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Octahedral Ruthenium Complex Selectively Stabilizes G-quadruplexes

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EXPERIMENTAL METHODS

All the chemicals were purchased from Sigma-Aldrich. All DNA sequences were purchased from Sangon (Shanghai, China). TRAPeze telomerase detection kit was purchased from Merck Millipore (Darmstadt, Germany). *Taq* DNA polymerase was purchased from Thermo Fisher Scientific (Waltham, Massachusetts, USA). Milli-Q water was used in all physical measurement experiments.

Synthesis of Ru^{II} complexes

[Ru(bpa)(DMSO)Cl]Cl_2 (Ru0). The ligand N,N-Bis-(1,10-phenanthrolin-2-yl)-amine (bpa) was prepared as previously reported.[43,44] bpa was protonated by dissolving in trifluoroacetic acid and precipitated with diethyl ether prior to usage in following synthesis. To prepare **Ru0**, *cis*-Ru(dmso)$_4$Cl$_2$ (72.0 mg, 0.15 mmol), bpa-H$^+$ (72.5 mg, 0.15 mmol) and ethylene glycol (5 mL) were heated at 190 °C for 2 h. The crude were obtained by removing the solvent under vacuum and washed with diethyl ether (yield: 85 %). ^{1}H NMR (300 MHz, DMSO) δ 10.31 (d, *J* = 4.9 Hz, 2H), 8.89 (d, *J* = 8.0 Hz, 2H), 8.75 (d, *J* = 8.7 Hz, 2H), 8.30 (m, 6H), 8.01 (d, *J* = 8.9 Hz, 2H), 1.68 (s, 6H).

[Ru(bpa)(NH_3)$_2$](PF_6)$_2$ (Ru1). Ru0 (36.9 mg, 0.05 mmol) was suspended in 4 mL MeOH and H_2O mixture (v/v 3:1), followed by addition of 28% ammonium hydroxide solution (1.0 mL). The mixture was refluxed overnight. Methanol was distilled out and the product was precipitated by adding an excessive amount of saturated KPF_6 solution. The solid was filtered and washed with diethyl ether to provide the final product. Crystal of **Ru1** was grown by diffusing diethyl ether into acetone at 4 °C and purple needle crystals were obtained in 40 % yield. ^{1}H NMR (500 MHz, Acetone-d$_6$) δ 10.59 (d, *J* = 4.9 Hz, 2H), 8.98 (d, *J* = 8.0 Hz, 2H), 8.83 (d, *J* = 8.5 Hz, 2H), 8.37 (m, 6H), 8.16 (d, *J* = 8.6 Hz, 2H). HR-MS (MeOH) *m/z* (%): 510.0970 (calc 510.0980 for C24H22N7Ru).

[Ru(bpa)(NH_3)(DMSO)](PF_6)$_2$ (Ru2). Ru0 (22.1 mg, 0.03 mmol) was suspended in 4 mL MeOH and H_2O mixture (v/v 3:1), followed by addition of 28% ammonium hydroxide solution (0.5 mL). The mixture was refluxed for 5 h. Methanol was distilled out, and the product was precipitated by adding an excessive amount of saturated KPF_6 solution. The solid was filtered and washed with diethyl ether to provide the final product. Purple needle crystals of **Ru2** were grown by diffusing diethyl ether into acetone at 4 °C (yield: 53 %). ^{1}H NMR (300 MHz, DMSO) δ 12.83 (s, 1H), 10.18 (d, *J* = 4.7 Hz, 2H), 9.06 (d, *J* = 8.2 Hz, 2H), 8.92 (d, *J* = 8.8 Hz, 2H), 8.39 (m, 6H), 8.10 (d, *J* = 8.9 Hz, 2H), 1.83 (s, 6H). HR-MS (MeOH) *m/z* (%): 571.0848 (calc 571.0854 for C26H25N6OSRu).

[Ru(bpa)(NH_2CH_3)$_2$]Cl_2 (Ru3). Ru0 (22.1 mg, 0.03 mmol) was suspended in 4 mL MeOH and H_2O mixture (v/v 3:1), followed by addition of 40% aqueous methylamine solution (100 μL). The mixture was refluxed overnight. After distilling the solvent, the obtained solid of **Ru3** was purified and recrystallized by diffusing diethyl ether into methanol at 4 °C (yield: 66 %). ^{1}H NMR (500 MHz, MeOD) δ 10.41 (d, *J* = 4.8 Hz, 2H), 8.94 (d, *J* = 8.0 Hz, 2H), 8.80 (d, *J* = 8.8 Hz, 2H), 8.36 (m, 6H), 8.10 (d, *J* = 8.8 Hz, 2H), 0.90 (t, *J* = 6.5 Hz, 6H). HR-MS (MeOH) *m/z* (%): 536.1126 (calc 536.1137 for C26H26N7Ru).

UV-vis absorption titration

Absorption spectra of Ru complexes were recorded on a Cary 100 UV/Vis spectrophotometer. For UV titration experiment, a Tris/KCl buffer (100 mM KCl, 10 mM Tris HCl, pH 7.4) was used and UV/Vis spectra were recorded after each addition of concentrated DNA stock (100 μM) to 25 μM Ru complex solutions in a quartz cuvette (path length = 1 cm) at 25 °C. Binding association constant K (M^{-1}) was calculated with Eq(1), [45,46]

$$\frac{D}{\Delta\varepsilon_{ap}} = \frac{D}{\Delta\varepsilon} + \frac{1}{\Delta\varepsilon \times K} \qquad \text{eq (S1)}$$

where D is the concentration of DNA, $\Delta\varepsilon_{ap} = |\varepsilon_A - \varepsilon_F|$, $\varepsilon_A = A_{obs}/[Ru]$, $\Delta\varepsilon = |\varepsilon_B - \varepsilon_F|$, $\varepsilon_B$and ε_F represented the extinction coefficients of the DNA-complex adduct and free complex that is in solution, respectively. The results were got from average of three replicates.

G4-FID assay

FID assay was performed on Varian Cary Eclipse fluorescence spectrophotometer in a 100 mM KCl, 10 mM Tris, pH 7.4 buffer. The concentration of all DNA used was 0.25 μM. Quadruplex DNA were added with 2 molar equivalents of thiazole orange (TO, 0.5 μM) and ds26 were added with 3 molar equivalents of TO (0.75 μM) according to the binding stoichiometry. Metal complexes were titrated to displace TO from DNA by following a 11-steps gradual addition (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0, 6.0, 8.0 and 10.0 molar equivalents of DNA). Fluorescence spectra are recorded after each addition and the TO displacement is evaluated by measuring the fluorescence area (FA, from 510 nm to 750 nm, excitation wavelength is 501 nm). The percentage of TO displacement (TOD) is determined using the following equation:

$$TODx = 100 - [(FAi/FA1)\, x\, 100] \qquad \text{eq (S2)}$$

with 1< i <12, where $FA1$ is the fluorescence area of thiazole orange upon binding to DNA and FAi are the fluorescence area of TO under various ratios of [**Ru1**]/[TO-DNA]. The percentage of TO displacement ($TODx$) is then plotted as a function of the concentration of **Ru1**.

Competition dialysis Assay

75 μM of different DNA structures, including duplex and G-quadruplexes (i.e. ds26, HT21, HT48, c-myc and c-kit2) in buffer (10 mM sodium cacodylate with 190 mM NaCl, pH 7.4) were annealed by heating at 95 °C for 5 min and slowly cooling down to room temperature before use. 100 μL of each oligonucleotide sample was incubated within Slide-a-Lyzer MINI dialysis unit which contains semipermeable membrane, held by floatation device, in contact with 1 μM of ruthenium complexes in a beaker. After incubation for 12 hours, 90 μL of each oligonucleotide solution was taken out, and 10 μL of 10 % sodium dodecyl sulphate (SDS) was added into each solution to dissociate the complexes from the oligonucleotides. The SDS treated solutions and the free complexes in the dialysis buffer were then analysed by UV/Vis spectroscopy to determine the amount of bound complexes on DNA structures (concentrations of ruthenium complex present inside minus that outside of the dialysis membrane).

CD titration

CD spectra were recorded on a Jasco J-1500 spectropolarimeter using a 1 cm path length cuvette. DNA stock solutions were diluted to 3 μM with 10 mM Tris (pH 7.4) with or without 100 mM KCl, respectively. CD spectra were recorded from 220-320 nm at a scan rate of 200 nm/min after each titration of complex to DNA solutions. All CD spectra were baseline-corrected and each curve represented of five averaged scans taken at 25 °C. Final

analysis of the data was carried out using Origin 8.0 (OriginLab Corp.).

Thermal melting assay

Thermal melting of G-quadruplexes and double stranded DNA were obtained by CD and UV melting, respectively. Due to T_m of duplex DNA cannot be detected by CD melting, we combined UV melting result of duplex DNA as a supplementary with CD melting results of G-quadruplex DNAs, to compare the stabilization effects of Ru complex on these DNAs. 100 μL of *c-myc* (3 μM in 10 mM Tris buffer, pH = 7.4, 0.1 mM KCl), c-kit2 (3 μM in 10 mM Tris buffer, pH = 7.4, 5 mM KCl), HT21 and HT48 (3 μM in 10 mM Tris buffer, pH = 7.4, 5 mM KCl), HT21 (3 μM in 10 mM Tris buffer, pH = 7.4, 5 mM NaCl) and 130 μL of ds26 (3 μM in 10 mM Tris buffer, pH = 7.4, 5 mM KCl) were annealed by heating at 95 °C for 5 min and gradually cooling to room temperature over couple hours. Ru complexes were added to DNA solution to yield a final concentration of 3 μM. For CD melting, ellipticity at 260 nm for *c-myc* and c-kit2, and at 295 nm for HT21 and HT48, were monitored upon temperature elevation from 15 °C to 90 °C with heating ramp of 0.5 °C/min. For UV melting, UV absorption of **ds26** at 260 nm and G-quadruplexes at 295 nm were recorded for the same temperature ramp as CD melting. Melting temperatures were analyzed by Origin 8.0 (OriginLab Corp.). Standard deviation over three repeat experiments were used as error bars.

Telomerase Repeat Amplification Protocol (TRAP) Assay

TRAP assay was performed by using the Millipore TRAPEZE® Telomerase Detection Kit and the protocols were slightly modified to fulfill the requirements in our experiment according to the previously report.[48] CHAPS Lysis Buffer was used to extract telomerase from HeLa cells. TRAP assay was performed as follows. The "Master Mix" solution (including 5.0 μL 10× TRAP reaction buffer, 1.0 μL 50× dNTP mix, 1.0 μL TS primer, 1.0 μL TRAP primer mix, 2 Units Taq polymerase, 48.0 μL and 1.0 μL cell extract) was prepared in an RNase-free tube. Xx μL of Master mix were combined with XX μL of Ru complexes solution freshly prepared in DEPC-treated water to achieved the final concentration of **Ru1** as 0.5, 1.0, 2.0, 4.0, 8.0 and 16.0 μM. The mix was incubated at 30 °C for 30 min for the initial extension of TS by telomerase. To amplify the extension products by PCR, the following procedure was used: 95 °C for 5 min to inactivate the telomerase, followed by 24 cycles at 95 °C for 30 s, 52 °C for 30 s and 72 °C for 30 s. Following PCR, 30 μL of TRAP reaction mixtures (containing 10 μL of 6 × loading dye) were analyzed by 10 % non-denaturing acrylamide gel in 0.5 × TBE (90 min at 400 volts).

Cytotoxicity

MDA-MB-231 (human breast carcinoma cells), HT1080 (human fibrosarcoma cells), HeLa (human cervical cancer cells), A549 (Human lung carcinoma), MCF-7 (human breast adenocarcinoma) and NIH 3T3 (mouse embryo fibroblast cells) were seeded in a 96-well culture plate (5000 cells per well), and incubated in DMEM medium supplemented with10% FBS, 1% L-glutamine and 1 % P/S at 37 °C with 5% CO_2 in a humidified atmosphere for 24 hours. The tested compounds were dissolved in bio-grade DMSO, diluted with DMEM medium to the required concentrations (contains <1% v/v DMSO) and added to the wells respectively. The plates were incubated at 37 °C in a 5% CO_2 incubator for 48 h continuously. After incubation, the medium was removed and 200 μL fresh medium contains MTT (20 μL, 5 mg/mL) was added to each well and the plate was incubated at 37 °C in 5% CO_2 for another 4 hours. Then the medium was removed and 150 μL DMSO was added into each well.

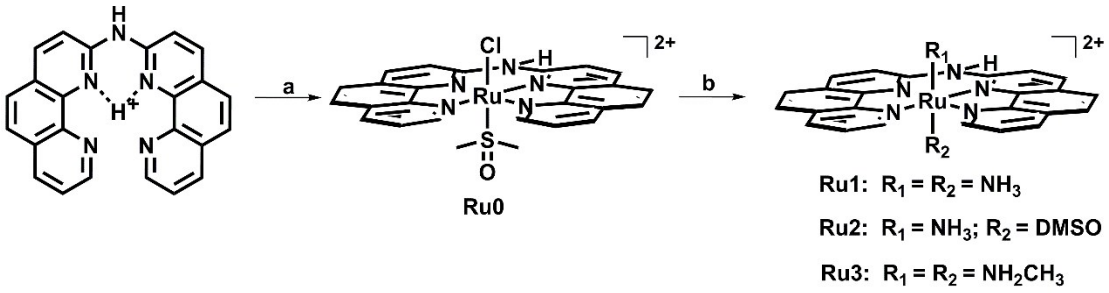
After the plates were shaken for 10 mins, the optical density of each well was measured on a plate spectrophotometer at a wavelength of 570 nm with background subtraction at 650nm.

Molecular docking of Pt complexes to GQ topological structures

In docking simulation, GOLD Suite v5.4 (CCDC Software Limited)[1] is used to dock the crystal structure of two complexes, **Ru1** and **Ru2**, into the basket (PDB ID: 2mcc) and hybrid G-quadruplex structures (PDB ID: 2mb3), respectively. The scoring function was calculated by CHEMPLP. In each simulation, all the torsional angles within 15 Å to the binding site, i.e. the center of the external G-quartet layer, was free to rotate. Subsequently genetic algorithm was employed to find the maximum fitness value among all possible configurations and translational positions of complexes around the binding center on G-quadruplexes. For each simulation, the docking solution with largest fitness value was chosen. The docked structures of ruthenium complexes on GQs were visualized and the H-bond lengths were measured in VMD (NIH Center for Macromolecular Modeling and Bioinformatics, at the Beckman Institute, University of Illinois at Urbana-Champaign)[2].

References

1. G. Jones, P. Willett and R. C. Glen, *J Mol Biol*, 1995, **245**, 43-53.
2. W. Humphrey, A. Dalke and K. Schulten, *J Mol Graph Model*, 1996, **14**, 33-38.
3. T. Taka, K. Joonlasak, L. Huang, T. R. Lee, S. T. Chang, W. Tuntiwechapikul, *Bioorg Med Chem Lett*, 2012, **22**, 518–522.
4. J. Reed, M. Gunaratnam, M. Beltran, A. P. Reszka, R. Vilar, S. Neidle, *Anal Biochem*, 2008, **380**, 99-105.



a) *cis*-$Ru(DMSO)_4Cl_2$, Ethlene glycol, 180 °C, 2 h; b)reflux with R_1 solution in MeOH/H2O (3:1).

Scheme S1. Synthetic routes for Ru complexes.

Table S1a. Crystal data, collection and structure refinement parameters for **Ru1.**

Chemical formula	$C_{36}H_{45}F_{12}N_7O_4P_2Ru$
Formula weight	1030.80 g/mol
Crystal system	triclinic
Space group	P -1
Crystal size (mm^3)	0.040 x 0.100 x 0.300
Temperature (K)	103(2)
a (Å)	8.4109(4)
b (Å)	11.4169(6)
c (Å)	11.7250(6)
α (deg)	74.801(2)
β (deg)	76.758(2)
γ (deg)	87.087(2)
V ($Å^3$)	1057.59(9)
Z	1
ρ_{calcd} ($g\ cm^{-3}$)	1.618
μ (mm^{-1})	0.547
F(000)	524
Data / restraints / parameters	5904 / 456 / 428
R_1 [I > 2σ(I)]	0.0365
wR_2 [I > 2σ(I)]	0.0723
R_1 (all data)	0.0506
wR_2 (all data)	0.0796
Goodness-of-fit on F^2	1.088

Table S1b. Selected Bond Lengths (Å) and Bond Angles (deg) for **Ru1**.

Ru1-N1	2.215(7)
Ru1-N2	1.865(6)
Ru1-N4	1.861(6)
Ru1-N5	2.215(6)
Ru1-N6	2.1237(17)
Ru1-N7	2.1237(17)
N1-Ru1-N2	80.0(4)
N1-Ru1-N4	179.6(6)
N1-Ru1-N5	99.9(4)
N1-Ru1-N6	89.7(3)
N1-Ru1-N7	90.3(3)
N2-Ru1-N4	100.4(5)
N2-Ru1-N5	178.5(6)
N2-Ru1-N6	90.5(4)
N2-Ru1-N7	89.5(4)
N4-Ru1-N5	79.7(4)
N4-Ru1-N6	90.2(4)
N4-Ru1-N7	89.8(4)
N5-Ru1-N6	91.0(3)
N5-Ru1-N7	89.0(3)
N6-Ru1-N7	180.00(11)

Table S1c. Crystal data, collection and structure refinement parameters for **Ru2.**

Chemical formula	$C_{29}H_{32}F_{12}N_6O_3P_2RuS$
Formula weight	935.67
Crystal system	monoclinic
Space group	P 1 21/c 1
Crystal size (mm^3)	0.020 x 0.040 x 0.300
Temperature (K)	103(2)
a (Å)	11.5273(4)
b (Å)	12.9572(4)
c (Å)	24.0256(7)
α (deg)	90
β (deg)	97.6261(17)
γ (deg)	90
V ($Å^3$)	3556.8(2)
Z	4
ρ_{calcd} ($g\ cm^{-3}$)	1.747
μ (mm^{-1})	0.695
F(000)	1880
Data / restraints / parameters	8994 / 602 / 616
R_1 [$I > 2\sigma(I)$]	0.0722
wR_2 [$I > 2\sigma(I)$]	0.1928
R_1 (all data)	0.1360
wR_2 (all data)	0.2432
Goodness-of-fit on F^2	1.084

Table S1d. Selected Bond Lengths (Å) and Bond Angles (deg) for **Ru2**.

Ru1-N1	2.105(5)
Ru1-N2	2.022(6)
Ru1-N4	2.020(5)
Ru1-N5	2.102(5)
Ru1-N6	2.130(5)
Ru1-S1	2.2294(16)
N1-Ru1-N2	80.5(2)
N1-Ru1-N4	171.5(2)
N1-Ru1-N5	107.7(2)
N1-Ru1-N6	88.7(2)
N2-Ru1-N4	91.5(2)
N2-Ru1-N5	169.9(2)
N2-Ru1-N6	87.9(2)
N4-Ru1-N5	79.9(2)
N4-Ru1-N6	88.1(2)
N5-Ru1-N6	86.6(2)
S1-Ru1-N1	87.73(15)
S1-Ru1-N2	89.29(16)
S1-Ru1-N4	95.11(15)
S1-Ru1-N5	96.68(14)
S1-Ru1-N6	175.74(15)

Table S2. Sequences of oligomers (primers) used in this work.

Oligomer	Sequence
c-myc	5’-TGGGGAGGGTGGGGAGGGTGGGGAAGG-3’
c-kit2	5’-CGGGCGGGCGCGAGGGAGGGG-3
HT21	5’-GGG(TTAGGG)$_3$-3’
HT48	5’-(TTAGGG)$_8$-3’
DS26	5’-CAATCGGATCGAATTCGATCCGATTG-3’

Table S3. The melting temperature incensements (ΔT_m) of different DNA structures in the presence of **Ru1**.

DNA	ΔT_m (°C)
HT21 (in Na^+)	26 (2)
HT21 (in K^+)	20 (1)
HT48 (in K^+)	23 (2)
c-myc	10 (2)
c-kit2	10 (2)
DS26	1.9 (0.4)

Table S4. The melting temperature incensement (ΔT_m) of DNA structures in the presence of **Ru2** and **Ru3**.

	ΔT_m (*c-myc*)	ΔT_m (HT21)	ΔT_m (ds26)
Ru2	7.2 (0.5)	9.6 (0.8)	1.2 (0.3)
Ru3	0.8 (0.3)	4.3 (0.3)	1.3 (0.1)

Table S5. TRAP assay (EC_{50}) and Cytotoxicity (IC_{50}) of **Ru1** towards various cancerous cell lines.[a]

Compound	Cytotoxicity (μM^{-1})					TRAP assay (μM^{-1})
	HT 1080	MDA-MB-231	HeLa	A549	MCF-7	
Ru1	26 (1)	25 (3)	24 (3)	17 (2)	51 (2)	1.9 (0.3)
TMPyP4	38 (6)	41 (5)	21 (4)	43 (1)[b]	90 (3)	8.9[c]

[a].Standard deviations were listed in parentheses.

[b] From reference.[3]

[c] A modified TRAP assay with eliminating $TMPyP_4$ before PCR step.[4]

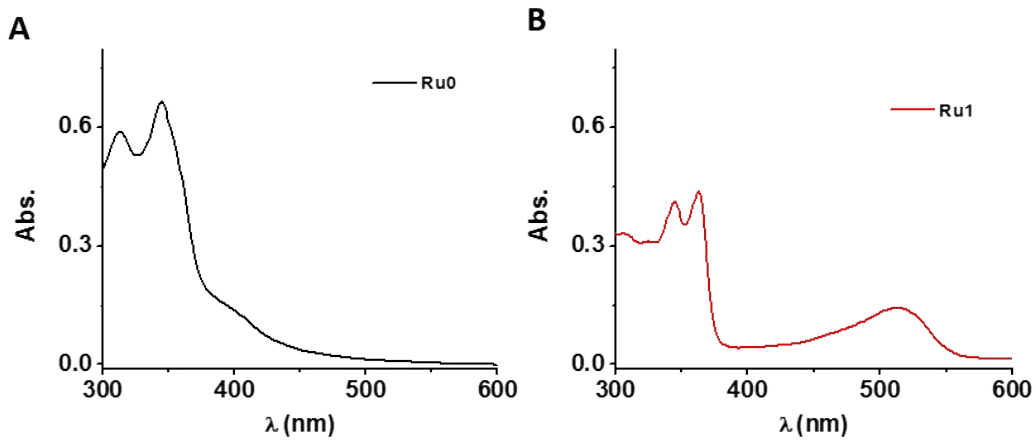


Figure S1. UV-vis absorption spectrum of **Ru0** (A) and **Ru1** (B).

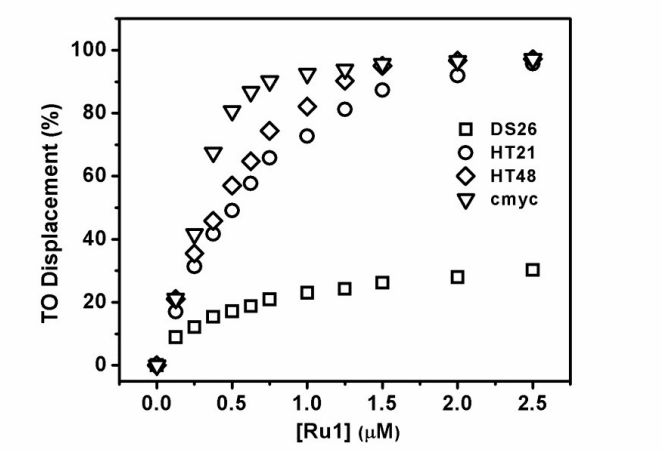


Figure S2. Plot of TO displacement percentage *vs*. concentrations of **Ru1** with different DNA sequences. Lines are the exponential fittings of TO displacement curves.

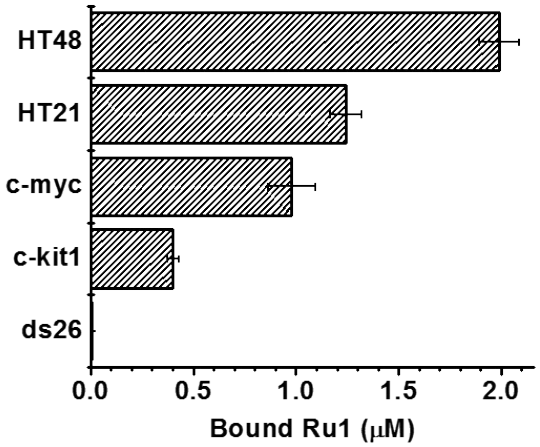


Figure S3. Competition dialysis of **Ru1** on various DNA secondary structures.

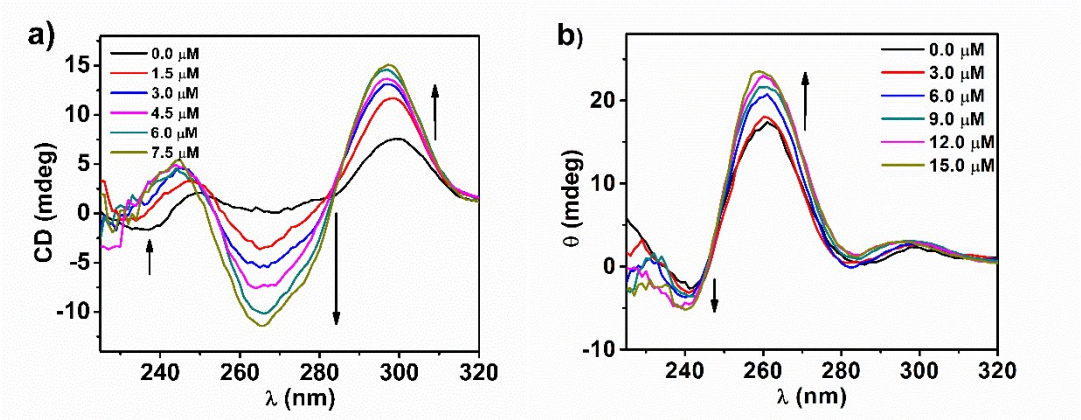


Figure S4. Circular dichroism spectra of GQ **HT21** (a) and GQ **cmyc** (b) upon titration of **Ru1** in 10 mM Tris (pH 7.4).

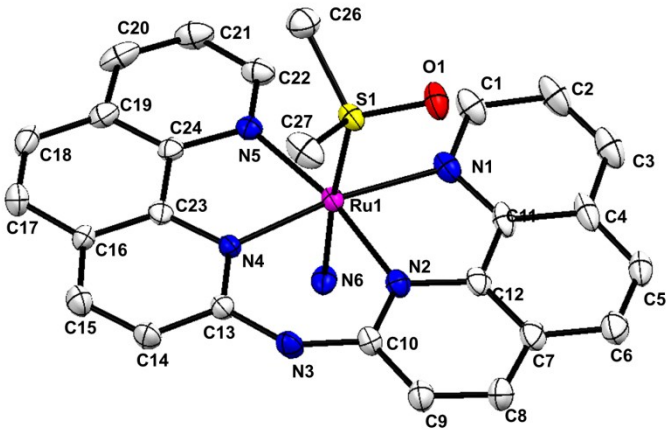
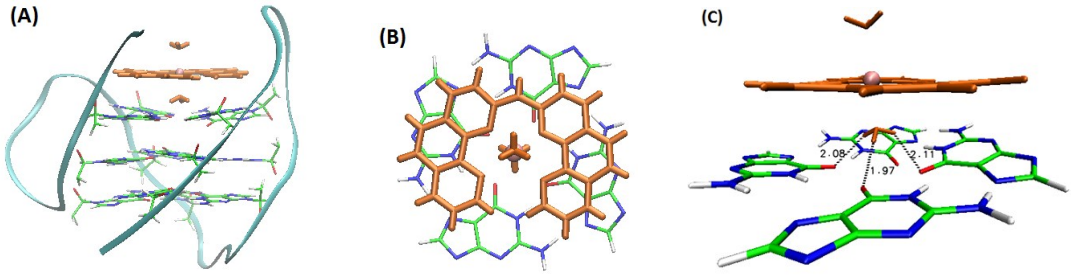


Figure S5. The crystal structure of **Ru2** with each atom numbering.



D)

GQ topology (PDB code)	Basket GQ (2mcc)	3+1 hybrid GQ (2mb3)	Parallel c-myc (1xav)
Ru1	94.21	88.06	60.57
Ru2	92.91	82.59	-

Figure S6. Molecular docking of **Ru1** (A-C) on 3+1 hybrid telomric GQ (PDB: 2mb3). (A) side view; (B) top view of the docking structure and (C) H-Bonds between NH_3 and G-quartet. Backbone is represented in blue ribon and Ru1 is shown in brown. (D) Fitting values of Ru complexes on various GQ topological structures from molecular docking.

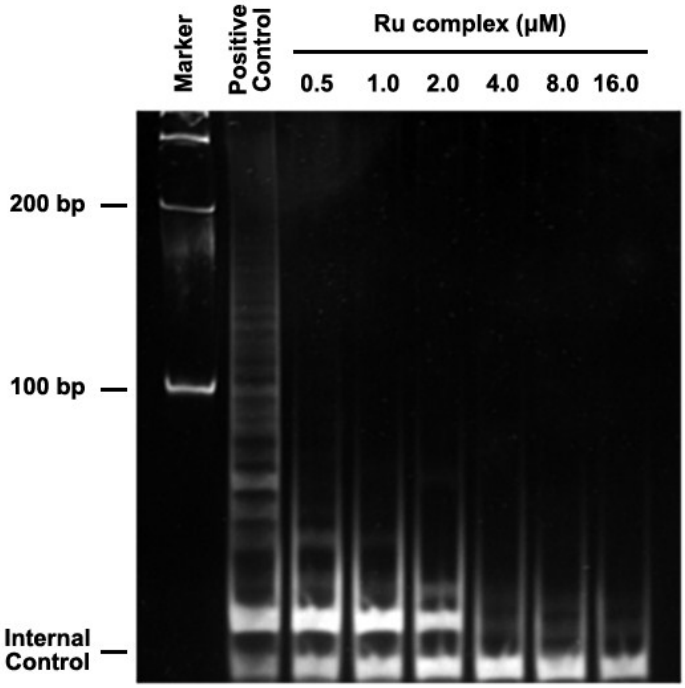


Figure S7. Inhibition of telomerase activity by different concentrations of **Ru1**. Ladder shows the product of telomerase elongation.

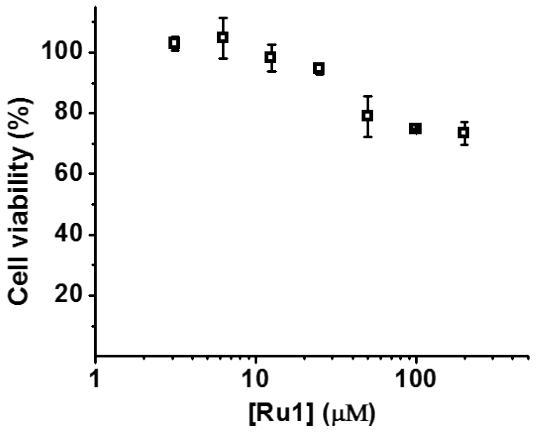


Figure S8. Viability of the normal NIH 3T3 cell (mouse embryo fibroblast cells) in the presence of 3, 6, 12, 25, 50, 100 and 200 μM **Ru1**.