Platinum(II) clovers targeting G-quadruplexes and their anticancer activities

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1 Experimental details

1.1 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 NMR (400 MHz) spectra were recorded on a Bruker AVANCE 400, Bruker, Germany. The mass spectra were recorded on a LCQ DECA XP, Thremo, USA. Elemental analysis was recorded on a Vario EL elemental analyzer. Fluorescence resonance energy transfer(FRET) themal melting curves were obtained on a Roche Light Cycler II real-time PCR machine. Job-plot and UV-Vis spectroscopy were carried out on UV3150 UV-vis spectrophotometer(Shimadzu, Japan). Steady-state fluorescence spectroscopy study were performed on FLS920 fluorescence spectrophotometer(Edinburgh,UK).Circular dichroism (CD) spectra were recorded on a JASCO J-810 CD spectrometer.Oligonucleotides purified by ultrapage were purchased from Sangon (China). Unless otherwise noted, materials obtained from commercial suppliers were used without further purification.

1.2 Synthesis and characterization of clover-like platinum porphyrins

The supramolecular self-assembly synthetic route is shown in scheme **1** and scheme **2**. The involved platinum complex side arms——Pt(DPA)Cl(DPA=2,2′-Dipicolylamine) and Pt(dien)Cl (dien= diethylenetriamine) are prepared according to a reported literature.[1] Commercially available TPyP4 (5,10,15,20-Tetra(4-pyridyl)porphyrin)is bought directly without synthesis and further purification.

Pt(DPA)Cl: MS(ESI): 430.2(M-Cl), 393.4(M-Cl-Cl+H). Elemental Analysis(%) for $C_{12}H_{13}Cl_2N_3Pt\cdot H_2O$ (483.3)，Calcd: C, 29.82; N, 8.70; H, 3.13. Found: C, 29.83; N, 8.79; H, 3.14.

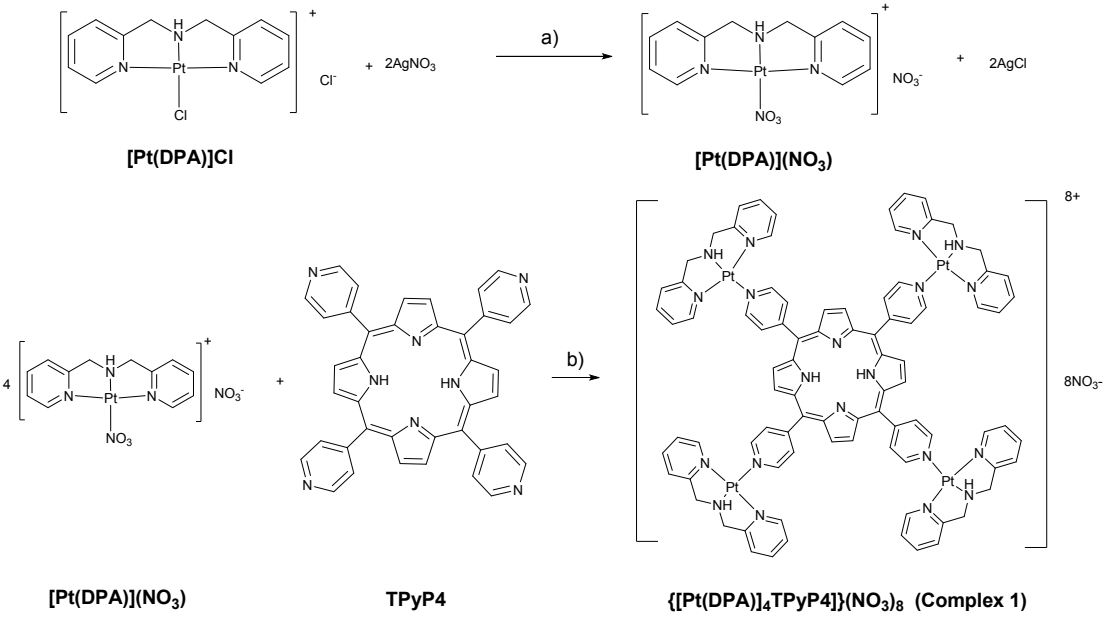
Pt(dien)Cl: MS(ESI): 334.3(M-Cl). Elemental Analysis(%) for $C_4H_{13}Cl_2N_3Pt$ (369.2), Calcd: C, 13.01; N, 11.38; H, 3.55. Found: C, 13.30; N, 11.14; H, 3.52.

Synthesis of $\{[Pt(DPA)]_4(TPyP4)\}\cdot(NO_3)_8$ (**1**):

An equivalent amount of Pt(DPA)Cl(0.12 mmol, 58 mg) and $AgNO_3$(0.24 mmol, 40.7 mg) are mixed and dissolved in 15 mL distilled water. White precipitate appears immediately and the mixture is heated at 60 degree and stirred under argon in the dark. After 36 h's reaction, the mixture in the flask is taken out and centrifuged so that AgCl precipitate could be removed from the desired supernant. It is encouraged to wash the precipitate with a tiny amount of water twice and collect the supernant in order to maintain the stoichiometry.

To the collected supernant, 10 mL trifluoroethanol solution containing TPyP4(0.03 mmol, 18.5 mg) is added dropwise under vigorous agitation. Another 10 mL trifluoroethanol is added in case of precipitation of the water-insoluble TPyP4. The reaction mixture is heated at 60 ℃ under argon in the dark. 48h later, concentrate the solution to approximately 5 mL, followed by the addition of 5 mL ethanol and 15 mL ether. Purple solid forms immediately and is centrifuged. The desired product is got by drying under vacuum at 45 degree overnight (72 mg, yield: 85%).

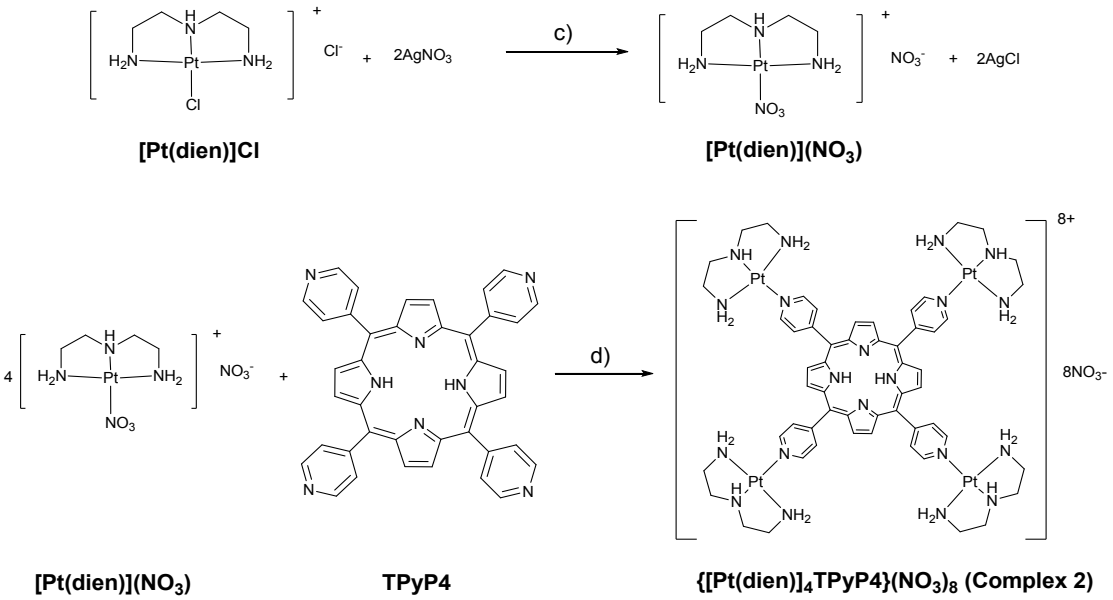
EA(%):$C_{88}H_{78}N_{28}O_{24}Pt_4 \cdot 8H_2O$ (2836.2) Calcd: C, 37.27; H, 3.34; N, 13.83. Found: C, 37.51; H, 3.48; N, 13.85. ^{1}H NMR (400 MHz, d_6-DMSO, δ/ppm) 9.9 (s, 4H), 9.5 (d, J = 13.3 Hz, 8H), 9.2 (s, 3H), 8.8 (d, J = 5.1 Hz, 8H), 8.4 (t, J = 7.8 Hz, 8H), 8.3 (d, J = 5.7 Hz, 8H), 8.0 (d, J = 7.8 Hz, 8H), 7.8 (d, J = 5.5 Hz, 8H), 5.2 (dd, J = 15.5, 8.6 Hz, 8H), 4.9 (dd, J = 20.2, 9.1 Hz, 8H).^{195}Pt NMR (400 MHz,D_2O, δ/ppm): -794.9, and K_2PtCl_4 used as an internal standard(δ=0) (Fig. S1†).



Scheme S1 The synthetic route of **1**. Reaction condition: a) H_2O, 60℃, 36 h, dark; b) H_2O /CF_3CH_2OH, 60 ℃, 48 h.

Synthesis of {[Pt(dien)]$_4$(TPyP4)}·$(NO_3)_8$ (**2**):

The synthesis method is similar to **1**. The desired product is water-soluble purple solid. (yield: 78%). EA(%): $C_{56}H_{78}N_{28}O_{24}Pt_4 \cdot 8H_2O$ (2451.8) Calcd: C, 27.43 N, 16.00 H, 3.86. Found: C, 27.71 N, 15.73 H, 4.013. ^{1}H NMR (400 MHz, D2O, δ/ppm): 9.1 (d, J = 5.9 Hz, 8H), 8.7 (s, 8H), 8.0 (s, 8H), 3.5 (dd, J = 12.2, 3.7 Hz, 8H), 3.3 (dtd, J = 25.3, 13.4, 3.9 Hz, 16H), 3.0 (dd, J = 11.3, 3.4 Hz, 8H). ^{195}Pt NMR (400 MHz,D_2O, δ/ppm): -1225.8, and K_2PtCl_4 used as an internal standard(δ=0) (Fig. S2†).



Scheme S2 The synthetic route of **2**. Reaction condition: c)H_2O, 45℃, 36 h, dark; d) H_2O / CF_3CH_2OH, 60 ℃, 48 h.

1.3 Oligonucleotides and sample preperation

Table S1oligonucleotide sequences used in PCR-stop assay

PCR system	DNA sequences	
c-myc	Pu27	5'-d[TGGGGAGGGTGGGGAGGGTGGGGAAGG]-3'
	Pu27mut	5'-d[TGGGGAGGGTGGAAAGGGTGGGGAAGG]-3'
Telomere	HTG21	5'-d[GGGTTAGGGTTAGGGTTAGGG]-3'
	HTG21mut	5'-d[GGGTTAGGGTTAAAGTTAGGG]-3'

Table S2oligonucleotide sequences used in CDstudy

Name	DNA sequences
Pu27	5’-d[TGGGGAGGGTGGGGAGGGTGGGGAAGG]-3'
HTG21	5´-d[AGGGTTAGGGTTAGGGTTAGGG]-3'

Table S3 oligonucleotide sequences used in FRET melting study

Name	DNA sequences
HTG21	5'-*FAM*-d[GGGTTAGGGTTAGGGTTAGGG]-*TAMRA*-3'
Pu27	5'-*FAM*-d[TGGGGAGGGTGGGGAGGGTGGGGAAGG]-*TAMRA*-3'
c-kit	5'-*FAM*-d[AGGGAGGGCGCTGGGAGGAGGG]-*TAMRA*-3'

bcl-2	5'-*FAM*-d[AGGGGCGGGCGCGGGAGGAAGGGGGCGGGAGCGGGGCTG]-*TAMRA*-3'
dsDNA	5'-*FAM*-d[TATAGCTA-HEG-TATAGCTATA]-*TAMRA*-3'

Table S4oligonucleotide sequences used in TRAP

Name	DNA sequences
TS primer	5'-d[AATCCGTCGAGCAGAGTT]-3'
ACX primer	5'-d[GCGCGG(CTTACC)$_3$CTAACC]-3'
TSNT(Internal Control)	5'-d[AATCCGTCGAGCAGAGTTAAAAGGCCGAGAAGCGAT]-3'
NT primer	5'-d[ATCGCTTCTCGGCCTTTT]-3'

1.4 Polymerase chain reaction (PCR)-stop assay

The templates and primers were listed in **Table S1**. The reaction were performed in 1×PCR buffer, containing 10 pmol of each template and primer, 0.2 mM dNTP, 2.5 U Taq polymerase, and various concentrations of complexes. The PCR reaction mixtures were incubated in a thermocycler with the following PCR procedure: 94 ℃ for 2 min, followed by 30 cycles (for Pu27 and Pu27mut) or 14 cycles (for HTG21 and HTG21mut)：94 ℃ for 30 s, 58 ℃ for 30 s and 72 ℃ for 30 s. PCR products were electrophoresed on 15% nondenaturing polyacrylamide gels in 1×TBE and visualized by silver stain.

1.5 CD spectroscopy study

CD spectra were recorded on CD spectrometer, using a 1cm path length quartz cuvette. The scans were performed at 25℃ over a wavelength range of 220-360 nm with a scanning speed of 200 nm/min, a response time of 1s, 0.2 nm data pitch, and 3.41 bandwidth. A blank sample containing only buffer was treated in the same manner and used as baseline. All the DNA sequences were dissolved in a 10 mM Tris-HCl(pH 7.4) buffer containing 100 mM KCl to achieve a 3 μM solution and were annealed by heating at 95℃ for 5 min and then slowly cooling to room temperature overnight.Aliquots of porphyrin ligands(3 mM in water) were added stepwise to achieve the desired equivalent proportions. The ligands alone did not show any significant signals in the observed wavelength range. The CD spectra represent an average of three scans and were smoothed. Final analysis and manipulation of the data was carried out using Origin 8.0.

1.6 FRET melting assay

The fluorescent labeled oligonucleotide were diluted in 60 mM potassium cacodylate buffer(pH 7.4) and annealed by heating to 95 ℃ for 5 min and slowly cooling down to room temperature overnight. Thermo-denature procedures were on a Roche Light Cycler II real-time PCR machine, using a total reaction volume of 25 μL,with a series of different concentrations of complexes and 200 nM labled sequences. Melting curves were determined by recording the fluorescence signal at 530 nm (excited at 470 nm) at intervals of 1 ℃ over the temperature range of 37~99 ℃, with a constant temperature being maintained for 30 s prior to each reading to ensure a stable state. Data analysis was dealed on Origin 8.0(OriginLab Corp.)

1.7 UV spectroscopy and fluoresence spectroscopy study

Absorption spectra were recorded on UV3150 UV-Vis spectrophotometer, using a 1cm path length quartz cuvette. The scans were performed at 25℃ over a wavelength range of 350-800 nm with a scanning speed of 500 nm/min, a response time of 1s, 0.2 nm data pitch, and 1.0 slit. All the DNA sequences were dissolved in a 10 mM Tris-HCl(pH 7.4) buffer containing 100 mM KCl to achieve a 3 μM solution and were annealed by heating at 95℃ for 5 min and then slowly cooling to room temperature overnight. Aliquots of DNA(0.4 mM in water) were added both the sample and the reference cuvettes stepwise to achieve the desired equivalent proportions, and the absorption spectrum is recoreded after each addition.

Fluorescence spectra were recorded on FLS920 fluorescence sphectrophotometer, using a 1 cm path quartz cuvette. The scans were performed at 25 ℃ over a wavelength range of 580~800 nm. All the DNA sequences were annealed overnight and aliquots of DNA were added stepwise to achieve the desired equivalent proportions and the emission spectrum is recorded after each addition.(λ_{ex}=431 nm, λ_{em}=654 nm)

1.8 Job-plot analysis

Job-plot analysis was performed according to a reported procedure. Stock solution 3 μM of **1** and **2** were prepared. Telomeric G-quadruplex HTG21 was dissolved and annealed to match the concentration of the stock solutions in 10 mM Tris-HCl and 100 mM KCl buffer(pH 7.4).The total concentrations of the complex solutions were maintained at 3 μM. Befor testing, the complex solutions were allowed to equilibrate for at least one hour in the dark. The data were collected at the Soret band of each complex, and the spectra of complex without DNA were measured as the reference and substracted out. The difference in absorbance was ploted against the molar ratio of complex, and the Job-plot was achieved[2].

1.9 Cell culture

HeLa (Human cervix carcinoma cell line), CNE-2(Human nasopharyngeal carcinoma cell line), HepG2 (Human hepatocellular liver carcinoma cell line) and A549 (Human lung adenocarcinoma cell line) and 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% FBS(Hyclone)and penicillin (Sigma-Aldrich, 100 U/mL)/streptomycin (Sigma-Aldrich, 100 mg/mL) at 37.0 °C in a humidified atmosphere containing 5% CO_2.

1.10 Cytotoxicity study(MTT assay)

The cells were harvested during the exponential phase and seeded equivalently into 96-well plates. The porphyrin **1** , **2** and TMPyP4, and a typical cytotoxin cis-platin as a positive control were added to the wells to achieve final concentrations. 20 μL of a stock MTT dye solution (5 mg/mL) was added to each well after incubation for 44 h. DMSO (100 μL) was added to dissolve the MTT formazan after an additional 4 h incubation time. The percentage of cell viability was calculated using the following equation: (mean OD of treated cells/mean OD of control cells) × 100%. Data are presented as the means of three independent experiments ± standard deviations. From MTT assay, appropriate subcytotoxic drug concentrations were selected, any concentration that induced obvious cell death was avoided in the following experiment.

1.11 Flow cytometry study

HeLa cells were seeded into 6-well plates and incubated for 24 h or 48 h. Different concentrations of **1** and **2** were added into the plates and incubated for 24 h. The cells were collected by digestion with trypsin and fixed in 1 mL of 70% ethanol at 4 ℃ for 12 h. After that, cells were washed twice with ice-cold PBS, incubated with 0.5 mg/mL RNase A, and stained with 200 μL 10 μg/mL propidium iodide(PI) or Annexin V-

FITC and PI. After incubation at 37 ℃ for 30 min in the dark, the cell suspensions were analyzed by flow cytometry. Data were analyzed by ModFit LT 2.0 software.

1.12 Telomere Repeat Amplification Protocol (TRAP)

Telomerase extract was prepared from HeLa cells and used for evry reaction in a final volume of 50 μL consisting 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, 0.25 μM ACX primer, 0.25 μM NT primer，2×10-22 mol TSNTtemplate, 1 U hot-start Taq polymerase and the complexes. The mixture was incubated at 30 ℃ for 30 min, followed by a 30-35 cycles of PCR reaction: 95 ℃ for 30 s. 50 ℃ for 30 s, and 72 ℃ for 60 s. PCR products were electrophoresed on a 8% polyacrylamide gel and visualized by Gel-red staining.

1.13 Western blot assay

Hela cells(5×10^4) were seeded in 6 cm culture dishes and exposed to subcytotoxic concentrations of drugs for 72 h. After incubation, the total protein was extracted by RIPA lysis buffer with the addition of protease inhibitor cocktails(Roche). The protein sample was electrophoresed on 10% polyacrylamide gel and transferred to PVDF membrane (Millipore, USA). The membrane were blocked in Tris-HCl buffered saline containing Tween-20 and 5% skim milk at room temperature for 1 h. Afterwards, the membranes were incubated with 5% milk blocking buffer containing 1:1000 dilution of either anti-c-myc monoclonal antibody (Santa Cruz Biotech.,USA) or anti-β-actinmonoclonal antibody(Santa Cruz Biotech.,USA) at 4℃ overnight. After a subsequent incubation with goat antimouse lgG-HRP(1:2000) for 1 h and treated with BeyoECL Plus chemiluminescence kit (Beyotime,China), bands were imaged using FluoChem Q system (ProteinSimple, USA)

2. Characterization of 1 and 2

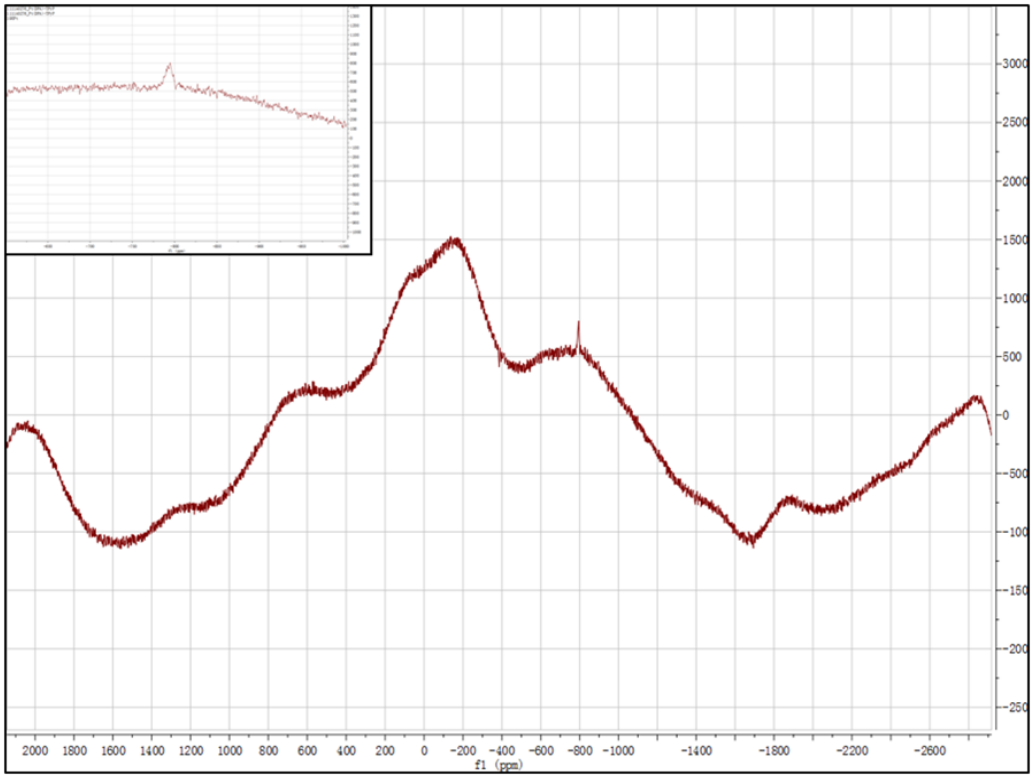


Fig. S1 The ^{195}Pt NMR (400 MHz,D_2O, δ/ppm) for **1**. The inset shows the ^{195}Pt NMR for **1** in the region of -600~-1400 ppm.

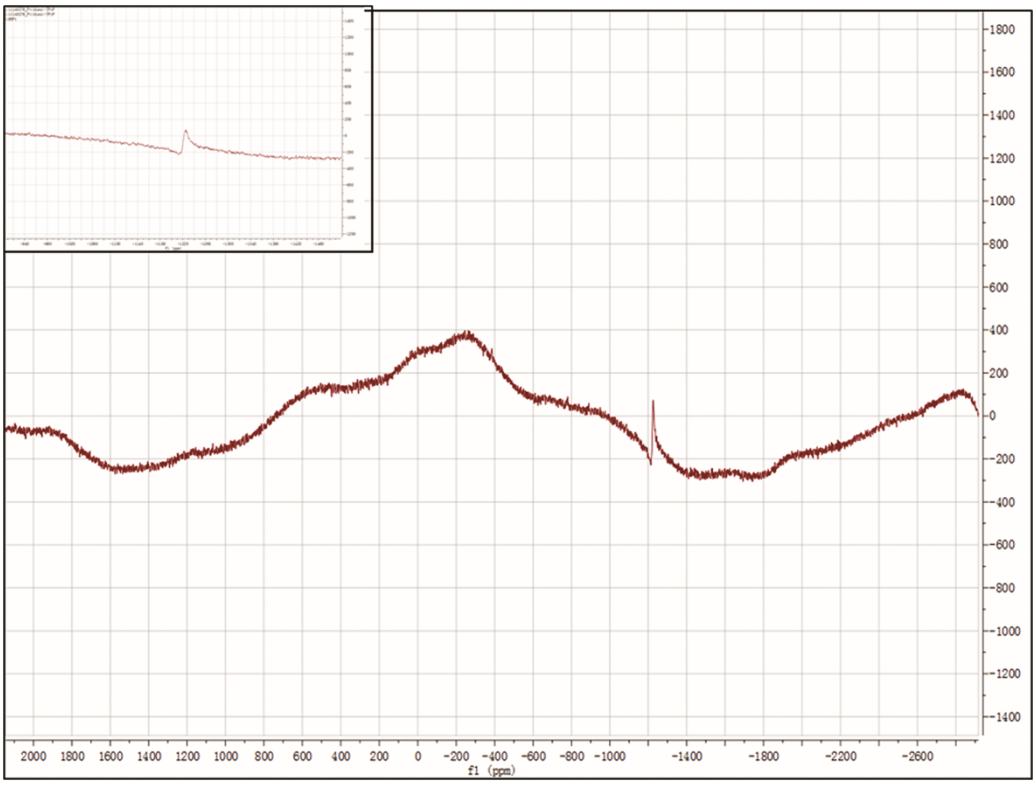


Fig. S2 The ^{195}Pt NMR (400 MHz,D_2O, δ/ppm) for **2**. The inset shows the ^{195}Pt NMR for **2** in the region of -1000~-1800 ppm.

3. FRET melting curves

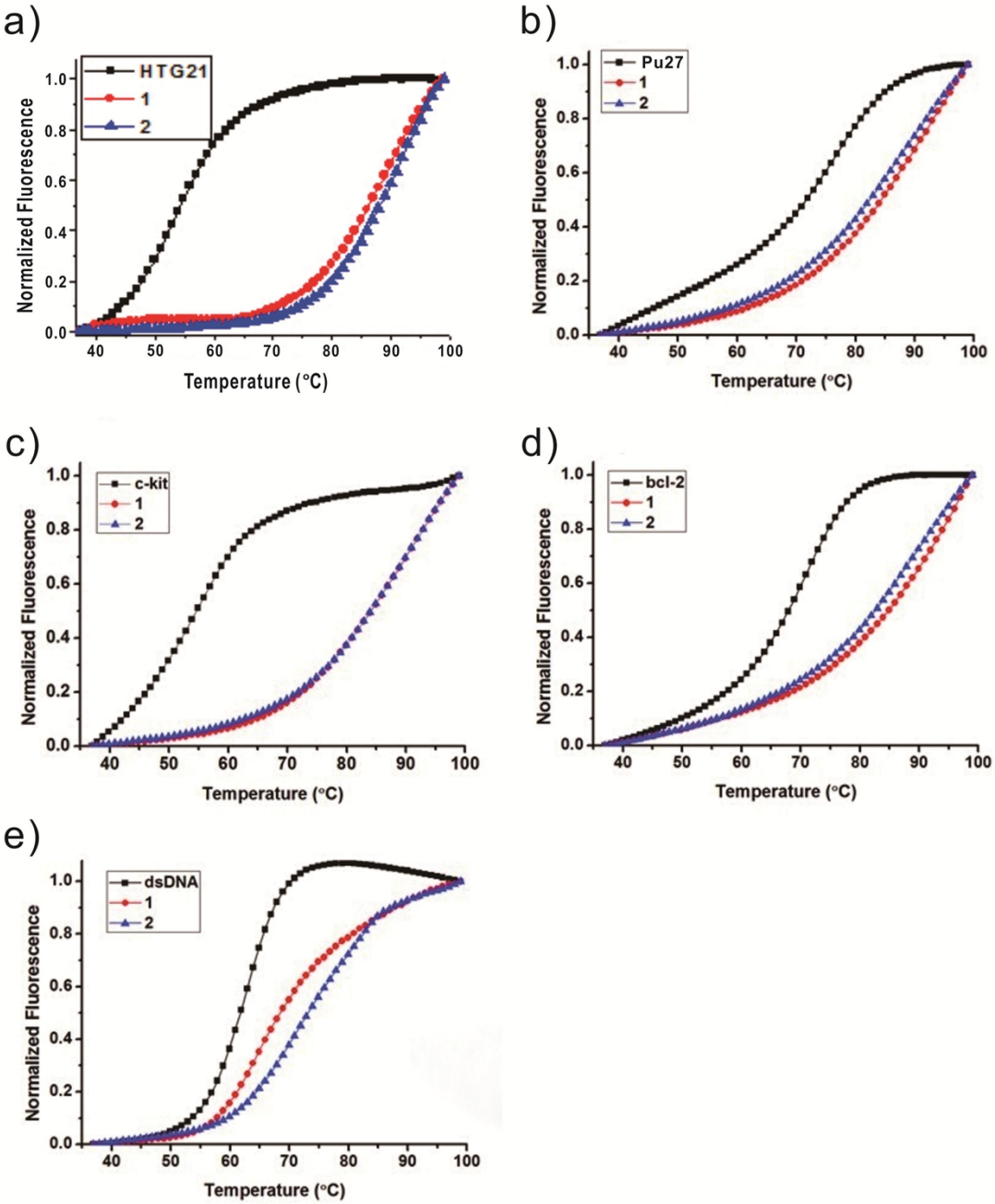


Fig. S3 FRET melting curves of **1**(0.8 μM) and **2**(0.8 μM) with G-quadruplex DNA (200 nM) and a duplex DNA (200 nM). Buffer: 60 mM potassium cacodylate (pH 7.4).

4. PCR-stop assay

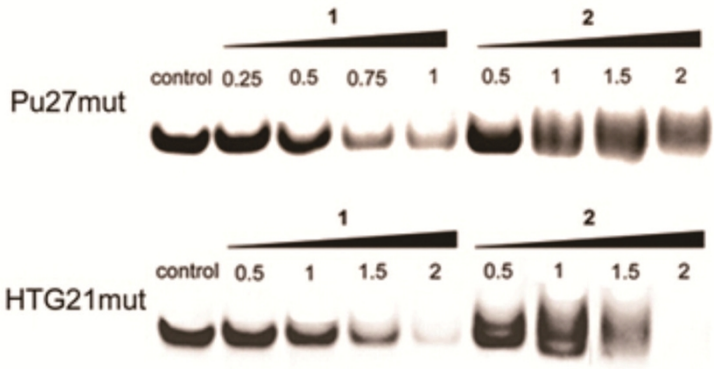


Fig. S4 PCR-stop assay. Reaction with a control primer (Pu27mut, HTG21mut), which cannot form G4 structures, was not affected. Control experiments also showed no inhibition of the Taq polymerase of **1** and **2**.

5. Absorption and emission spectroscopy study

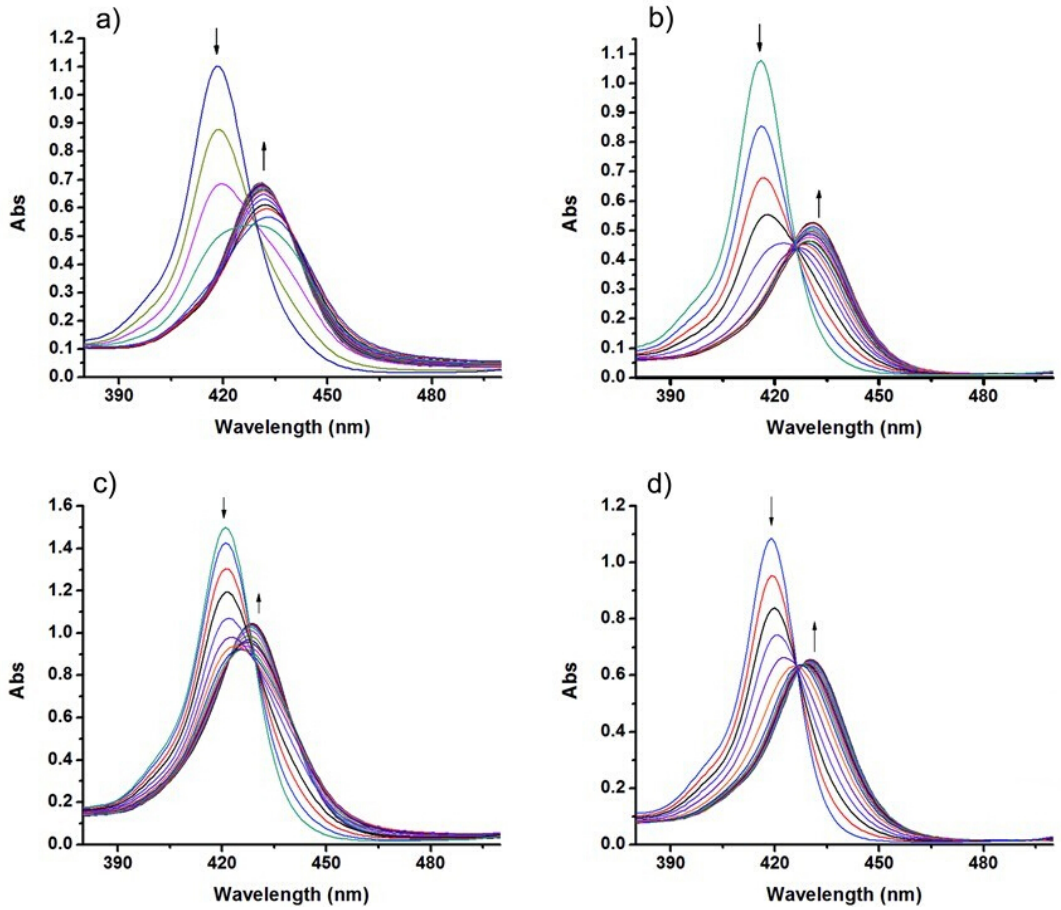


Fig. S5 Absorption spectra of **1** and **2** (3 μM) in buffer with increasing amounts (0~3 μM) of G-quadruplex DNA. a) **1** and HTG21; b) **2** and HTG21; c) **1** and Pu27; d) **2** and Pu27. (Buffer: 10 mM Tris-HCl with 100 mM KCl, pH7.4)

Table S5 Profile of the bathochromic shift and hypochromicity

	λ(free) / nm	λ(bound)/ nm	Δλ / nm	hypochromicity/%
1+22AG	419	432	12	37.8
1+Pu27	419	432	12	45.5
2+22AG	416	431	15	51.3
2+Pu27	417	433	16	53.2

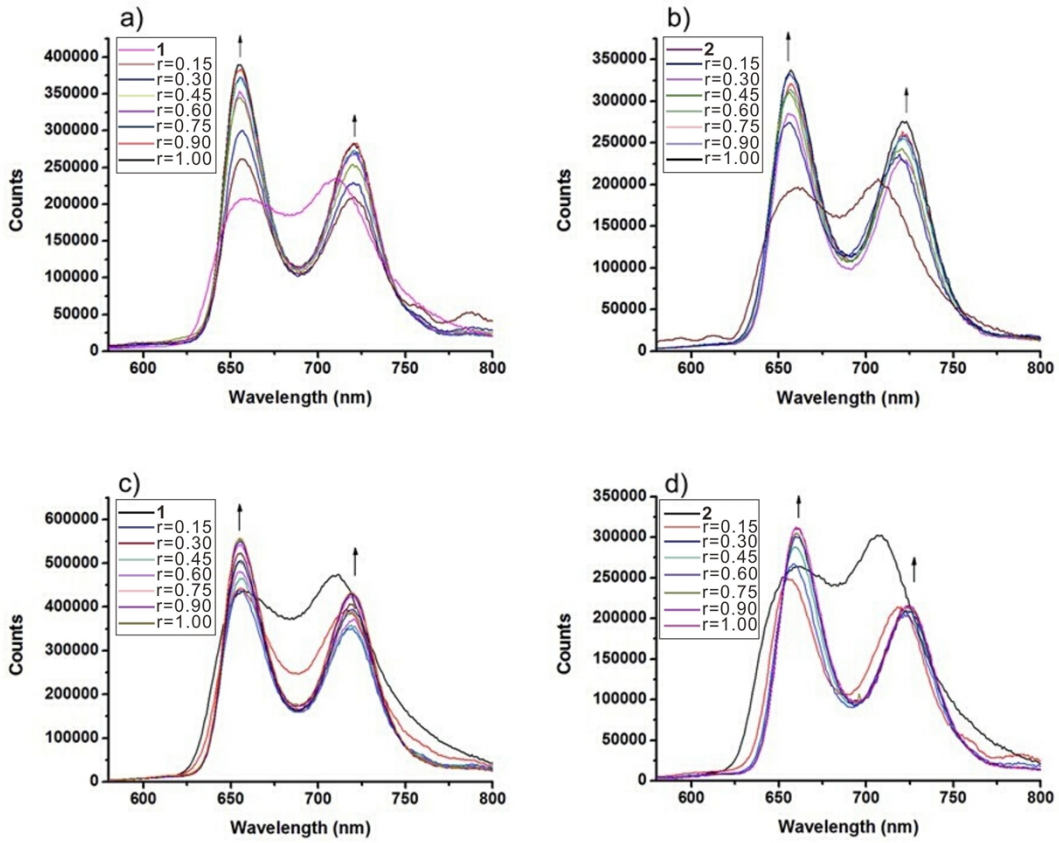


Fig. S6 Emission spectra of **1** and **2** (3 μM) in buffer with increasing amounts (0~3 μM) of G-quadruplex DNA. The arrows indicate the increasing amounts of G-quadruplex DNA (r = DNA strand concentration / complex). a) **1** and HTG21; b) **2** and HTG21; c) **1** and Pu27; d) **2** and Pu27. (Buffer: 10 mM Tris-HCl with 100 mM KCl, pH7.4)

6. Job-plot Analysis

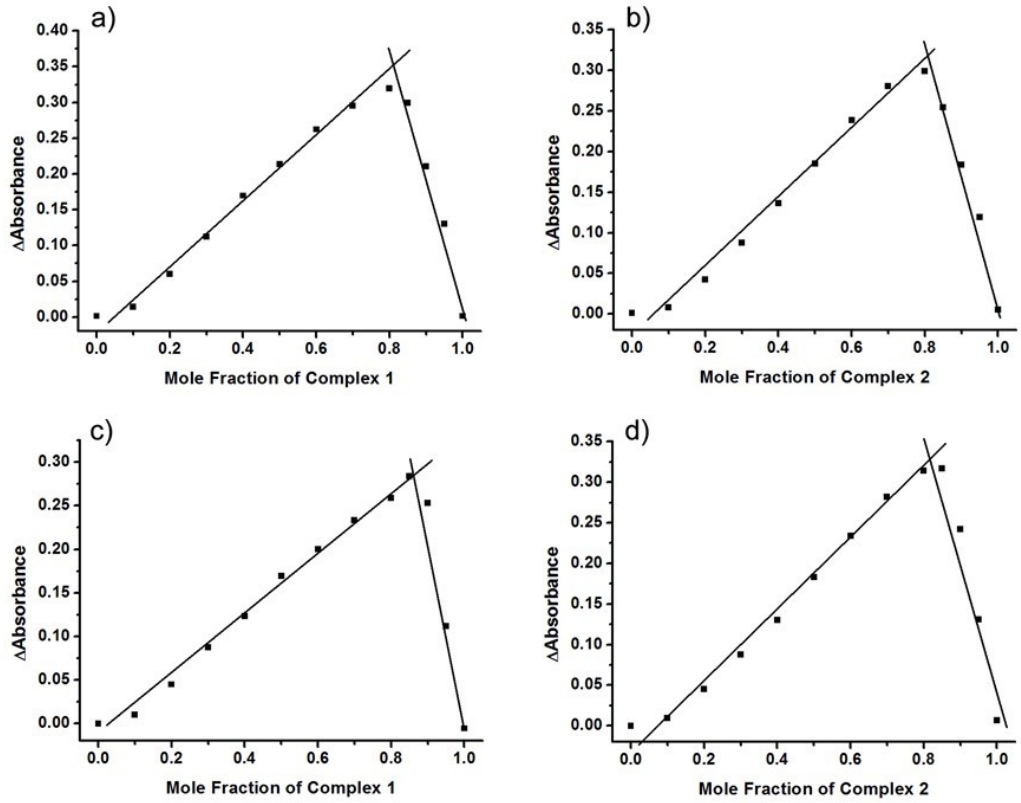


Fig. S7 Job Plot of binding of **1** and **2** to HTG21 andPu27. a) **1** with HTG21; b) **2** with HTG21; c) **1** with Pu27; d) **2** with Pu27. (Buffer: 10 mM Tris-HCl with 100 mM KCl, pH7.4)

7. Cytotoxicity of 1 and 2

Table S6 IC_{50} values determined by MTT assay

Complex	IC_{50} (μM)			
	HeLa	HepG2	CNE-2	A549
1	4.8 ± 2.5	9.8 ± 2.6	15.5 ± 2.3	2.8 ± 2.1
2	24.4 ± 5.2	50.5 ± 2.5	30.2 ± 3.6	23.5 ± 4.6
TMPyP4	41.6 ± 3.6	70.8 ± 4.1	49.4 ± 3.4	40.4 ± 1.6

7. Reference

1. C. F. Weber and R. van Eldik, *Eur. J. Inorg. Chem.*, 2005, **2005**, 4755-4761.

2. C. Wei, G. Jia, J. Zhou, G. Han and C. Li, *Phys. Chem. Chem. Phys.*, 2009, **11**, 4025-4032; C. Wei, L. Wang, G. Jia, J. Zhou, G. Han and C. Li, *Biophys. Chem.*, 2009, **143**, 79-84.