 g, 0.50 mmol) was added. The solution was heated to 60 ^{o}C for 3 days under N$_2$. The solution was concentrated to approximately 2 mL, and ethanol (25 mL) was added to precipitate the desired complex. The precipitate was collected by filtration, washed with ethanol and ether, and dried under a vacuum. The slow evaporation of a concentrated aqueous solution of **1** yielded pink crystals that were suitable for X-ray analysis. Yield: 54%. Elemanal. Calcd (%) for C$_{40}$H$_{56}$N$_{24}$O$_{24}$Pt$_4$·5H$_2$O (2127.53): C, 22.58; H, 3.13; N, 15.80. Found: C, 22.59; H, 3.19; N, 15.68. ^{195}Pt NMR (500 MHz, D$_2$O): -1080 ppm.

Synthesis of [Pt(NH$_3$)$_2$(quinoxaline)]$_4$(NO$_3$)$_8$ (2): This was prepared using the procedure as for **1**, except that *cis*-Pt(NH$_3$)$_3$Cl$_2$ (0.150 g, 0.50 mmol), and at 40 ^{o}C for 4 days under N$_2$. Yield: 63%. Elemanal. Calcd (%) for C$_{32}$H$_{48}$N$_{24}$O$_{24}$Pt$_4$·5H$_2$O (2021.24): C, 19.00; H, 2.89; N, 16.61. Found: C,19.36; H, 2.93; N, 16.53. ^{195}Pt NMR (500MHz, D$_2$O): -878 ppm.

Table S1. Crystallographic data of **1**

Complex	**1**		
Formula	C$_{40}$H$_{66}$N$_{24}$O$_{29}$Pt$_4$	c (Å)	16.3334(8)
Molecular weight	2127.53	α (Å)	97.317(1)
Description	Block, wine-purple	β (Å)	91.554(1)
Temperature (K)	293(2)	γ (Å)	106.389(1)
Crystal size (mm)	0.11 × 0.04 × 0.03	Volume Å^3	3249.9(3)
λ (Å)	0.71073	Z	2
Crystal system	Triclinic	R1[a][I > 2σ(I)]	0.0707
Space group	P-1	WR2[a]	0.2044
a (Å)	14.3774(8)	GOF[b]	1.085
b (Å)	14.5747(8)		

[a] $R1 = \sum \| |F_0| - |F_c| \| / \sum |F_0|$, $wR2 = \left\{ \sum \left[w\left(F_0^2 - F_c^2\right)^2 \right] / \sum \left[w\left(F_0^2\right)^2 \right] \right\}^{1/2}$

[b] $GOF = \left\{ \sum \left[w\left(F_0^2 - F_c^2\right)^2 / (n-p) \right] \right\}^{1/2}$ where n is the number of data and p is the number of parameters refined.

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Table S2. Important bond lengths (Å) and bond angles (^{0}) for **1**

Pt(1)-N(1)	2.023(11)	Pt(1)-N(9)	2.024(13)
Pt(1)-N(10)	2.030(13)	Pt(1)-N(8)	2.046(12)
Pt(2)-N(2)	2.031(12)	Pt(2)-N(3)	2.045(12)
Pt(2)-N(11)	1.992(13)	Pt(2)-N(12)	2.021(13)

N(1)-Pt(1)-N(9)	172.6(5)	N(1)-Pt(1)-N(10)	90.4(5)
N(9)-Pt(1)-N(10)	83.4(5)	N(1)-Pt(1)-N(8)	95.2(5)
N(9)-Pt(1)-N(8)	91.2(5)	N(10)-Pt(1)-N(8)	173.8(5)
N(2)-Pt(2)-N(12)	177.5(5)	N(3)-Pt(2)-N(2)	89.8(5)
N(3)-Pt(2)-N(11)	178.9(9)	N(3)-Pt(2)-N(12)	92.7(5)
N(11)-Pt(2)-N(2)	93.8(5)	N(11)-Pt(2)-N(12)	83.7(6)

Table S3. 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	7.14×10^3	4.33×10^{-3}	1.65×10^6	6.06×10^{-7}	4.42
c-myc	3.85×10^3	5.10×10^{-3}	7.58×10^5	1.32×10^{-6}	5.50
bcl2	5.16×10^3	2.53×10^{-2}	2.04×10^5	4.90×10^{-6}	3.65
dsDNA	4.32×10^4	1.08×10^0	4.02×10^4	2.49×10^{-5}	3.58
		2			
htelo	9.47×10^3	1.77×10^{-3}	5.38×10^6	1.86×10^{-7}	4.00
c-myc	5.05×10^3	2.95×10^{-2}	1.71×10^5	5.85×10^{-6}	2.46
bcl2	5.61×10^3	1.12×10^{-2}	5.03×10^5	1.99×10^{-6}	1.89
dsDNA	3.56×10^4	7.87×10^{-1}	4.52×10^4	2.21×10^{-5}	3.86

[a] Kinetic constants (k_a and k_d are the corresponding association and dissociation rate constants) determined from ProteOn analysis by using two states fitting of 4~6 min association and 3 min disassociation. [b] K_A determined from k_a / k_d, K_D determined from k_d / k_a. [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.

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Table S4. Thermodynamics of the Pt(II) square-quadruplex interaction [a]

Complex	n[b]	K_b/M^{-1}	ΔH/cal mol^{-1}	ΔS/cal mol^{-1}	ΔG/cal mol^{-1}
1	6	1.15×10^7	1.53×10^4	83.5	-9.59×10^3
2	6	1.00×10^9	3.19×10^4	148.0	-1.22×10^4

[a,b] The binding curves obtained for **1** and **2** were well fitted using a sequential binding sites model. The experimental error for each thermodynamic property is <5%; n refers to the stoichiometry of the Pt(II) squares/cubes molecules interacting in each binding event, respectively.

Table S5. The IC_{50} values determined by MTT assay [a]

Complex	IC_{50} (μM)				
	HeLa	HepG2	MCF-7	A549	A549/cisR
1	13.2 ± 2.7	15.6 ± 2.2	15.8 ± 1.5	20.9 ± 2.1	22.9 ± 3.5
2	5.9 ± 1.4	7.1 ± 0.9	6.3 ± 0.5	9.1 ± 1.2	6.5 ± 0.7
cisplatin	10.7 ± 3.6	14.1 ± 2.5	25.1 ± 3.4	12.5 ± 1.6	64.6 ± 5.9

[a] IC_{50} values were drug concentrations necessary for 50% inhibition of cell viability. The percentage of cell viability was calculated using the equation: (mean OD of treated cells/mean OD of control cells) × 100%. Data were presented as means ± standard deviations obtained in at least three independent experiments and drug treatment period was 48 h.

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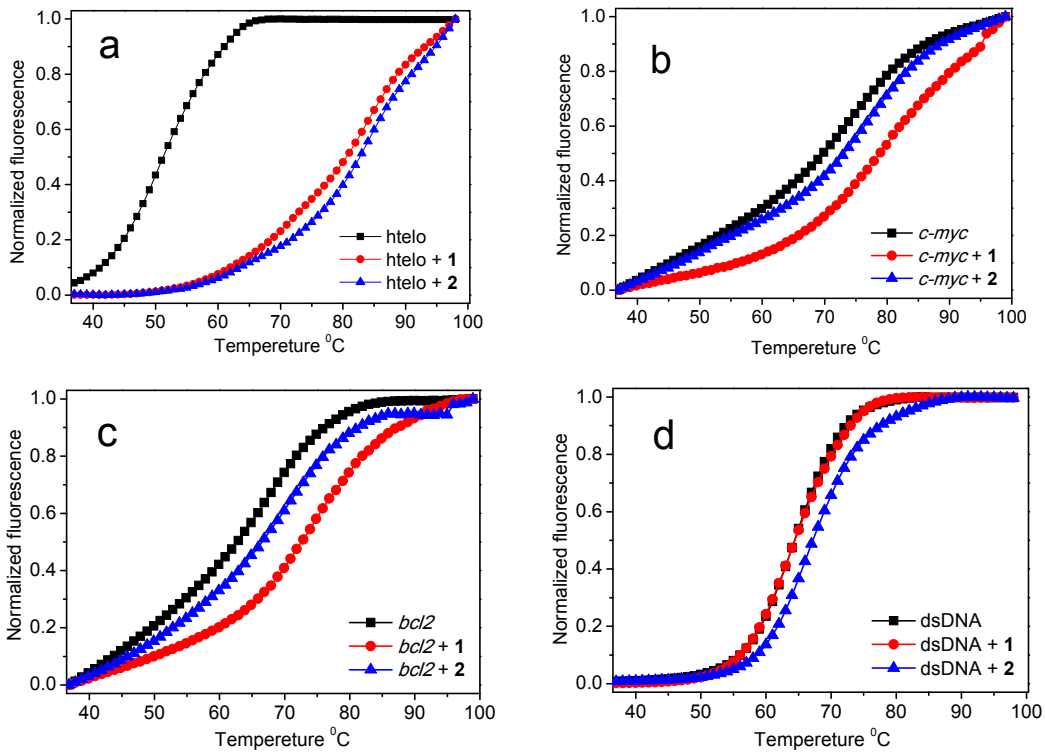

Fig. S1. FRET stabilization curves of **1** (0.5 μM) and **2** (0.5 μM) with all three quadruplex DNA and the duplex DNA. The concentration of quadruplex DNA were 400 nM in 60 mM potassium cacodylate buffer (pH 7.4). a, the human telomeric G-quadruplex; b, *c-myc* promoter G-quadruplex; c, *bcl2* promoter G-quadruplex; d, the duplex DNA (dsDNA).

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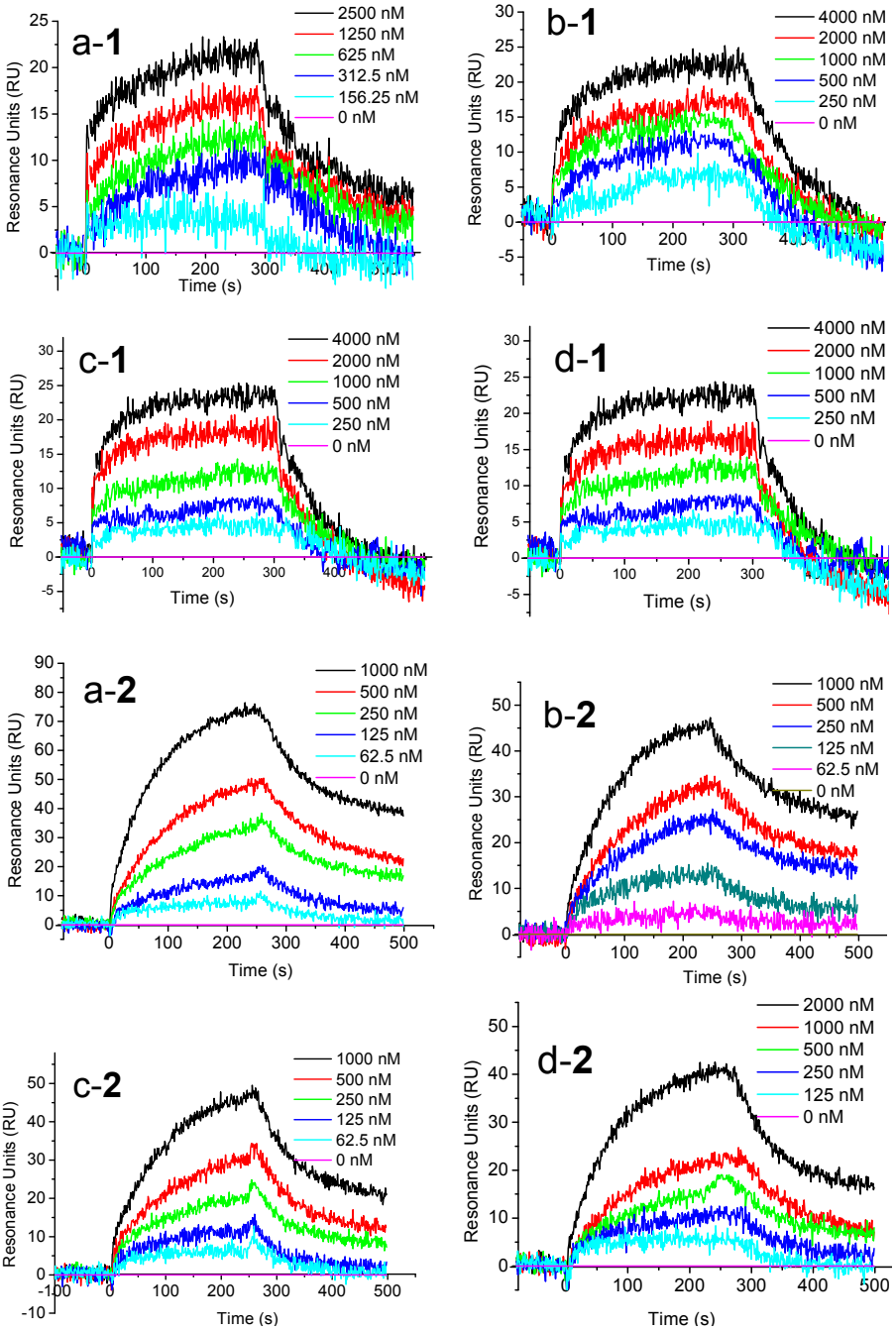

Fig. S2. SPR sensorgram overlay for binding of **1** (a-**1**, b-**1**, c-**1**, d-**1**) and **2** (a-**2**, b-**2**, c-**2**, d-**2**) to the immobilized G-quadruplex in the presence of 50 mM Tris-HCl, pH 7.4, 100mM KCl, 0.005% Tween-20 at 25 ^{o}C. **a,** the human telomeric G-quadruplex; **b,** c-myc G-quadruplex; **c,** bcl2 G-quadruplex; **d,** the duplex DNA.

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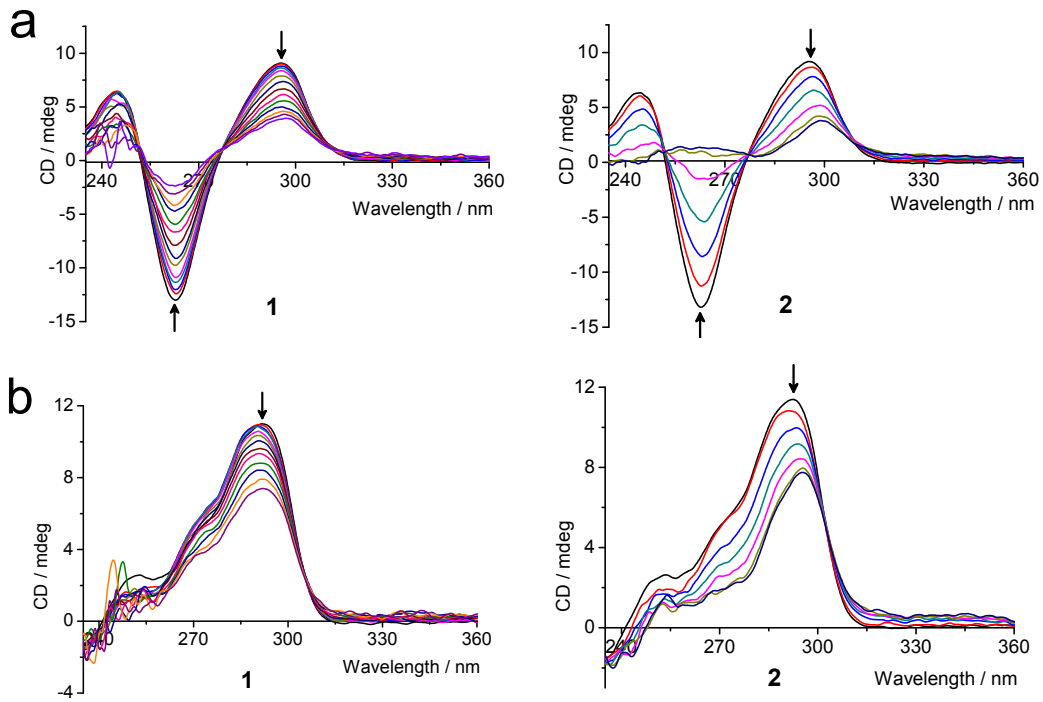

Fig. S3. CD spectra of G-quadruplex induced by **1** and **2** the presence of the Na^+ and the K^+ cations. (r = C_{ML}/C_{DNA}). (a), CD spectra of the complexes with a 3 μM G-quadruplex in 10 mM Tris-HCl, pH 7.4, 100 mM NaCl at room temperature. **1**: r =0 ~ 13; **2**: r =0 ~ 6. (b), CD spectra of the complexes with a 3 μM G-quadruplex in 10 mM Tris-HCl, pH 7.4, 100 mM KCl at room temperature. **1**: r =0 ~ 12, **2**: r =0 ~ 6.

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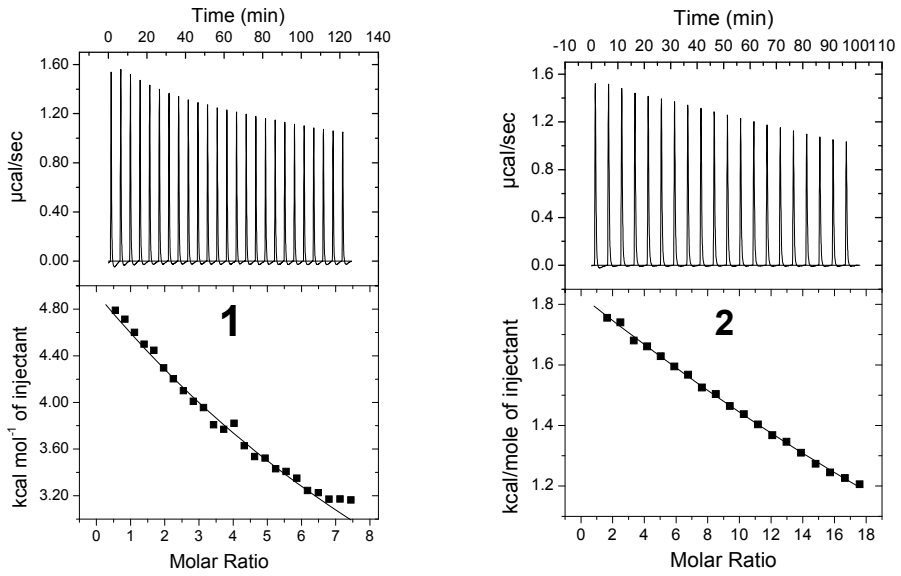

Fig. S4. ITC data for the binding of **1** (K_b = 6.32 × 10^2 M^{-1}) and **2** (K_b = 1.28 × 10^3 M^{-1}) to the buffer. The data were obtained in a buffer of 100 mM KCl and 10 mM Tris-HCl (pH 7.0) at 25.0 °C.

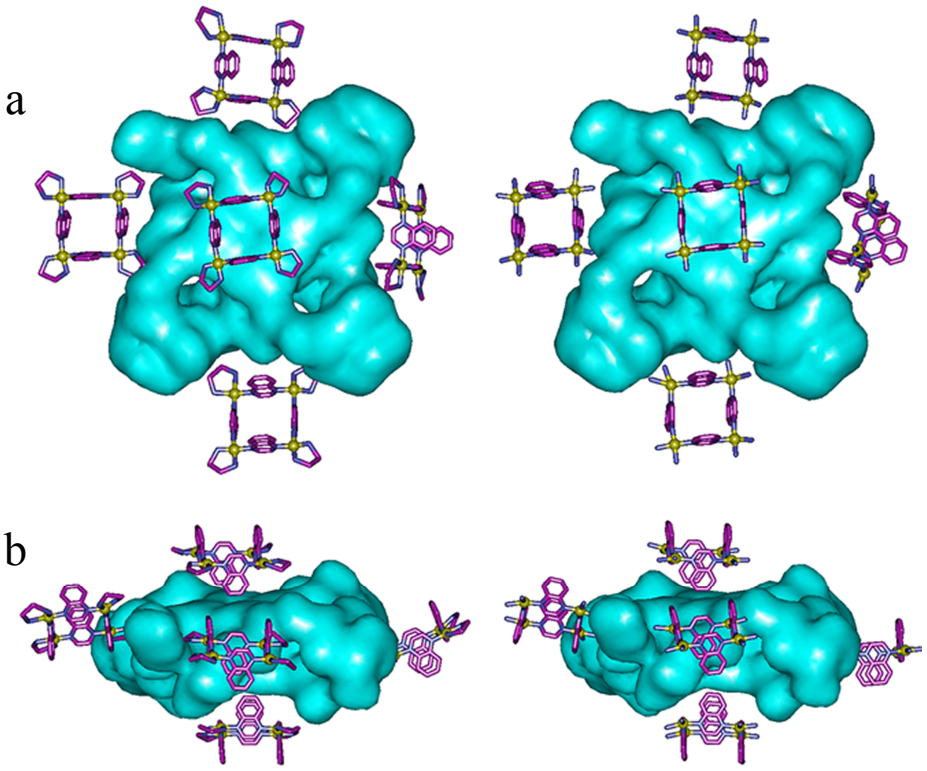

Fig. S5. The most favourable binding modes of **1** (left) and **2** (right) towards 22-mer G4-DNA. (a) Top view. (b) Side view. The crystal structure of the native 22-mer G4-DNA[6] (PDB ID 1KF1) and the crystal structures of **1** are used. **1** and **2** are in red and pink (shown as a stick and ball model).

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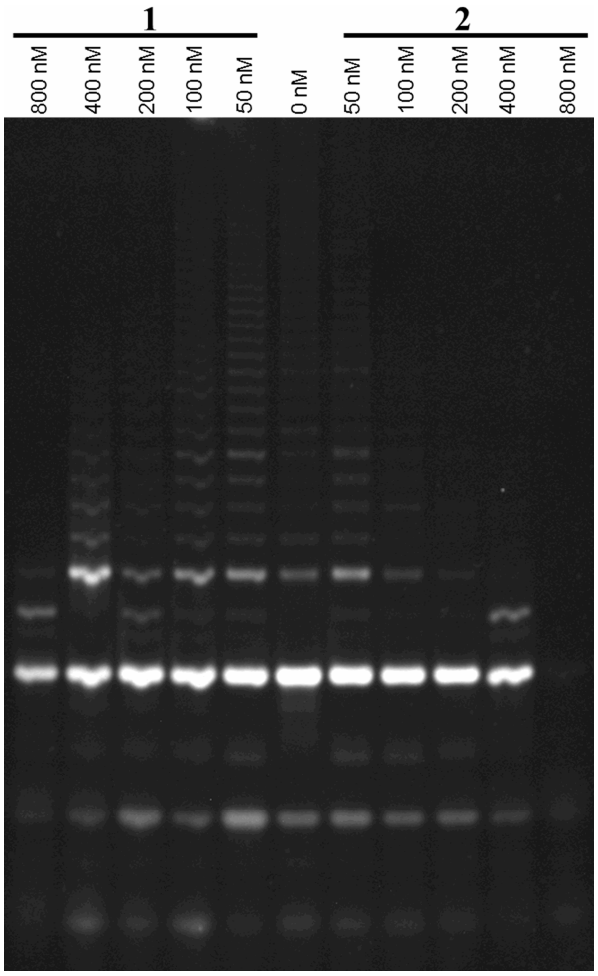

Fig. S6. TRAP assay of **1** and **2**, showing ladders generated by the action of telomerase on a TS primer (PCR amplified).

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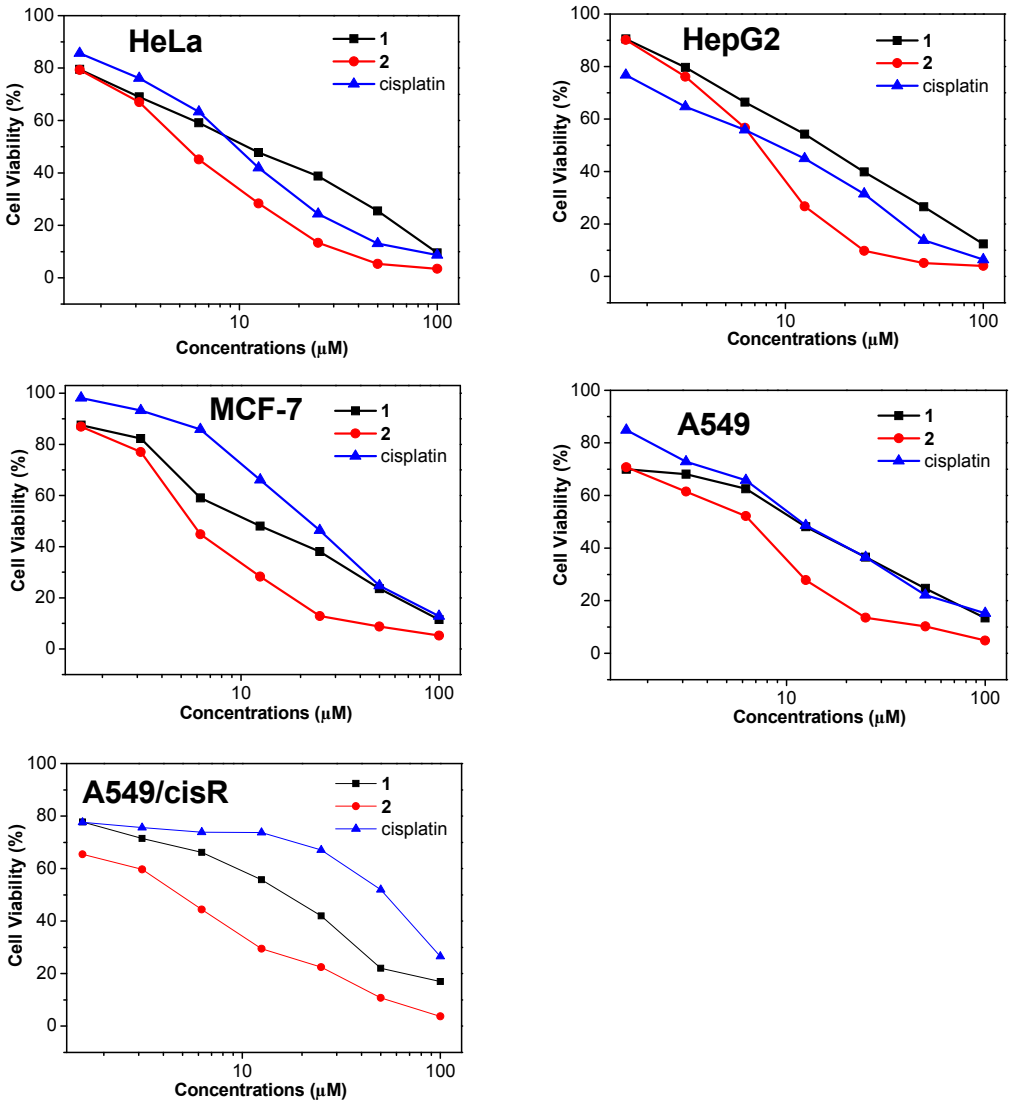

Fig. S7. Concentration-dependent cytotoxic effects of Pt(II) squares/cubes and cisplatin on indicated cell lines determined by MTT assay after 48 h treatment. Data were presented as means of three independent experiments ± standard deviations and the duration of treatment was 48 h.

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