Supporting Information for

Mixed valance copper (I,II) complexes based on 2-alkylthio-5-arylmethylene-4H-imidazolin-4-ones with unexpected structure: synthesis, polymerase inhibition, nuclear localization and anticancer activity.

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Structures

Figure S1. The crystal structures of complexes **2**, **4**, **5** and **6**.

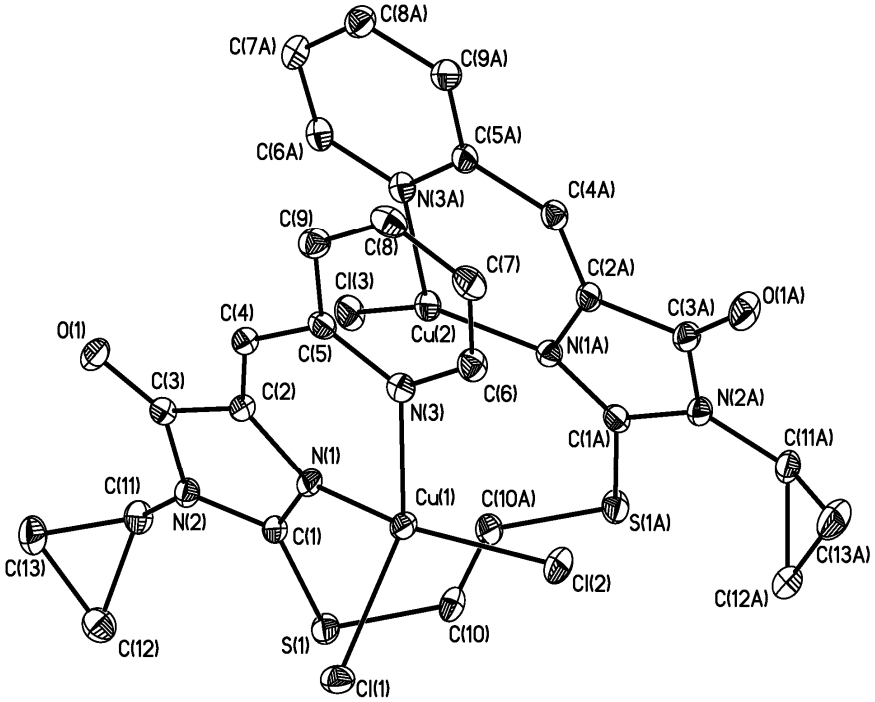
X-Ray crystallography data for complexes **2**, **4**, **5**, **6** have been deposited at the Cambridge Crystallographic Data Centre and copies can be obtained on request, free of charge, by quoting the publication citation and the deposition number CCDC 875592, CCDC 875592, CCDC 931655, CCDC 931656, respectively.

X-Ray crystallography data for complex **2** presented in the main text.

Table S1. Crystal data and structure refinement for complex **2.**

Identification code	OK12	
Empirical formula	$C_{26}\ H_{24}\ Cl_3\ Cu_2\ N_6\ O_2\ S_2$	
Formula weight	750.06	
Temperature	100(2) K	
Wavelength	0.71073 Å	
Crystal system	Monoclinic	
Space group	P 21/c	
Unit cell dimensions	a = 14.508(2) Å	α= 90°.
	b = 15.845(3) Å	β= 110.362(3)°.
	c = 13.610(2) Å	γ = 90°.
Volume	2933.2(8) $Å^3$	
Z	4	
Density (calculated)	1.699 Mg/m^3	
Absorption coefficient	1.903 mm^{-1}	
F(000)	1516	
Crystal size	0.25 x 0.20 x 0.20 mm^3	
Theta range for data collection	1.97 to 28.00°.	
Index ranges	-18<=h<=19, -20<=k<=20, -17<=l<=17	
Reflections collected	21536	
Independent reflections	7064 [R(int) = 0.0666]	
Completeness to theta = 28.00°	99.8 %	
Absorption correction	Semi-empirical from equivalents	
Max. and min. transmission	0.701 and 0.646	
Refinement method	Full-matrix least-squares on F^2	
Data / restraints / parameters	7064 / 0 / 370	
Goodness-of-fit on F^2	1.010	
Final R indices [for 4951 rfln with I>2sigma(I)]		R1 = 0.0457, wR2 = 0.1011
R indices (all data)	R1 = 0.0771, wR2 = 0.1174	
Largest diff. peak and hole	0.861 and -0.581 e. $Å^{-3}$	

X-Ray crystallography data for complex **4**.



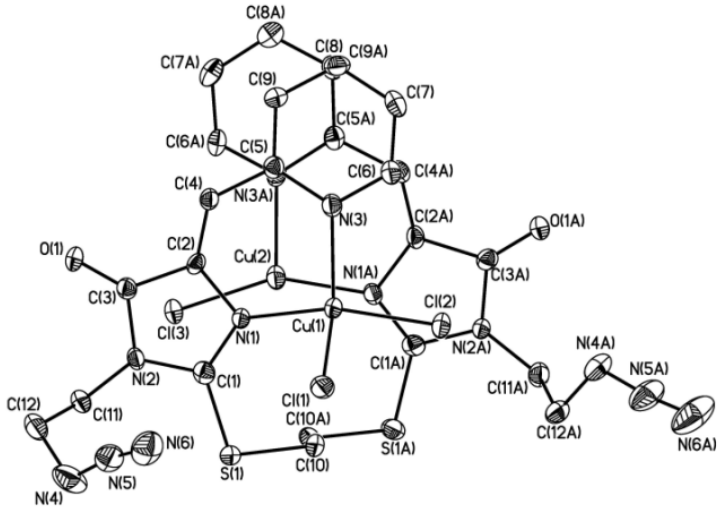
General view of the complex **4** in representation of atoms via thermal ellipsoids at 50% probability level. Hydrogen atoms are not shown. Crystallographic data have been deposited at the Cambridge Crystallographic Data Centre and copies can be obtained on request, free of charge, by quoting the publication citation and the deposition number CCDC 875592.

Table S2. Crystal data and structure refinement for complex **4.**

Identification code	OK98	
Empirical formula	$C_{26}\ H_{24}\ Cl_3\ Cu_2\ N_6\ O_2\ S_2$	
Formula weight	750.06	
Temperature	100(2) K	
Wavelength	0.71073 Å	
Crystal system	Monoclinic	
Space group	P 21/c	
Unit cell dimensions	a = 14.3168(6) Å	α= 90°.
	b = 15.8994(6) Å	β= 108.8600(10)°.
	c = 13.2058(5) Å	γ = 90°.
Volume	2844.63(19) $Å^3$	
Z	4	
Density (calculated)	1.751 Mg/m^3	
Absorption coefficient	1.962 mm^{-1}	
F(000)	1516	

Crystal size	0.20 x 0.20 x 0.15 mm^3
Theta range for data collection	1.97 to 29.00°.
Index ranges	-19<=h<=19, -21<=k<=21, -18<=l<=18
Reflections collected	34452
Independent reflections	7555 [R(int) = 0.0302]
Completeness to theta = 29.00°	100.0 %
Absorption correction	Semi-empirical from equivalents
Max. and min. transmission	0.753 and 0.690
Refinement method	Full-matrix least-squares on F^2
Data / restraints / parameters	7555 / 0 / 370
Goodness-of-fit on F^2	1.006
Final R indices [I>2sigma(I)]	R1 = 0.0254, wR2 = 0.0771
R indices (all data)	R1 = 0.0324, wR2 = 0.0819
Largest diff. peak and hole	0.500 and -0.515 e. $Å^{-3}$

X-Ray crystallography data for complex **5**.



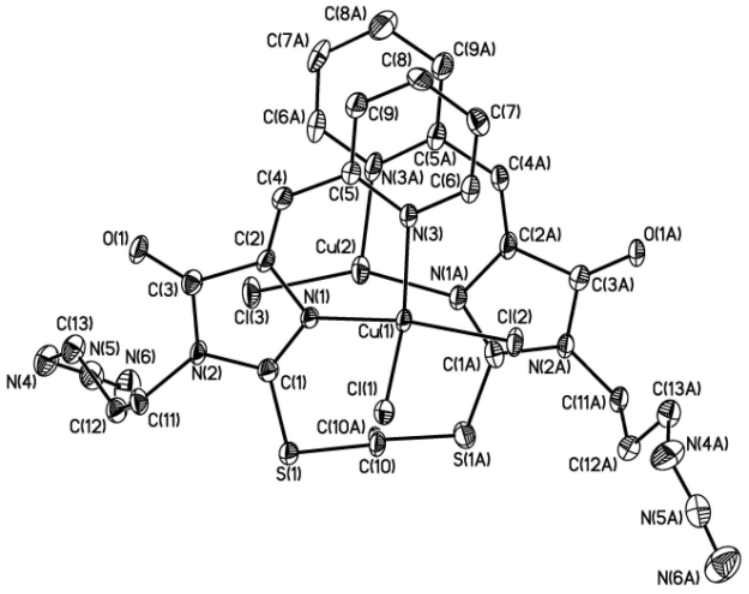
General view of the complex **5** in representation of atoms via thermal ellipsoids at 50% probability level. Hydrogen atoms are not shown. Crystallographic data have been deposited at the Cambridge Crystallographic Data Centre and copies can be obtained on request, free of charge, by quoting the publication citation and the deposition number CCDC 931655.

Table S3. Crystal data and structure refinement for complex **5.**

Identification code	VG-82
Empirical formula	$C_{24}\ H_{22}\ Cl_3\ Cu_2\ N_{12}\ O_2\ S_2$
Formula weight	808.09
Temperature	100(2) K
Wavelength	0.71073 Å

Crystal system	Monoclinic	
Space group	P 21/c	
Unit cell dimensions	a = 14.441(3) Å	α= 90°.
	b = 15.869(3) Å	β= 105.063(4)°.
	c = 13.737(3) Å	γ = 90°.
Volume	3040.0(10) $Å^3$	
Z	4	
Density (calculated)	1.766 Mg/m^3	
Absorption coefficient	1.848 mm^{-1}	
F(000)	1628	
Crystal size	0.35 x 0.10 x 0.02 mm^3	
Theta range for data collection	1.94 to 27.00°.	
Index ranges	-18<=h<=18, -20<=k<=20, -17<=l<=17	
Reflections collected	29600	
Independent reflections	6638 [R(int) = 0.0934]	
Completeness to theta = 27.00°	100.0 %	
Absorption correction	Semi-empirical from equivalents	
Max. and min. transmission	0.960 and 0.569	
Refinement method	Full-matrix least-squares on F^2	
Data / restraints / parameters	6638 / 0 / 406	
Goodness-of-fit on F^2	1.001	
Final R indices [for 4432 rfln with I>2sigma(I)]		R1 = 0.0410, wR2 = 0.0835
R indices (all data)	R1 = 0.0812, wR2 = 0.0991	
Largest diff. peak and hole	0.538 and -0.464 e. $Å^{-3}$	

X-Ray crystallography data for complex **6**.



General view of the complex **6** in representation of atoms via thermal ellipsoids at 50% probability level. Hydrogen atoms are not shown. Crystallographic data have been deposited at the Cambridge Crystallographic Data Centre and copies can be obtained on request, free of charge, by quoting the publication citation and the deposition number CCDC 931656.

Table S4. Crystal data and structure refinement for complex **6.**

Empirical formula	$C_{26}\ H_{26}\ Cl_3\ Cu_2\ N_{12}\ O_2\ S_2$	
Formula weight	836.14	
Temperature	100(2) K	
Wavelength	0.71073 Å	
Crystal system	Monoclinic	
Space group	P 21/c	
Unit cell dimensions	a = 14.7078(16) Å	α= 90°.
	b = 16.0864(18) Å	β= 110.377(2)°.
	c = 14.5895(16) Å	γ= 90°.
Volume	3235.8(6) $Å^3$	
Z	4	
Density (calculated)	1.716 Mg/m^3	
Absorption coefficient	1.740 mm^{-1}	
F(000)	1692	
Crystal size	0.35 x 0.10 x 0.02 mm^3	
Theta range for data collection	1.95 to 27.00°.	
Index ranges	-18<=h<=18, -20<=k<=20, -18<=l<=18	
Reflections collected	33552	
Independent reflections	7069 [R(int) = 0.0875]	
Completeness to theta = 27.00°	99.9 %	

Absorption correction	Semi-empirical from equivalents
Max. and min. transmission	0.960 and 0.581
Refinement method	Full-matrix least-squares on F^2
Data / restraints / parameters	7069 / 0 / 424
Goodness-of-fit on F^2	1.008
Final R indices [for 4650 rfln with I>2sigma(I)]	R1 = 0.0402, wR2 = 0.0838
R indices (all data)	R1 = 0.0820, wR2 = 0.1002
Largest diff. peak and hole	0.782 and -0.580 e. $Å^{-3}$

Table S5. Cyclic Voltamperometry Data for Copper Complexes.

All reduction potentials are with respect to SCE, and a 200 mV s^{-1} scan rate was used for all the measurements.

N	Complex	$E_{1/2}$(V)[a]	ΔE_p(mV)
2	AllThd*Cu_2Cl_3	0.24	120
3	PhThd*Cu_2Cl_3	0.48	100
4	CycThd*Cu_2Cl_3	0.42	80

[a] Half-wave reduction potential is given by $E_{1/2} = (E_{pa} + E_{pc})/2$; [b] The difference in potential is given by $\Delta E_p = E_{pa} - E_{pc}$, where E_{pa} and E_{pc} are the anodic and cathodic peak potentials, respectively.

Table S6. IC_{50} values for complex **2** in MTT cytotoxicity experiments in different cell lines.

	IC_{50}, μM MDA-MB-231	IC_{50}, μM HepG2	IC_{50}, μM PC3
AllThd*Cu_2Cl_3	4,14±0,26	5,04±1,10	3,97±0,13

Table S7. Concentrations of Cu^{2+} in cells and media after complex **2** or corresponding concentrations of $CuCl_2$ treatment measured by ICP-MS**.**

Sample	C [Cu^{2+}], µM (Measured)	C [Cu^{2+}], µM (Theoretical)
Cells incubated with 9 µM complex **2**	218,6	
Cells incubated with 3 µM complex **2**	62,6	
Cells incubated with 18 µM $CuCl_2$	18,8	
Cells incubated with 6 µM $CuCl_2$	10,8	
Cells incubated without substance	6,25	
Media with 9 µM complex **2**	15,9	18
Media with 3 µM complex **2**	5,9	6
Media with 18 µM $CuCl_2$	13,3	18
Media with 6 µM $CuCl_2$	6,6	6
Media without substance	0,3	

Table S8. Tumor growth inhibition in mice after injection of complex **2.**

The inhibition of tumor growth in mice lines of C57BL / 6 (female) with adenocarcinoma 755 after five intraperitoneal injection of complex **2**.

Dose, mg/kg	7 days after treatment		14 days after treatment	
	V (mm^3), average of tumor volume	Inhibition of tumor growth, %	V (mm^3), average of tumor volume	Inhibition of tumor growth, %
24	1092.3±248,3	73.5	4170.2±776,8	59.5
12	2218.8±942,7	46.1	6577.8±2685,2	36.1
Control	4119.2±1395,6	-	10290.4±3235,4	-

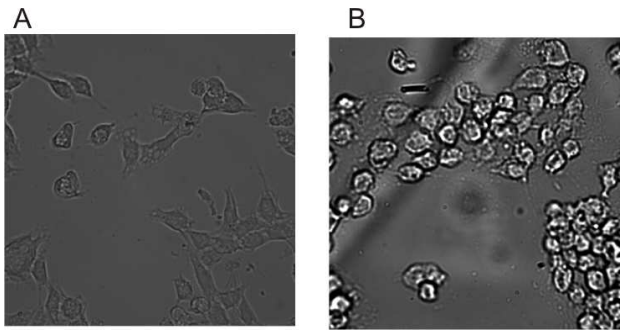


Figure S2. Complex **2** induces morphological changes of cells.

A - untreated HEK293 cells. B – HEK293 cells treated with complex **2**.

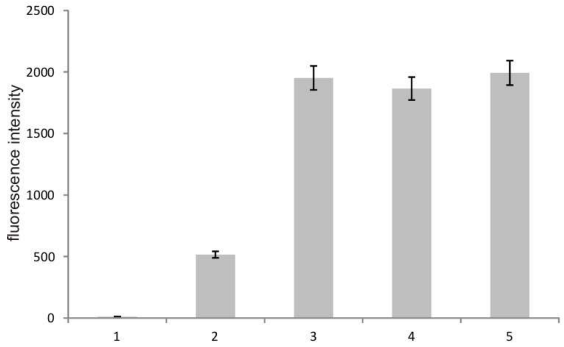


Figure S3. Generation of intracellular Reactive Oxygen Species (ROS) after complex **2** and ligand **L2** treatment of HEK293 cells. 1 – untreated HEK293 cells, 2 – HEK293 cells treated with CM-H_2DCFDA probe, 3 – HEK293 cells treated with H_2O_2 and CM-H_2DCFDA probe, 4 – HEK293 cells treated with complex **2** (2,6 μM) and 5 – HEK293 cells treated with ligand **L2** (16 μM) and CM-H_2DCFDA.

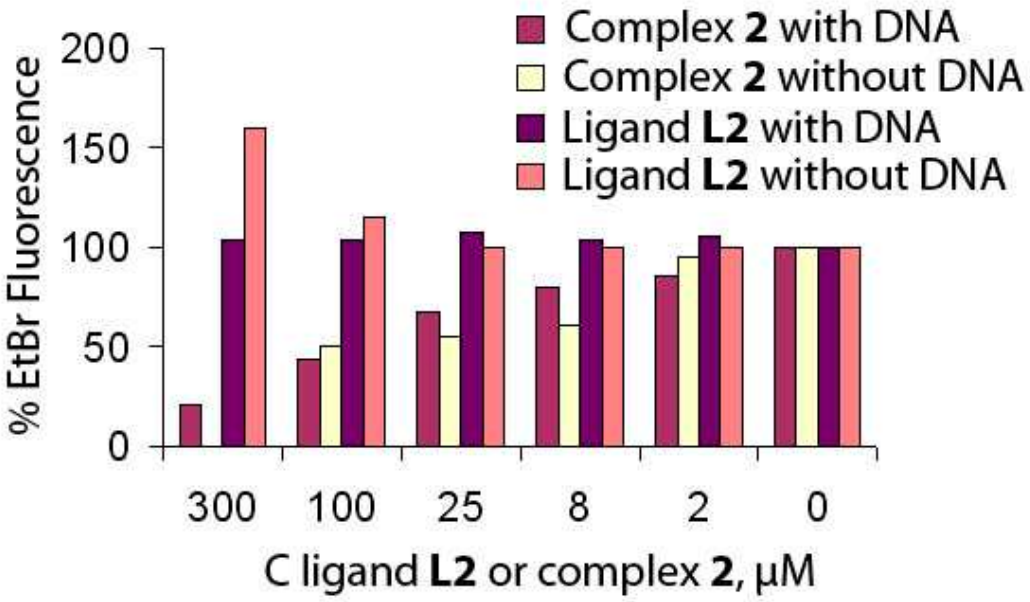


Figure S4. DNA-binding assay based on ethidium bromide fluorescence emission quenching. Ethidium bromide (EtBr) displacement: the percentage of 1,3 μM EtBr fluorescence in presence or absence of 25 mM DNA at different concentrations of complex **2** or ligand **L2**.

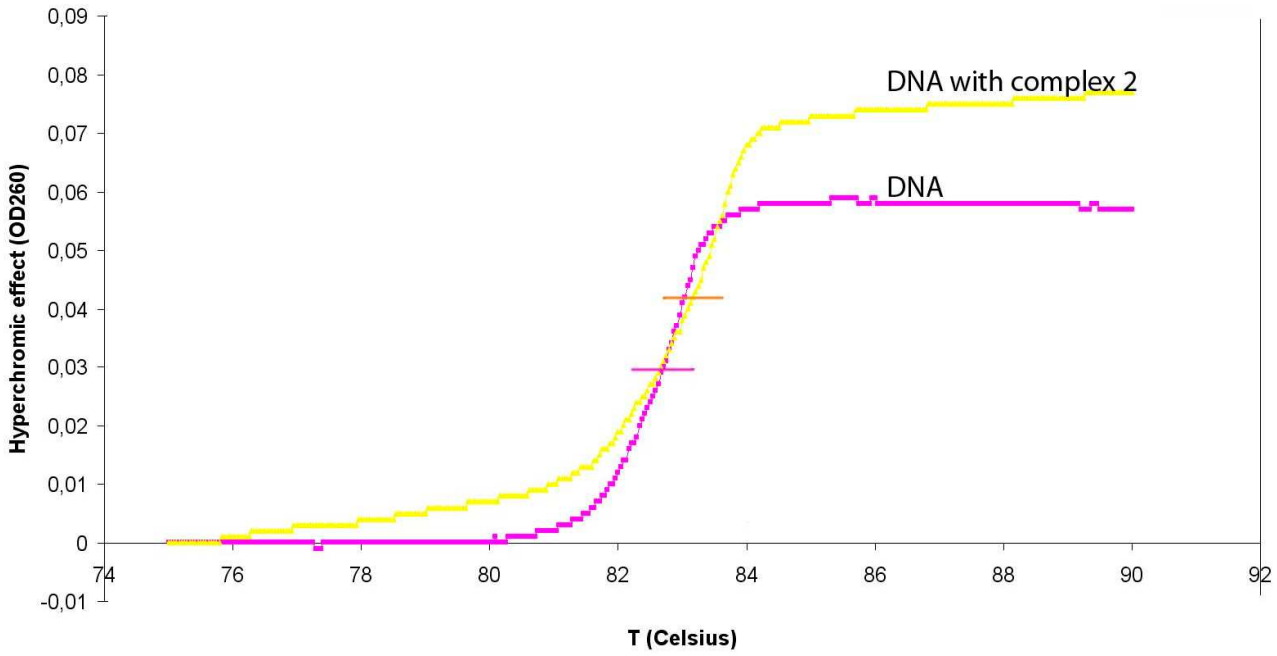


Figure S5. DNA-DNA duplex stabilization test.

Measuring the hyperchromic effect of DNA melting in the presence and absence of 20 μM complex **2**. No reliable changes in melting temperature of 124 bp duplex DNA in presence of up to 20 μM complex **2** compared to melting without substance.

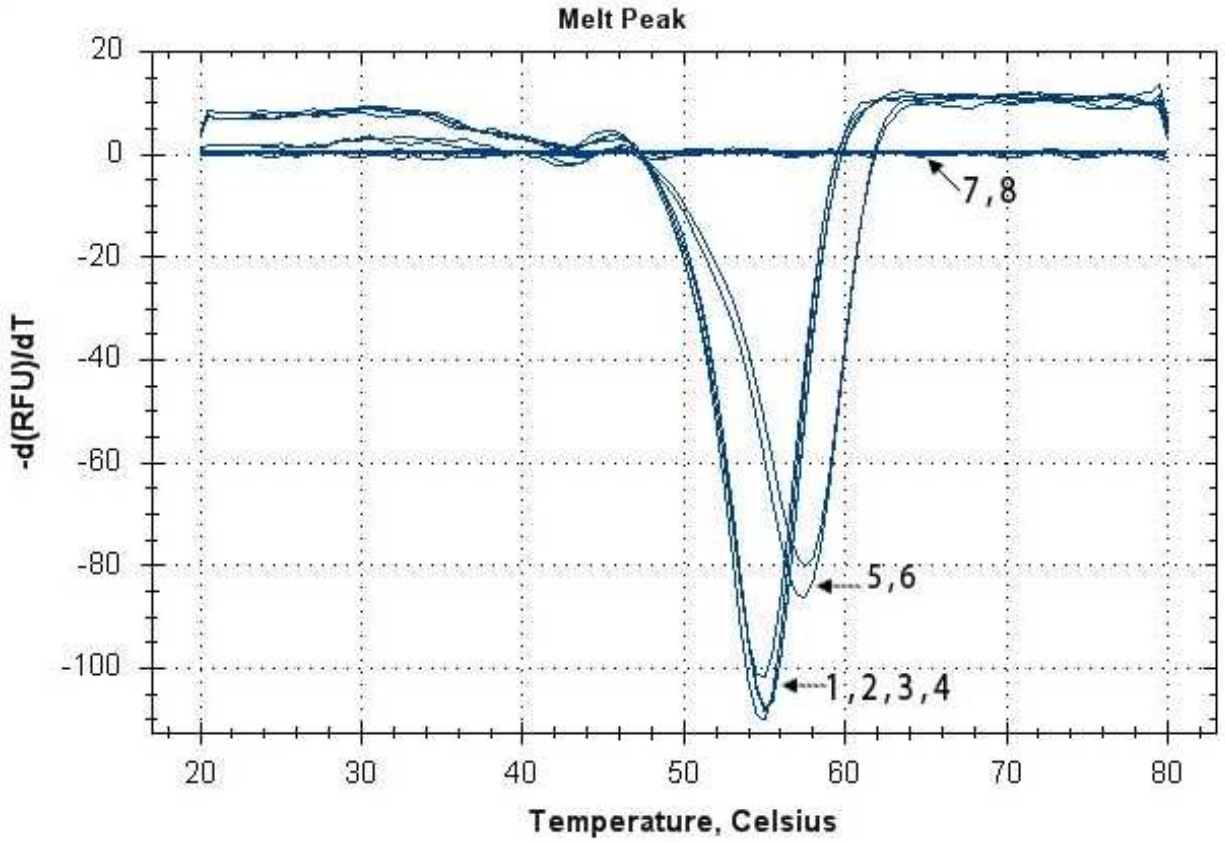


Figure S6. DNA-RNA duplex stabilization test.

Graph "1","2" – melting curve of DNA-RNA duplex, "3","4"– melting of DNA-RNA duplex in presence of 20 μM complex **2**, "5","6" – melting of DNA-RNA duplex in presence of 20 μM TMPyP (5,10,15,20-tetrakis(N-methyl-4-pyridyl)-21H,23H-porphine), which binds nucleic acid duplex [1], "7","8" – probe without DNA-RNA duplex in the same conditions. There is no stabilization of DNA-RNA-duplex in presence of 20 μM complex **2**.

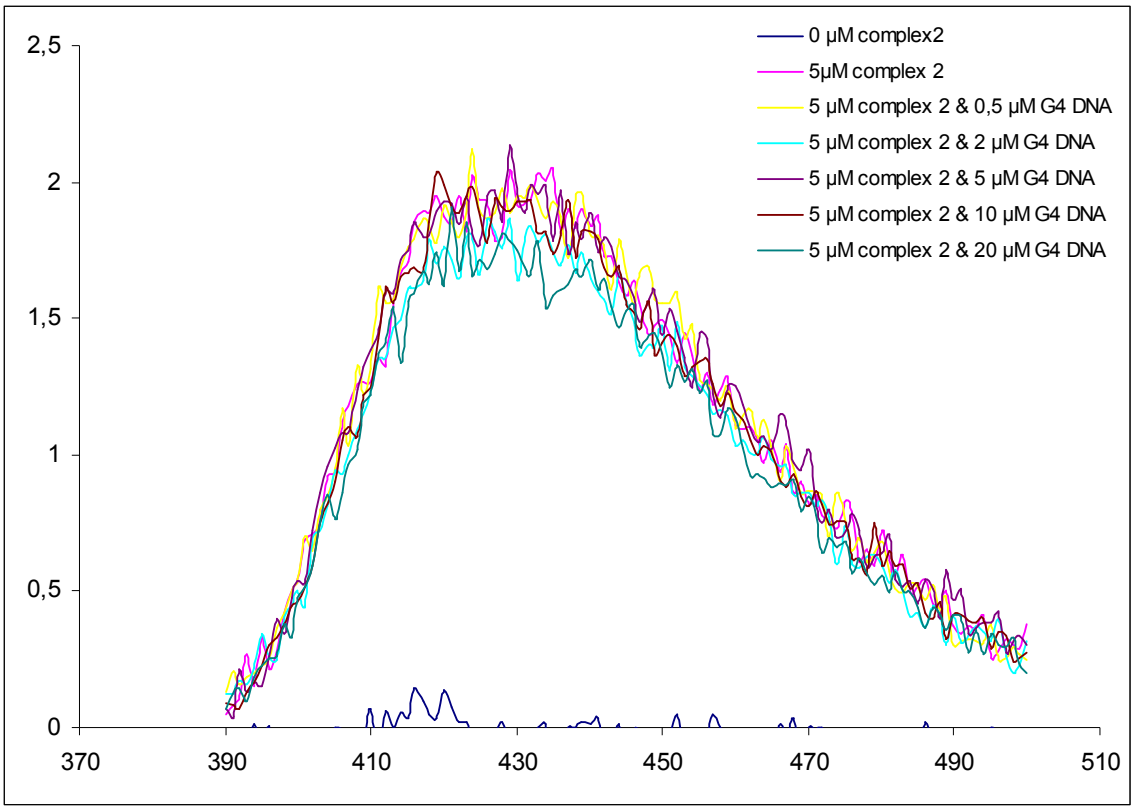


Figure S7. DNA-quadruplex interaction test.
Fluorescence spectra of complex **2** upon titration from 0,5 to 20 µM of quadruplex DNA.

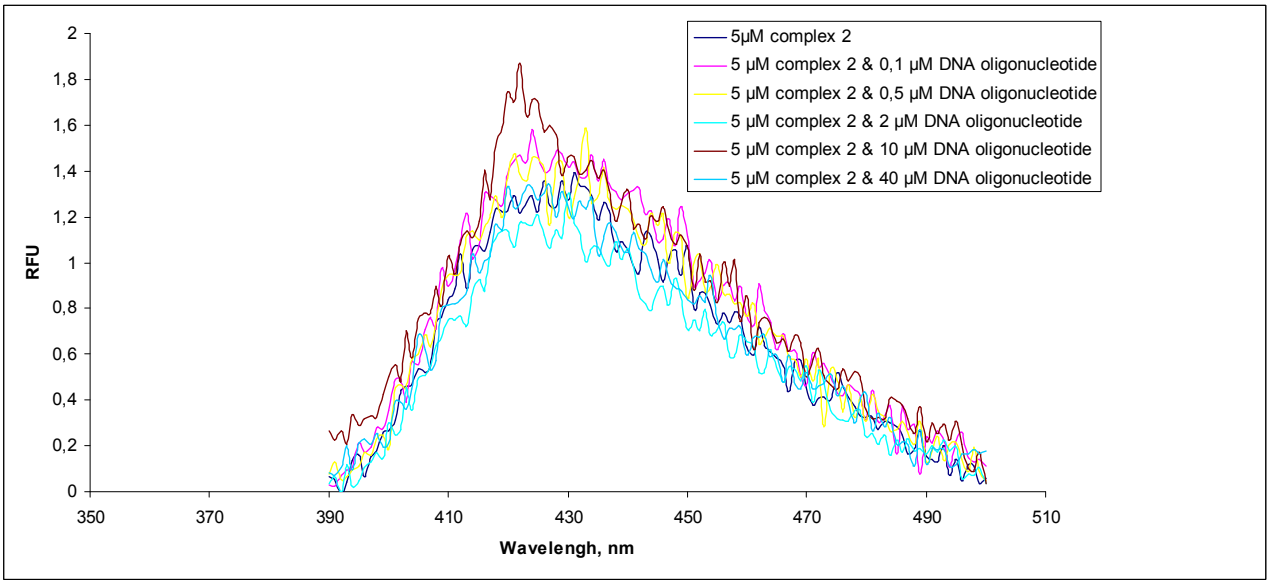


Figure S8. ssDNA interaction test.
Fluorescence spectra of complex **2** upon titration with 0,1-20 µM of DNA-oligonucleotide.

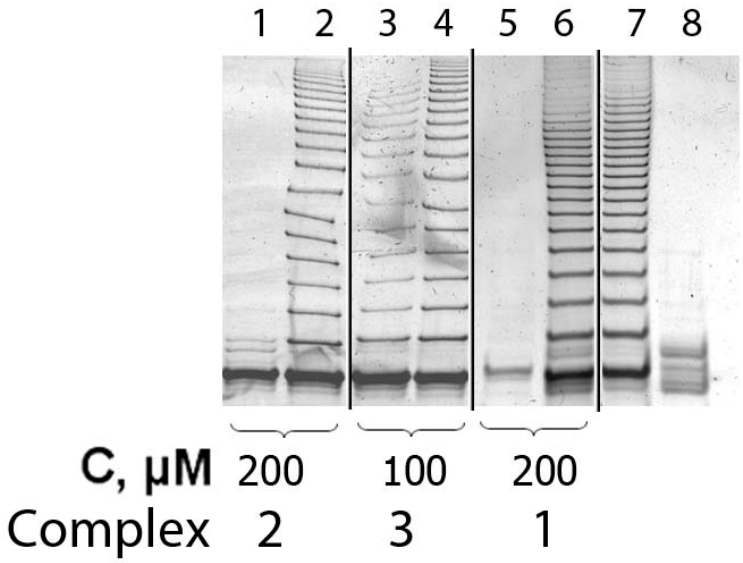


Figure S9. Analysis of telomerase activity by TRAP assay in presence complex **2**.

Examples of TRAP assay screenings for inhibitors. Substances were primary tested at 200 µM or maximal concentration in case of low solubility in water. "1,3,5" – substances were added in the telomerase reaction. "2,4,6" - substances were added after telomerase reaction, before PCR. "7" – control without inhibitors, "8" – contamination control without telomerase-containing extract.

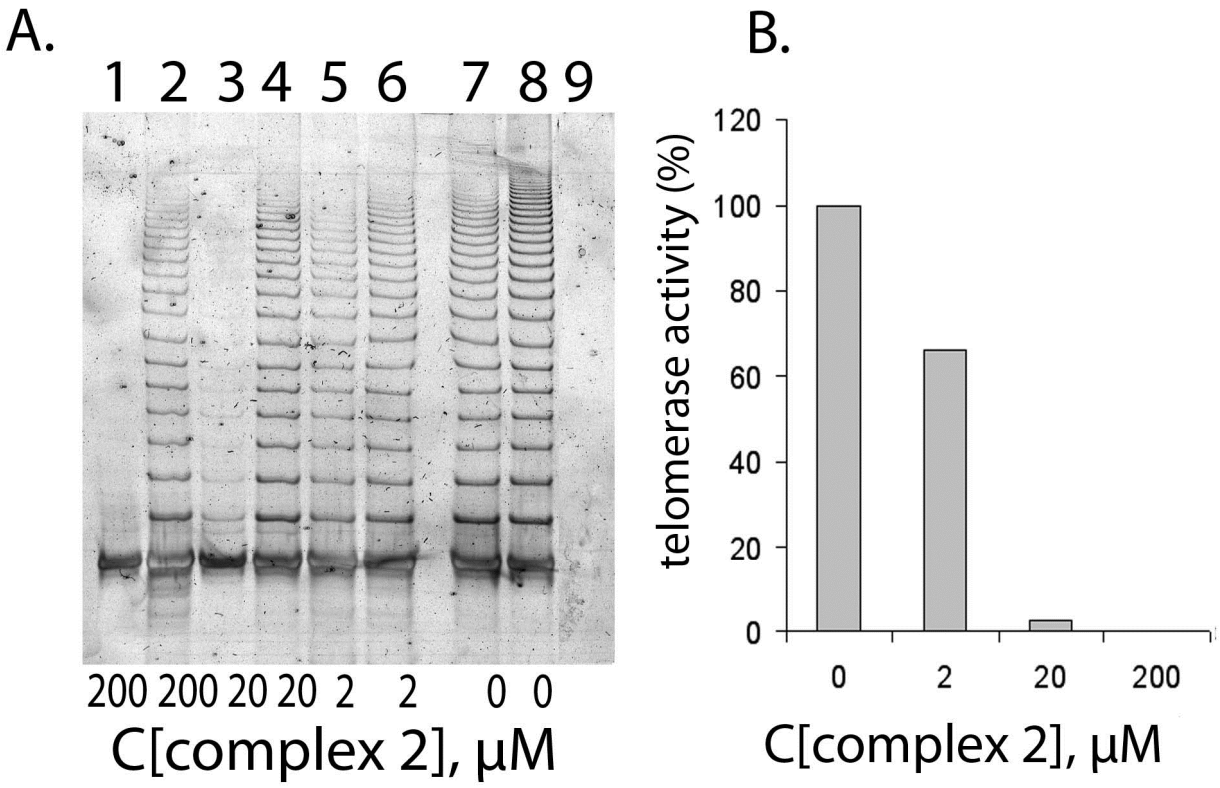


Figure S10. IC50 evaluation of complex **2** for telomerase activity by TRAP assay.

A. Gel electrophoresis of TRAP assay products. 1, 3, 5 – products of the telomerase reaction in presence of complex **2**; 2, 4, 6 – complex **2** in DMSO was added after telomerase reaction, before PCR. 7 – the same DMSO volume in reaction. 8 – products of telomerase reaction, 9 – contamination control without telomerase-containing extract. B. Densitometry analysis of telomerase activity.

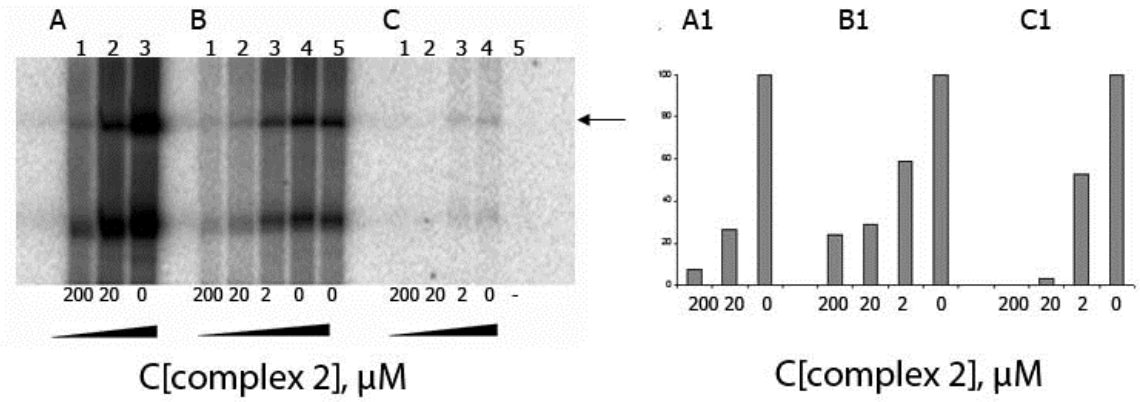


Figure S11. Analysis of HIV reverse transcriptase activity in presence complex **2.**

Reverse transcription by HIV reverse transcriptase in the presence of complex **2**. A - 1 activity unit HIV RT, B - 0,25 activity unit HIV RT, C - 0,0625 activity unit HIV RT . Concentration of complex **2** marked on the bottom panel. "-" - without HIV RT in the reaction. Arrow shows analyzed band. A1, B1, C1 are densitometry results of A, B, C part.

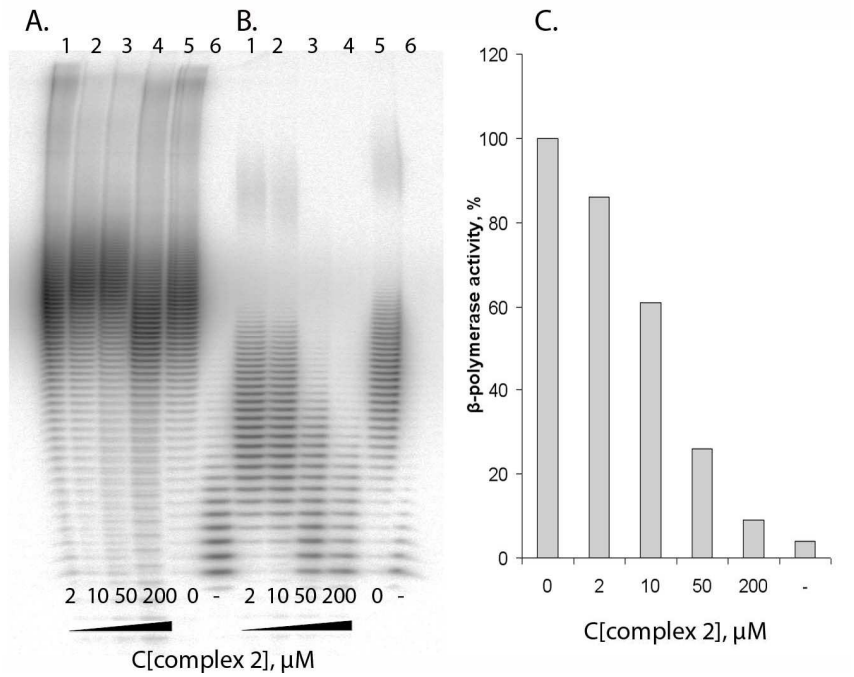


Figure S12. Analysis of calf thymus DNA polymerase *β* activity in presence complex **2** with visualization by PAGE separation

Calf thymus DNA polymerase *β* activity was analyzed by radiophotography of PAGE separation of reaction products. A, B – reaction products of 3.5 µM and 0.35 µM calf thymus polymerase *β*, respectively, in the presence of complex **2** in different concentrations. C – calf thymus polymerase *β* activity, calculated with scintillation measurement of the samples from B part of the gel. 0 - calf thymus polymerase *β* without inhibition. 2-200 - calf thymus polymerase *β* products in the presence of complex **2** in concentrations from 2 to 200 µM. "-" - background with heat-inactivated calf thymus polymerase *β*. IC 50 for 0.35 µM calf thymus polymerase *β* is 17 µM.

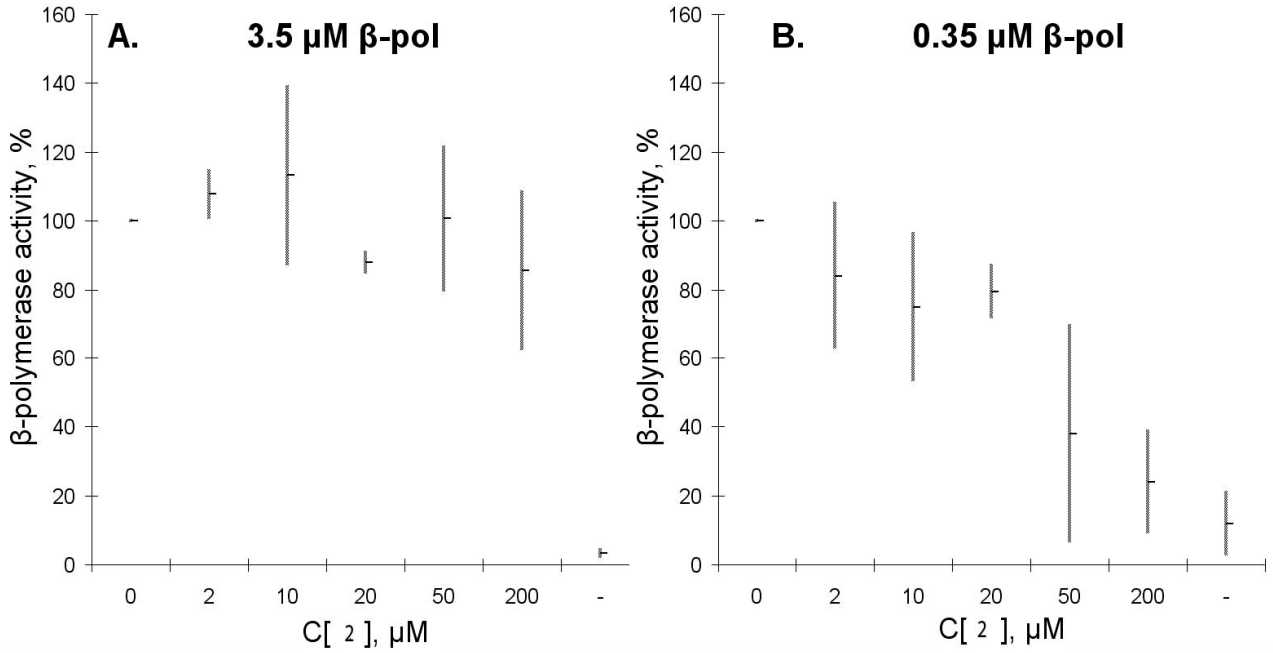


Figure S13. Measure of calf thymus DNA polymerase *β* activity by scintillation calculation in the presence complex **2.**

A - 3.5 μM calf thymus DNA polymerase *β*, B - 0.35 μM calf thymus DNA polymerase *β*. 0 - activity of calf thymus DNA polymerase *β*. 2-200 - calf thymus DNA polymerase *β* activity in presence of 2 in concentrations from 2 to 200 μM.."-" - scintillation background with heat-inactivated calf thymus DNA polymerase *β*. IC 50 for 0.35 μM calf thymus DNA polymerase *β* is 20 μM.

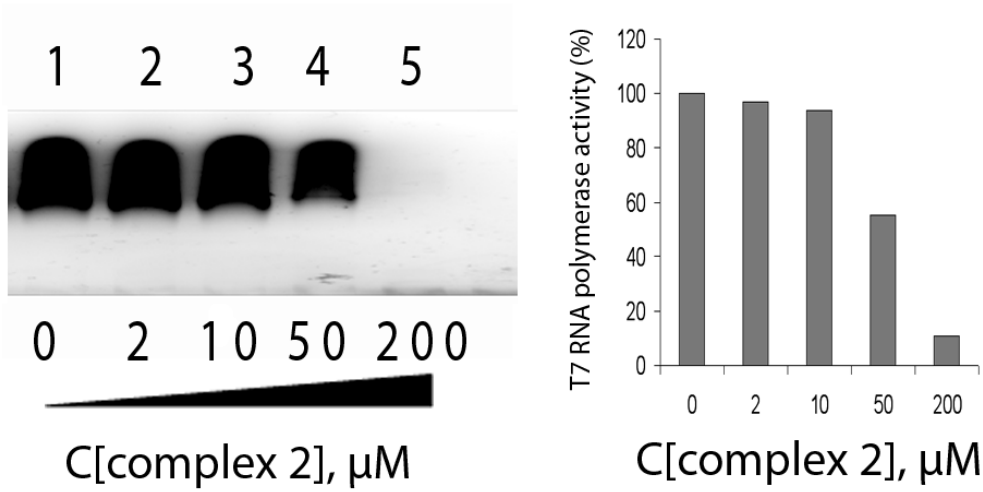


Figure S14. Analysis of T7 RNA polymerase activity in the presence complex **2.**
T7 RNA polymerase products were separated in agarose gel after reaction in presence of different concentrations complex **2**. "2-5" - 2-200 µM of complex **2**, "1" - reaction without complex **2**.

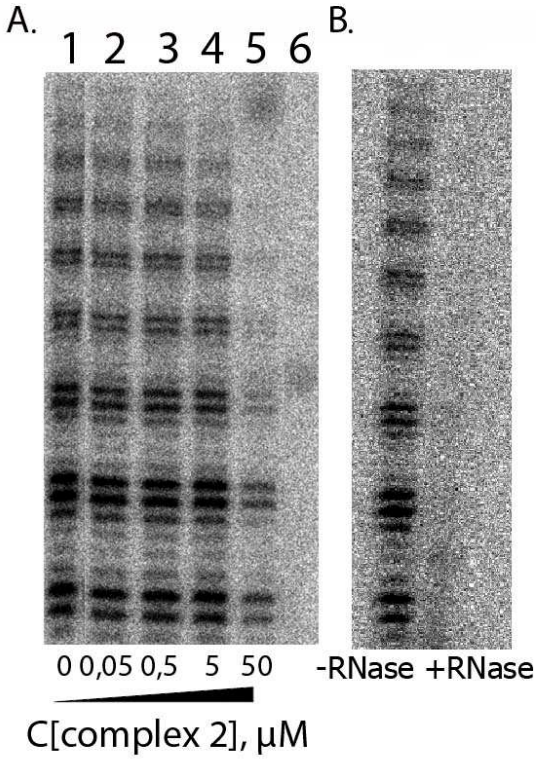


Figure S15. Analysis of telomerase activity by direct assay in the presence complex **2.**

A - direct telomerase assay products in the presence of complex **2** in concentrations from 0.05 to 50 μM separated by denaturing PAGE. 0 – reaction without complex **2**, only solvent was added in reaction, "-" - reaction without telomerase-containing extract. B - Telomerase activity of cell extracts with and without RNase A treatment.

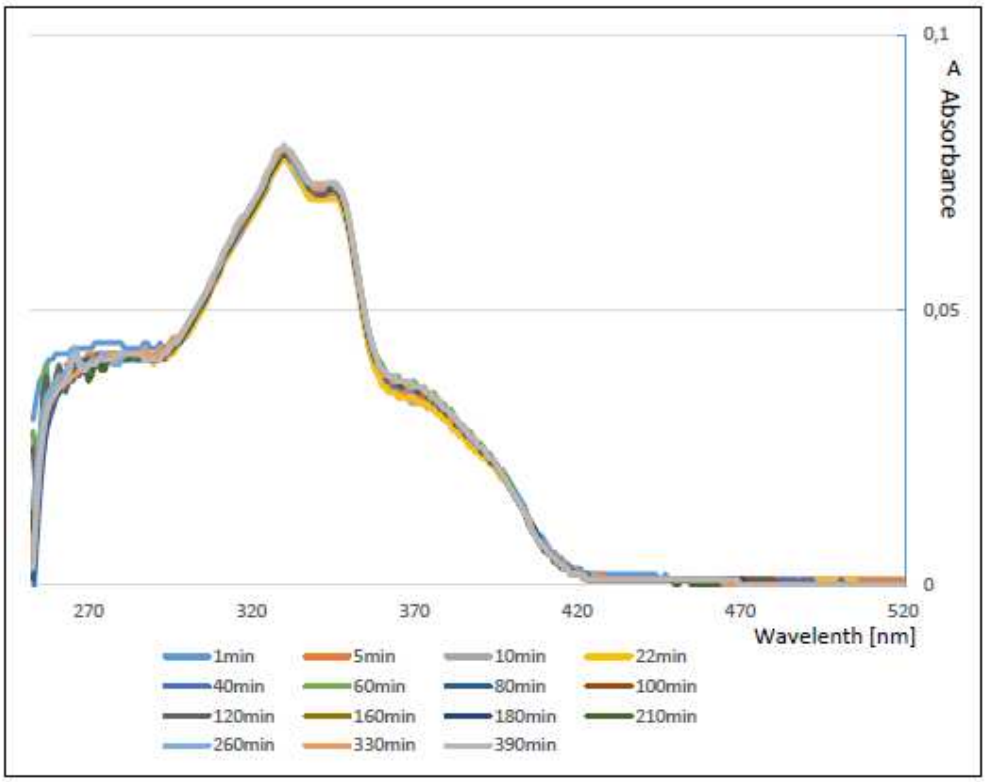


Figure S16. UV-visible absorbance spectra of complex **2** (2μM solution) in the presence of TRAP buffer.

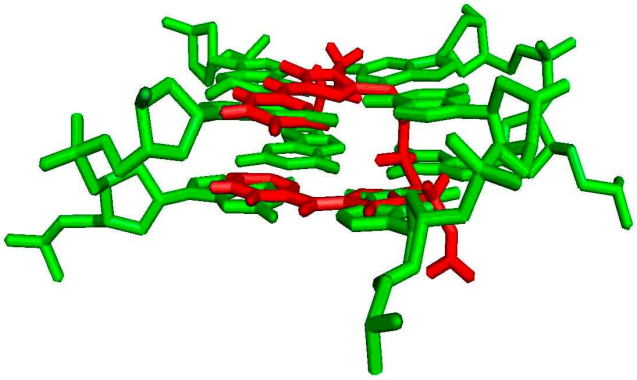


Figure S17. Alignment of complex **2** on G-quadruplex.

Manual alignment of complex **2** (red) on G-quadruplex structure (green , PDB id 1KF1) with PyMOL soft.

Supplementary materials and methods.

Chemicals

Unless otherwise noted, all preparations were carried out in reagent grade solvents. All chemicals used in the synthesis were obtained from Acros or Sigma-Aldrich and were used without further purification.

Solvents were deoxygenated/distilled/purified by bubbling through a stream of argon or by conventional methods and dried over molecular sieves.

Taq polymerase was obtained from Fermentas, T7 RNA polymerase was obtained from Ambion and Fermentas. HIV reverse transcriptase was obtained from Worthington Biochemical, calf DNA polymerase β was a kind gift of Prof. O.I. Lavrik. Radiolabeled NTPs and dNTPs were obtained from Institute of Bioorganic Chemistry of the Russian Academy of Sciences. All other enzymes and chemicals for molecular biology tests were obtained from Fermentas or Invitrogen and were used without further purification.

Analytical thin-layer chromatography

Analytical thin-layer chromatography (TLC) was performed on Merck silica gel aluminium plates with F-254 indicator. Compounds were visualized by irradiation with UV light or iodine staining.

Pre-synthesis

2-thioxo-3-phenyl-5-(pyridine-2-ylmethylene)-3,5-dihydro-4H-imidazole-4-on was synthesized from 2-thioxo-imidazol-4-one according to literature procedure [2]. Initial substituted 2-thiohydantoines were synthesized by known methods [3].

Physical Measurements

Elemental analyses were carried out on a Vario MICRO cube CHNS/O Elementar. ^{1}H-NMR and ^{13}C-NMR were recorded on a Bruker Avance400 (400 MHz) using CDCl3 or (CD3)2SO as solvent. Data are reported in the following order: chemical shift (δ) values are reported in ppm with the solvent resonance as internal standard (CDCl3: δ = 7.26 ppm for 1H, δ = 77.16 ppm for 13C; (CD3)2SO: δ = 3.30 ppm for 1H, δ = 39.52 ppm for 13C); multiplicities are indicated br s (broadened singlet), s(singlet), d (doublet), t (triplet), q (quartet) m (multiplet); coupling constants (J) are given in Hertz (Hz).Temperature was kept constant using a variable temperature unit within the error limit of ±1 K.

Details of electrochemical experiments:

Electrochemical studies were carried out on a PI-50-1.1. potentiostat in MeCN or water. Glassy-carbon (2mm in diameter in MeCN; 4 mm in diameter in water) disks polished by Al_2O_3 were used as

working electrodes; a 0.05 M Bu_4NClO_4 solution in MeCN served as supporting electrolyte; Ag/AgCl/KCl (satur.) was used as a referenced electrode. All measurements were carried out under argon. The samples were dissolved in pre-deairated solvents.

X-ray crystal structure determinations:

X-ray diffraction was measured with a CAD4 diffractometer at 293 K [graphite monochromator, l(MoKα) = 0.71073 Å, w-scan with a step of 0.3°]. The structure was solved by a direct method SIR2002 (solution, [4]) and JANA2000 (refinement, [5]). Atomic coordinates, bond lengths, bond angles and thermal parameters have been deposited at the Cambridge Crystallographic Data Centre (CCDC). These data can be obtained free of charge via www.ccdc.cam.uk/ conts/retrieving.html (or from the CCDC, 12 Union Road, Cambridge CB2 1EZ, UK; fax: +44 1223 336 033; or deposit@ccdc.cam.ac.uk).

Telomerase activity measurement assays

TRAP assay [6] was performed with some modifications [7].

Extract preparation for TRAP

Cells were trypsinazed and precipitated by centrifugation (10 min, 2000 g). Then they were washed with phosphate–salt buffer (1.7 mM KH_2PO_4, 5.2 mM Na_2HPO_4, and 150 mM NaCl) and resuspended in lysis buffer: 10 mM Trishydroxymethylaminomethane-HCl (Tris–HCl), pH 7.5; 10 mM $MgCl_2$; 1 mM ethyleneglycoltetraacetate (EGTA), 5 mM β-mercaptoethanol, 5% glycerol, 0.5% 3-((3-Cholamidopropyl)dimethylammonio)-1-pro-panesulfonate (CHAPS); 0.1 mM phenylmethylsulfonylfluoride (PMSF), 1 ml per 1 million cells. Further, the samples were incubated for 30 min in ice and centrifuged for 10 min at 4°C and 16000 g. The supernatant was separated and the extract was aliquoted and frozen in liquid nitrogen.

TRAP

48 μl of TRAP mixtures were prepared, containing TRAP buffer [20 mM of Hepes-N-Hydroxyethylpiperazine-N-ethane-sulfonate (Hepes-KOH)]; 1.5 mM $MgCl_2$; 63 mM KCl; 1mM EGTA; 0.1 mg/ml of bovine serum albumin; 0.005% v/v polyoxyethylene (20) sorbitan monolaurate; 20 μM dNTP; 1.6 μM TS oligonucleotide (AATCCGTCGAGCAGAGTT). 1 μl of inhibitor solution was added in sample probes. Control probes didn't contain inhibitor. Then cellular extract from 1000 cells were added to the sample and control probes and reaction mixtures were incubated for 30 min at 25°C. Finally samples were placed in ice.

At the second stage inhibitor solution was added to the control probes. Then two units of *Taq* DNA polymerase (Fermentas) and 0.1 μg of ACX oligonucleotide (GCGCGGCTTACCCTTACCCTTACCCTTACCCTAACC) were added in all probes. PCR was

performed according to the following scheme: 35 s (94°C), 35 s (50°C), 90 s (72°C) (29 cycles, Mastercycler (Eppendorf)).

15 μl of the PCR was mixed with 3 μl of loading dye and applied to a 15% polyacrylamide gel (acrylamide: bis-acrylamide 1 : 19, TBE 1x (0.1 M Tris-HCl; 0.1 M H_3BO_3; 2 mM Na_2 EDTA)); 0.1% TEMED (N,N,N',N'-Tetramethylethylenediamine), 0.1% ammonium persulphate). TBE 1x was used as electrode buffer. The electrophoresis was performed until the xylene cyanol marker reached 20 cm. The gel was stained with Sybr green I solution (concentrate in dimethyl sulfoxide (Sigma–Aldrich) and diluted 10^6 times with 0.1 M Tris-HCl buffer, pH 8.3) and captured using a PhosphorImager (GE Typhoon TLA 9500). Gel densitometry was estimated with ImageQuant program. We calculate IC50 with linear approximation of concentration-inhibition effect between two nearest to IC50 data points.

Telomerase extract preparation for direct assay

Extract preparation was based on [8]. HEK293T cells were transfected with 24 μg of plasmid DNA and Lipofectamine 2000 (Invitrogen) in 10cm petri dish following manufacturer's protocol. The mass ratio of hTERT- and hTR- expressing plasmids is 1:1 (12 μg pBabe-hygro-hTERT and 12μg pMND-Banshee-U1-hTR [9]) (a kind gift of J. Lingner). 2 days post-transfection, cells were detached by trypsinization, washed once in PBS and lysed in 400 μl of CHAPS lysis buffer (10 mM Tris-HCl pH 7.5, 1 mM MgCl2, 1 mM EGTA, 0.5% CHAPS, and 5 mM β-mercaptoethanol). After incubation on ice for 1h, cell lysate was centifuged at 4°C for 5 min at 13000 g. The supernatant was centrifuged at 4°C for 30 min at 100000 g, then supernatant was aliquoted, quick frozen in liquid nitrogen and stored at -80°C.

Direct telomerase activity assay

The reaction mixture (20 μL) contained 50 mM Tris-OAc (pH 8.5), 50 mM KCl, 1 mM $MgCl_2$, 5 mM β-mercaptoethanol, 1 mM spermidine, 1 μM 5'-biotinylated telomeric primer (TTAGGG)3, 37 MBq/ml [α-^{32}P]dGTP, 2μM dGTP, 1 mM dATP and 1 mM dTTP with 4 μL of cell lysate, which were incubated at 37˚C for 1 h. Reactions were terminated by adding 20 μL of prewashed Dynabead suspension in 10mM Tris-HCl, pH 7.5, and 2 M KCl. The reaction product was immobilized to Dynabeads by mixing on rotate wheel for 20 min at room temperature. The immobilized reaction products were separated from the suspensions using a magnet (Dynal MPC), and the beads were washed at least 4 times with washing buffer (1 M NaCl, 10 mM Tris-HCl, pH 7.5) and once with TE (10 mM Tris-HCl [pH 8.0], 1 mM EDTA). The bead-reaction product complex was resuspended in 10 μL of formamide dye and incubated at 90˚C for 5 min, and analyzed on 10% polyacrylamide-urea sequencing gels [8, 10].

DNA polymerase assays

***Taq* DNA polymerase.**

PCR control with *Taq* DNA polymerase (Fermentas) was performed with each telomerase assay (see "TRAP assay").

Telomerase-independent PCR controls were performed with three dilutions of *Taq* DNA polymerase: 4 u per 50 μl of the PCR mix (7.2 μg/ml protein), 0.4 u per 50 μl of the PCR mix (0.72 μg/ml protein), 0.04 u per 50 μl of the PCR mix (0.072 μg/ml protein). PCR was made from plasmid DNA, with product 1000 bp with 1xPCR buffer with $MgCl_2$ (Fermentas), 20 μM dNTP, 25 cycles.

Calf DNA polymerase *β* scincillation protocol

For first calf DNA polymerase *β* assay we used next conditions as discribed in [11] with little modifications: 50 mM Tris–HCl pH 8,0, 50 мМ NaCl, 10 mM $MgCl_2$, 7 mM β-mercaptoethanol, 2 OD/ml partially hydrolyzed calf thimus DNA, 30 μM dNTP (each), 1,7 MBq/ml [α-^{32}P]-dGTP, 0,4 μl of inhibitor solution or DMSO, 3.5 μM, 0.35 μM or 0.035 μM of calf DNA polymerase *β* (a kind gift of prof. O.I. Lavrik). Similar mix with heat-inactivated calf thymus DNA polymerase *β* we used as negative control. Samples were deproteinized with phenol equal volume protein extraction and added 1/10 volume of 3M NaOAc and three volume of ethanol, incubated -20ºC one hour and centrifuged 15 min, 10000g. Then pellet was washed with 70% EtOH and counted with scintillation counter. We calculate IC_{50} with linear approximation of concentration-inhibition effect between two nearest to IC_{50} data points.

Calf thymus DNA polymerase *β* gel protocol

In alternative calf DNA polymerase *β* assay we used next conditions: 50 mM Tris–HCl pH 8,0, 50 mM NaCl, 12 mM $MgCl_2$, 7 mM β-mercaptoethanol, 1 pmol/μl dA_{45}/dT_{18}, 30 μM dNTP (each), 1,7 MBq/ml [α-^{32}P]-dTTP, 3.5 μM or 0.35 μM of calf DNA polymerase *β* (a kind gift of prof. O.I. Lavrik). Similar mix with heat-inactivated calf DNA polymerase *β* we used as negative control. Samples were deproteinized, ethanol precipitated and centrifuged 15 min, 10000 g, washed with 80% EtOH. Samples were measured with scintillation counter, and then separated with 10% denaturing PAGE. After drying gel were photographed with PhosphorImager. We calculate IC_{50} with linear approximation of concentration-inhibition effect between two nearest to IC_{50} data points.

T7 RNA polymerase assay

T7 RNA polymerase assay was made in next conditions: 20 mM Tris–HCl pH 7.9, 1 mM spermidine, 22 mM $MgCl_2$, 3.6 μM NTP (each), 5 μM DTT, 10 ng/μl pUC18-tm plasmid, 0,3 μl of inhibitor solution or DMSO per 10 μl mix, 4 u of T7 RNA polymerase (Fermentas). Mix was incubated 2 hours at 37°C. We used similar mix with heat-inactivated T7 RNA polymerase as negative control. After reaction samples were mixed with equal volume "stop-solution" (0,01% xylene cyanol и 0,01% bromophenol blue, 25 mM EDTA, 80%

formamide), applied to 6% polyacrylamide gel. After electrophoresis gel was stained with methylene blue and photographed. Gel densitometry was estimated with ImageQuant program. We calculate IC50 with linear approximation of concentration-inhibition effect between two nearest to IC_{50} data points.

HIV reverse transcriptase assay

HIV reverse transcriptase assay based on [12] with some modifications was used. We annealed 1 pmol primer 33* (complement to 2591-2611 positions of 23S ribosomal RNA from *E.coli*, radiolabeled) with 23S ribosomal RNA from *E.coli* (0.5 pmol) in 10 µl 50 mM Tris-HCl pH 8.3, 40 mM KCl.

10 µl of annealed mix were mixed with 10 µl of extension mix that result in reaction conditions: 20 mM Tris-HCl pH 8.3, 0.8 mM NTP (each), 16 mM KCl, 2.4 mM $MgCl_2$). 0.4 µl of inhibitor solution or DMSO per 10 µl reaction and 0,4µl HIV reverse transcriptase solution (1, 0.25 or 0.0625 unit per reaction) were added.

Mix was incubated 1 hour at 37°C. After reaction samples were mixed with equal volume "stop-solution" (0,01% xylene cyanol and 0,01% bromophenol blue, 25 mM EDTA, 80% formamide), ¼ volume applied to 6% polyacrylamide gel. After electrophoresis gel was dried and radioactivity was detected by PhosphorImager. We calculate IC_{50} with linear approximation of concentration-inhibition effect between two nearest to IC_{50} data points.

MTT (Cell Proliferation/Viability) Assay

The cytotoxic effects were evaluated using MTT assay [13]. 4000 cells/well were seed in 96-well plates (Greiner) in DMEM media in presence of 10% FBS (fetal bovine serum), incubated overnight for cell attachment. Then several dilutions of testes substances were added an incubated 24h. After incubation with substances, media was changed to fresh and incubated additional 72 h. Then cells were incubated with MTT 2-3 hours, media was removed, precipitate was dissolved in DMSO and optical density was scanned at 555 nm with reference 670 nm. Data was processed with Graphpad software. Data are presented as mean ±SD (n=3) for each drug concentration.

Nucleic acid interaction tests

Fluorescent DNA interaction detection

We used weak own fluorescence of complex **2** substance (excitation maximum 375 nm, emission maximum 430 nm) to detect interaction with DNA. We titrated complex **2** with single-stranded DNA (R30_14chr2: ATGATAGTCTCCGTGGTTTGGCTGT) or G4-DNA-quadruplex (prepared with 100 mM KCl from HuG4: TTAGGGTTAGGGTTAGGGTTAGGG, excess salt was removed with EtOH precipitation). Mix contained 20 mM sodium phosphate buffer (pH 7.3), 0.5 µM complex 2 and 0.1-20 µM of DNA. After each DNA addition mix were incubated 5-10 min and scanned for emission in 390-

500 nm range with excitation wave 375 nm (we used fluorescence spectrophotometer Varian Cary Eclipse).

G4-DNA-quadruplex preparation: 10 μM HuG4 oligonucleotide was incubated with 100 mM KCl, then NaOAc was added up to 200 mM, three volume EtOH added and incubated for 1 hour at -20 C. Precipitate was rinsed three times with ice-cold 70 % EtOH, dried 15 minutes on the room temperature and dissolved in deionized water.

DNA-duplex stabilization measurement

We have melted DNA duplex in the absence and in the presence of 20 μM complex **2** and detected hyperchromic effect in temperature interval 50-95ºC. heating with and one minute incubation, after it next step of heating. DNA 124 bp-length duplex with sequence CTAACCCTAACCCACATTCTAACCCCAACCATAGTAAGTACTATAATTAATTAAAGTATATT GCTCAGTAGTTAAAGGGATAGTTACACTGCTCATTATGTCACCTTTAAATAAAGGTAA with or without complex **2** was heated for 1ºC in 30 seconds and 1 minute incubation next step of heating was performed. We used spectrophotometer Hitachi U2900 with thermostabilized cell.

RNA-DNA-duplex stabilization measurement

We prepared 10 μl mix containing 2 μl in 5x anneal buffer (250 mM Tris-HCl pH 8.3, 200 mM KCl), 2 μl 0,1 mM complex 2 in 1% DMSO (Dimethyl sulfoxide), 1,29 μl total ribosomal RNA 3.1 pmol/μl, 1 μl 2 μM FAM-23 A1618 5' – (FAM) GTG TCG GTT TGG GGT ACG ATT TG – 3', 2 μl 2 μM BHQ1-18 A1618 5' – TTC TCT ACC TGA CCA CCT (BHQ1) – 3' (FAM is Fluorescein amidite, BHQ is the Black Hole Quencher). Than we measured Fam fluorescence during melting with high resolution at C1000 Thermal Cycler (BioRad). The temperature was changed according to protocol: 80ºC 3 min, 79 ºC 20 sec, 78 ºC 20 sec, etc down to 20 ºC, then 20 ºC 30 sec, 20,5 ºC 30 sec, 21 ºC 30 sec, 21,5 ºC 30 sec, etc up to 80 ºC, detecting FAM fluorescence each step [14].

Ethidium bromide displacement DNA interaction test

DNA Interaction studies with ethidium bromide displacement was made in according to [15].

DNA degradation measurements

We prepared 15 μl mix, containing 150 ng pUC18 plasmid DNA, 20 mM Hepes-KOH (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid with KOH) pH 7.8, and 2-200 μM of complex **2**, ligand **L2** or $CuCl_2$. Mixtures were incubated at 37˚C 4 hours and analyzed in 1% agarose gel [14]. Gel densitometry was estimated with ImageQuant program. We calculate IC_{50} with linear approximation of concentration-inhibition effect between two nearest to IC_{50} data points.

Inductively coupled plasma-mass spectrometry (ICP-MS)

MCF7 or MCF7ADR (Doxorubicin resistant MCF7) cells were cultivated in DMEM.

Complex **2** at 3 or 9 μM was added (10 mM stock solution in DMSO) to the monolayer of the cells (80%), and cells were incubated in 5 % CO2 37°C 4 hours, rinsed with Hank's buffer. $CuCl_2$ at 6 or 18 μM was added (10 mM stock solution in water and equial to Complex 2 volumes of DMSO) to the monolayer of the cells (80%), and cells were incubated in 5 % CO2 37°C 4 hours, rinsed with Hank's buffer. Cells were removed by treatment with trypsin-EDTA solution (0.25% trypsin), diluted 4 times with media containing 10 % FBS, pelleted at 300 g, washed and resuspended in Hank's buffer. 400000 cells (10 μl) or equal volume of media (10 μl) were diluted with 300 μl of distilled water. Samples were mixed and treated with probe sonicator (30 seconds, 80% amplitude) then 10 minutes in bath sonicator. Then 15 μl aqua regia was added and the sample was incubated at 70°C overnight. Samples were diluted 5 times with distilled water. 63C2+ and 65C2+ copper content in the samples was determined by PerkinElmer Nexion 300Q ICP Mass Spectrometer. Calibration curve for copper content calculation was build with 10-100 ppm copper standards (Inorganic Ventures), all samples data were less then 100 ppm.

Cell cycle analysis

HEK 293 cells were treated with complex **2** at concentration 1,3, 2,6 and 6,5 μM or ligand **L2** at concentration 16 and 32 μM for 3 h. The cells were washed twice with PBS and harvested using 1X trypsin-EDTA. The cells were washed again with PBS and cell cycle analysis was performed with BD Cycletest™ Plus kit (BD Biosciences) according to manufacturer's recommendations. Monoparametric cell cycle analysis was performed at 1000 cells for each sample with a flow cytometer (FACSAriaIII BD Biosciences) and data were quantified with FACS Diva software.

Determination of reactive oxygen species (ROS) formation

HEK 293 cells were treated with complex **2** at concentration 1,3, 2,6 and 6,5 μM or ligand **L2** at concentration 16 and 32 μM at 15 min simultaneously with of the general Reactive Oxygen Species (ROS) Detection Reagent CM-H2DCFDA (Invitrogen). The treatment of cells by CM-H2DCFDA was performed according to manufacturer's recommendations. The cells were washed with PBS, harvested using 1X trypsin-EDTA and resuspended in DMEM with 10% of serum. Analysis of ROS generation was performed by flow cytometer (FACSAriaIII BD Biosciences) at FITC-channel and analyzed using the FACS Diva software.

Analysis of complex and ligand distribution inside the cell

HEK 293 cells were seeded at coverslips and treated with azide derivative of complex **5** at concentration 1,3, 2,6 and 6,5 μM or ligand **L5** at concentration 10 and 20 μg/ml at 3 h. Cells were fixed by 3,7% paraformaldehyde in PBS. After that cells were permeabilized by 0,5% Triton X-100 in PBS and processed by diSulfo-Cy5 alkyne (Cyandye LLC) in the presence of Click-iT cell reaction buffer kit (Invitrogen) according to the manufacturer's recommendations and counterstained by DAPI (Invitrogen).

Coverslips were mounted by Mowioil (Polysciences, Inc). Cells were analyzed by Nikon Ti2000 fluorescent microscope in DAPI and Cy5 channels.

***In vivo* study**

At the first the maximum tolerated dose (MTD) of complex **2** has defined by five times the intraperitoneal injection, using 10% DMSO. For the antitumor activity study of complex 2 was administered to animals at doses of 12 mg / kg (1/2 MTD) and 24 mg / kg (MTD). Antitumor activity was evaluated by determination of tumor growth inhibition (TGI). MTD and TGI were determined according to manual on experimental (preclinical) study of new pharmacological substances (Ed. RW Habriev, Moscow, Medical, 2005).

Strain of transplanted tumor (Ca 755) in the frozen state (in liquid nitrogen, in ampoules in a volume of 1 ml) were obtained from Blokhin Cancer Research Center (Russia) and transported in liquid nitrogen. Tumor cells (Ca 755) thawed at 37^0C and immediately 0.5 ml suspensions injected subcutaneously to mice of the corresponding lines C57BL / 6 (female). Study of antitumor activity of the complex was performed after two passages in mice.

Treatment was started 48 hours after inoculation. Complex 2 was administered intraperitoneally five times at intervals of 24 hours. Each experimental group consists of ten animals.

LIST OF ABBREVIATIONS

All - allyl
MTT - Methylthiazol Tetrazolium
TUNEL - **T**dT-mediated d**U**TP **N**ick **E**nd **L**abeling
CM-H_2-DCFDA - 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate
SWNTc- single walled carbon nanotubes
Dox - doxorubicine
C57bl - C57 black
DAPI - 4',6-diamidino-2-phenylindole
dsDNA - double-stranded DNA
PI - Propidium iodide
TRAP – telomere repeats amplification protocol
ORTEP - Oak Ridge Thermal Ellipsoid Plot
CCDC - Cambridge Crystallographic Data Centre
ICPMS - Inductively coupled plasma mass spectrometry
EtBr - Ethidium bromide
TMPyP -5,10,15,20-tetrakis(N-methyl-4-pyridyl)-21H,23H-porphine
HIV RT - Human immunodeficiency virus reverse transcriptase
PAGE - Polyacrylamide gel electrophoresis
EGTA - ethyleneglycoltetraacetate
CHAPS - 3-((3-Cholamidopropyl)dimethylammonio)-1-pro-panesulfonate
PMSF - phenylmethylsulfonylfluoride
Hepes - Hydroxyethylpiperazine-N-ethane-sulfonate
TEMED - N,N,N',N'-Tetramethylethylenediamine
dGTP - Deoxyadenosine triphosphate
dATP - Deoxyguanosine triphosphate

dTTP - Deoxythymidine triphosphate
dNTP - Deoxynucleotide Solution Mix
FAM - Fluorescein amidite
BHQ - Black Hole Quencher
FBS - Fetal bovine serum
Aqua regia - Mixed concentrated nitric acid and hydrochloric acid in a volume ratio of 1:3

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