

Electronic Supplementary Information

Dinuclear [$\{(p\text{-cym})\text{RuCl}_2(\mu\text{-phpy})\}(\text{PF}_6)_2$ and heterodinuclear [(ppy) $_2$ Ir($\mu\text{-phpy})$ Ru($p\text{-cym})$ Cl](PF $_6$) $_2$ complexes: synthesis, structure and anticancer activity†

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Experimental

Materials

The precursor complexes $[\text{Ru}(p\text{-cym})\text{Cl}(\mu\text{-Cl})_2]^1$ and $[\text{Ir}(\text{ppy})_2\text{Cl}(\mu\text{-Cl})_2]^2$ and 5,6 diamino 1,10 phenanthroline³ were prepared by following the reported procedures. The ligand 2,3-di(pyridin-2-yl)pyrazino[2,3-f][1,10] phenanthroline (phpy) ligand have been prepared with slight modification of the reported procedure.⁴ All chemicals were purchased from commercial sources and used as received. Solvents were dried by conventional methods and distilled prior to use. All culture media and supplements for cell work were purchased from Gibco Invitrogen Corporation (CA, USA). The primary antibodies for LC3, baclin-1, Atg5, Atg7, and β -actin were purchased from Cell Signaling Technology (Beverly, MA, USA). The horseradish peroxidase-conjugated secondary antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Instrumentation

Conductivity measurements were done by using OAKton PC2700 conductivity bridge. UV-Vis spectra were obtained by using a Perkin Elmer Lambda 35 spectrophotometer. Fluorescence measurement of the complex **2** was done using Edinburgh photonics FLS980 fluorescence spectrometer at 298 K in acetonitrile. The quantum yield of **2** was calculated using $[\text{Ru}(\text{bpy})_2]^{2+}$ as standard ($\Phi = 0.095$).⁵ FTIR spectra were recorded using Bruker Alpha FTIR spectrophotometer with samples prepared as KBr pellets. Elemental analyses have been carried out with a Euro –E 3000 elemental analyser. Electrospray ionisation (ESI) MS spectra were acquired on a Flexar SQ 300 MS PerkinElmer mass spectrometer. ¹H NMR spectra were recorded on a Bruker Avance III 400 spectrometer using DMSO-*d*₆ solvent. Electrochemical measurements were recorded in dinitrogen atmosphere using a CHI 6205 electrochemical analyser using NEt_4ClO_4 as the supporting electrolyte (0.1 mol dm^{-3}) and the solute concentration was $10^{-3} \text{ mol dm}^{-3}$. For electrochemical measurements a glassy carbon

working electrode, Pt wire counter electrode and saturated calomel electrode were used. The half-wave potential E_{298}° was set equal to $0.5(E_{pa} + E_{pc})$, where E_{pa} and E_{pc} are anodic and cathodic cyclic voltammetric peak potentials, respectively. In this cell, Fc/Fc⁺ couple had an $E_{1/2}$ value of 0.15 V.

Crystallography

Single crystals of **2** were grown by slow diffusion followed by evaporation of acetonitrile/tetrahydrofuran solution at room temperature. Single crystal X-ray structural studies were performed on a Bruker D8 venture. Data were collected at 296 (2) K using Mo K_{α} radiation ($\lambda_{\alpha} = 0.71073 \text{ \AA}$). The strategy for the data collection was evaluated by using the APEX 10 software. The data were collected by the standard 'phi-omega scan techniques, and were scaled and reduced using SAINT and XPREP software. The structures were solved by direct methods using SHELXS-2013 and refined by full matrix least-squares with SHELXL-2013, refining on F^2 .⁶ The positions of all the atoms were obtained by direct methods. All non-hydrogen atoms were refined anisotropically. The remaining hydrogen atoms were placed in geometrically constrained positions and refined with isotropic temperature factors, generally $1.2U_{eq}$ of their parent atoms.

CCDC reference number for **2** is 986366.

Synthesis of ligand and metal complexes

Synthesis of 2,3-di(pyridin-2-yl)pyrazino[2,3-f][1,10] phenanthroline (phpy): 5, 6 diamino 1, 10 -phenanthroline (100 mg, 0.48 mmol) and 2, 2'-bipyridyl (110 mg, 0.5 mmol) in 1:1 mole ratio were taken in methanol and heated to reflux for 5 h. After completion, the volume of the reaction mixture was reduced to 3 mL under vacuum and kept in freezer overnight. The precipitate thus obtained was filtered and washed with cold methanol and dried under vacuum to yield pure phpy ligand. Yield: 135 mg (73%). ¹H NMR: (400MHz, (CD₃)₂SO at 298K), δ (ppm): 7.27 (t, 2H, *J* = 4 Hz), 7.78 (t, 2H, *J* = 4 Hz), 7.91 (t, 2H, *J* = 4 Hz), 8.19 (d, 2H, *J* = 1.2 Hz), 8.37 (d, 2H, *J* = 1 Hz), 9.28 (d, 2H, *J* = 4 Hz), 9.57 (d, 2H, *J* = 4 Hz).

Synthesis of [Ru(*p*-cym)Cl]₂(μ-*phpy*)](PF₆)₂ (1**):** Phpy ligand (40 mg, 0.10 mmol) and [Ru(*p*-cym)Cl(μ-Cl)]₂ precursor (73 mg, 0.12 mmol) were taken in 40 mL methanol and refluxed in air for 8 h. After completion, the solvent of the reaction mixture was removed under vacuum and redissolved in minimum acetonitrile. A saturated aqueous solution of KPF₆ was added to it and a faint greenish yellow precipitate was obtained. The pure greenish yellow complex **1** was isolated by filtration followed by thorough washing with cold distilled water and dried in air. Yield: 80 mg (62%). Anal. Calc. for C₃₄H₂₈N₆ClF₆PRu₂ (**1**): C, 43.40; H, 3.48; N, 6.90. Found: C, 44.30; H, 3.27; N, 7.43. Molar conductivity [*M*/*Ω*⁻¹ cm² dm³ mol⁻¹] in acetonitrile: 204. MS (ESI+): *m/z* 1072.95 corresponding to [**1** - PF₆]⁺ (calculated molecular mass 1072.87). ¹H NMR: (400MHz, (CD₃)₂SO at 298K), δ (ppm): 0.89 (d, 6H, *J* = 4Hz), 1.01 (d, *J* = 8 Hz), 1.20 (m, 1H), 1.55 (s, 3H), 2.19 (s, 3H), 2.3 (m, 1H), 4.99 (d, 2H, *J* = 12 Hz), 5.63 (d, 2H, *J* = 16 Hz), 6.13 (d, 2H, *J* = 20 Hz), 6.42 (d, 2H, *J* = 16 Hz), 8.16 (d, 2H, *J* = 8 Hz), 8.36 (t, 2H, *J* = 4 Hz), 8.38 (d, 2H, *J* = 4 Hz), 9.7 (t, 2H, *J* = 8 Hz), 10.09 (t, 2H, *J* = 4 Hz). IR ν (cm⁻¹): 845. λ_{max}/nm (ε/dm³ mol⁻¹cm⁻¹) in acetonitrile: 580 (450), 374 (10680), 298 (29620), 276 (33920), 204 (58340).

Synthesis of [(ppy)₂Ir(μ-phpy)Ru(*p*-cym)Cl](PF₆)₂ (2**):** Phpy ligand (40 mg, 0.10 mmol) and [(ppy)₂IrCl(μ-Cl)]₂ precursor (68 mg, 0.05 mmol) were taken in 20 mL methoxyethanol and refluxed in air for 16 h. The volume of the solution was reduced to half and a saturated aqueous solution of KPF₆ was added to yield precipitate. The isolated mononuclear iridium complex (50 mg, 0.05 mmol) and [Ru(*p*-cym)Cl(μ-Cl)]₂ (17.5 mg, 0.029 mmol) precursor were taken in 2:1 ratio in methanol and refluxed for 12 h. The volume of the solution was reduced under vacuum and a saturated solution of aqueous KPF₆ was added to it which gave a brownish yellow precipitate. It was further filtered, washed with sufficient amount of cold distilled water and dried in air to yield pure **2** (40 mg, 56%). Anal. Calc. for C₅₆H₄₄N₈ClF₁₂P₂IrRu: C, 46.46; H, 3.06; N, 7.74. Found: C, 45.93; H, 3.27; N, 7.40. Molar conductivity [$\Lambda_M/(\Omega^{-1} \text{ cm}^2 \text{ dm}^3 \text{ mol}^{-1})$] in acetonitrile: 198. MS (ESI+): *m/z* 1302.81 corresponding to [**2**-PF₆]⁺ (calculated molecular mass for 1302.73). ¹H NMR: (400MHz, (CD₃)₂SO at 298K) δ (ppm): 1.02 (d, 6H, *J* = 8 Hz), 1.2 (m, 1H), 1.56 (s, 3H), 4.9 (d, 2H, *J* = 20 Hz), 5.69 (d, 2H, *J* = 4 Hz), 6.29 (d, 2H, *J* = 8 Hz), 6.98 (m, 4H), 7.09 (m, 4H), 7.46 (d, 1H, *J* = 8 Hz), 7.80 (d, 1H, *J* = 4 Hz), 7.85-7.93 (m, 4H), 7.97 (d, 1H, *J* = 8 Hz), 8.00 (d, 1H, *J* = 8 Hz), 8.14 (d, 1H, *J* = 4 Hz), 8.16 (d, 1H, *J* = 4 Hz), 8.14 (d, 1H, *J* = 4 Hz), 8.25-8.37 (m, 6H), 8.42 (d, 1H, *J* = 4 Hz), 9.33 (d, 1H, *J* = 4 Hz), 9.74 (t, 1H, *J* = 8 Hz). IR ν (cm⁻¹): 845. $\lambda_{\text{max}}/\text{nm}$ ($\epsilon/\text{dm}^3 \text{ mol}^{-1}\text{cm}^{-1}$) in acetonitrile: 470 (2400), 371 (23300), 304 (55184), 262 (92030).

Cell lines, cell culture and drug treatment.

The breast (MCF7), the androgen-insensitive (PC3), human ovarian (SKOV) and endometrial (Ishikawa) cancer cell line were obtained from American Type Culture Collection (Manassas, VA, USA). The cells were maintained as monolayers at 37 °C in a humidified atmosphere

containing 5% CO₂/95% air in Dulbecco's Modified Eagle's Medium (Gibco, Rockville, MD, USA) containing 10% heat-inactivated fetal bovine serum (FBS, Gibco), 1.25 mM HEPES (Gibco) and 100 U/mL penicillin/streptomycin (Gibco). After 48 h incubation, culture medium was replaced with treatment medium containing 1% FBS and the desired concentration of chemicals.

Cytotoxicity assay.

The cytotoxicities of the compounds **1** and **2** were determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT, 5mg/ml, Sigma). The cells were seeded in 96-well plates at a density of 2×10^3 cells per well. After 48 h incubation, cells were treated with various concentrations of compound and cultured for 24 h and 48 h, respectively. At the end, 15 μ L of MTT reagent was added to each well and incubated in the dark for 4 h at 37 °C. The supernatant was aspirated and formazan crystals were dissolved in 100 μ L of DMSO at room temperature for 10 min with gentle agitation. The absorbance was measured using the VERSA Max Microplate Reader (Molecular Devices Corp., CA, USA) at 540 nm. Data were analysed from three independent experiments then normalised to the absorbance of wells containing media only (0%) and untreated cells (100%).

Flow cytometry analysis.

The breast cancer cells (MCF-7) were exposed to the compound **3** for 48 h. At the end of the treatment period cells were washed, harvested, washed in 1% bovine serum albumin (BSA) and fixed with 95% ice-cold ethanol containing 0.5% Tween 20 for at least 30 min at -20 °C. The cells (1×10^6) were washed in 1% BSA, stained with propidium iodide (10 μ g/mL) in PBS, containing 100 μ g/mL RNase and incubated in the dark for 30 min at room temperature. Flow cytometry of DNA content was performed on a flow cytometry system (Becton

Dickinson, CA, USA) and data were analysed using Cylchred version 1.0.2 software.

Western blot analysis.

After 48 h of incubation, compound **2** treated cells (5 μ M) were harvested by trypsinisation and washed twice with cold PBS. Cells were suspended in PRO- PREPTM protein extract solution (iNtRON, Seongnam, KR) and placed on ice for 30 min. The suspension was collected after centrifugation at 10,000 x g for 15 min at 4 °C. Protein concentrations were measured using protein assay kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. Cell lysates (21-40 μ g) were resolved on 6-15% SDS-polyacrylamide gel, transferred to a PVDF membrane (PVDF, Millipore, Billerica, MA, USA) and probed sequentially with LC3, baclin-1, Atg5, Atg7 and β -actin antibodies. Proteins were visualised with horseradish peroxidase-conjugated secondary antibody using an enhanced chemiluminescence (ECL)-plus kit (GE healthcare) for detection.

Acridine Orange Staining.

Acridine orange staining was performed by following the published protocol.⁷ Briefly, Cells were grown in cover glass bottom dishes at a density of 1×10^5 cells per dish, cultured for 24 h, then incubated with the indicated drug treatment in DMEM containing 1% FBS for 12 h. The media were removed and the cells were stained with acridine orange (1 μ g/ml) at 37 °C for 15 min. The staining solution was removed, washed with PBS and immediately analyzed in the flow cytometer and examined under a fluorescence microscope (Zeiss LSM 510 META, Jena, Germany), using 490 nm band-pass blue excitation filters and a 515 nm long-pass barrier filter with 400x magnification.

	[2](PF ₆) ₂ · 3C ₇ H ₈ · C ₃ H ₈ O ₂
empirical formula	C ₈₀ H ₇₃ ClF ₁₂ IrN ₈ O ₂ P ₂ Ru
Fw	1797.15
radiation	MoK _α
wavelength	0.71073 Å
temp./ K	150 (2)
cryst system	Monoclinic
space group	P 21/c
<i>a</i> /Å	19.0145 (11)
<i>b</i> /Å	16.8284 (10)
<i>c</i> /Å	25.3054(15)
<i>α</i> (deg)	90
<i>β</i> (deg)	104.40 (2)
<i>γ</i> (deg)	90
<i>V</i> / Å ³	7842.9 (8)
Crystal size (mm)	0.11 x 0.10 x 0.05
<i>Z</i>	4
<i>μ</i> / mm ⁻¹	2.042
<i>D</i> _{calcd} / g cm ⁻³	1.522
F(000)	3604
<i>θ</i> range	2.212- 28.415
Data/restraints/parameters	19649 / 46 / 939
R1,wR2 [I>2σ(I)]	0.0911
R1,wR2 (all data)	0.2066
Largest diff. peak hole (eÅ ⁻³)	2.617, -2.530

Table T1 Selected crystallographic data for complex $[2](PF_6)_2 \cdot 3C_7H_8 \cdot C_3H_8O_2$.

Table T2 Important bond distances (Å) and bond angles (°) for $[2](PF_6)_2 \cdot 3C_7H_8 \cdot C_3H_8O_2$.

Bond lengths		Bond angles	
Ru(1)-N(1)	2.129(9)	N(1)-Ru(1)-N(2)	86.8(3)
Ru(1)-N(2)	2.135(8)	N(1)-Ru(1)-Cl(1)	85.5(2)
Ru(1)-Cl(1)	2.389(3)	N(2)-Ru(1)-Cl(1)	85.6(2)
Ru(1)-C(2)	2.225(11)	N(5)-Ir(1)-N(6)	77.3(3)
Ru(1)-C(3)	2.207(11)	N(5)-Ir(1)-N(7)	88.3(3)
Ru(1)-C(4)	2.192(12)	N(5)-Ir(1)-N(8)	95.4(3)
Ru(1)-C(5)	2.205(12)	N(5)-Ir(1)-C(45)	96.9(3)
Ru(1)-C(6)	2.181(11)	N(5)-Ir(1)-C(46)	171.5(3)
Ru(1)-C(7)	2.188(11)	N(6)-Ir(1)-C(46)	95.0(3)
Ru(1)-C _{centroid}	1.687	N(6)-Ir(1)-N(7)	98.6(3)
Ir(1)-N(5)	2.136(7)	N(6)-Ir(1)-N(8)	86.6(3)
Ir(1)-N(6)	2.139(7)	N(6)-Ir(1)-C(45)	174.2(3)
Ir(1)-N(7)	2.030(8)	N(7)-Ir(1)-C(45)	81.2(4)
Ir(1)-N(8)	2.035(8)	N(7)-Ir(1)-C(46)	96.4(4)
Ir(1)-C(45)	2.026(9)	N(7)-Ir(1)-N(8)	174.2(3)
Ir(1)-C(46)	1.990(8)	N(8)-Ir(1)-C(45)	93.9(3)
		N(8)-Ir(1)-C(46)	80.6(4)
		C(45)-Ir(1)-C(46)	90.8(3)

Table T3 Electrochemical data^a for complexes **1** and **2**.

Complexes	E_{298}° [V] ^b				
	Ox ₂	Ox ₁	Red ₁	Red ₂	Red ₃
1	1.60 (E_{pa}) ^c	1.37 (E_{pa}) ^c	-0.87 (E_{pc}) ^c	-1.08 (E_{pc}) ^c	-1.15(E_{pc}) ^c
2	1.63 (E_{pa}) ^c	1.03 (E_{pa}) ^c	-1.10 (E_{pc}) ^c	-1.60 (60)	-1.895 (110)

^aFrom cyclic voltammetry in CH₃CN/ 0.1M Et₄NClO₄ at 50 mV s⁻¹. ^bPotentials in V *versus* saturated calomel electrode, referenced to Fc⁺/Fc ($E_{1/2} = + 0.15$ V) as internal standard. ^c E_{pa} and E_{pc} are anodic and cathodic peak potentials, respectively.

Fig. S1. Electrospray (+ve) mass spectra of complexes **1** and **2** in CH₃OH.

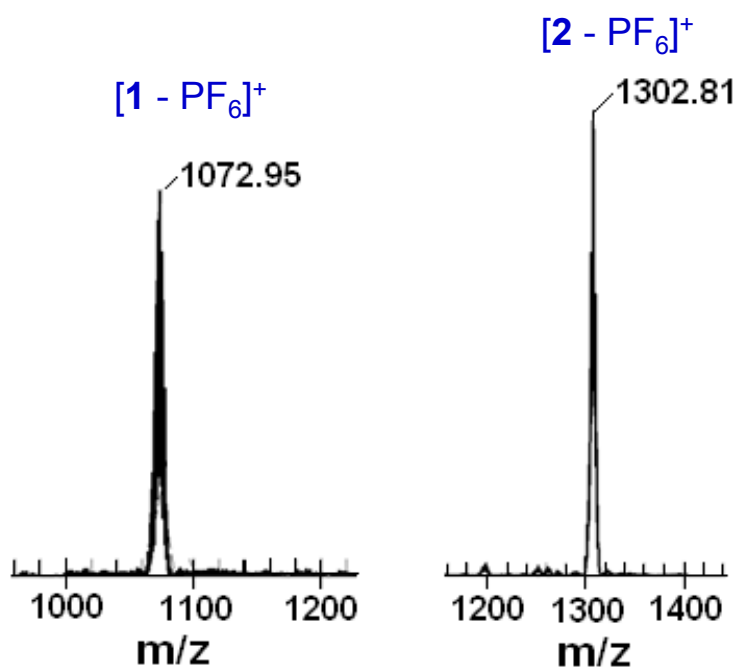


Fig. S2 ^1H NMR spectra of complexes **1** and **2** recorded in $\text{DMSO } d_6$.

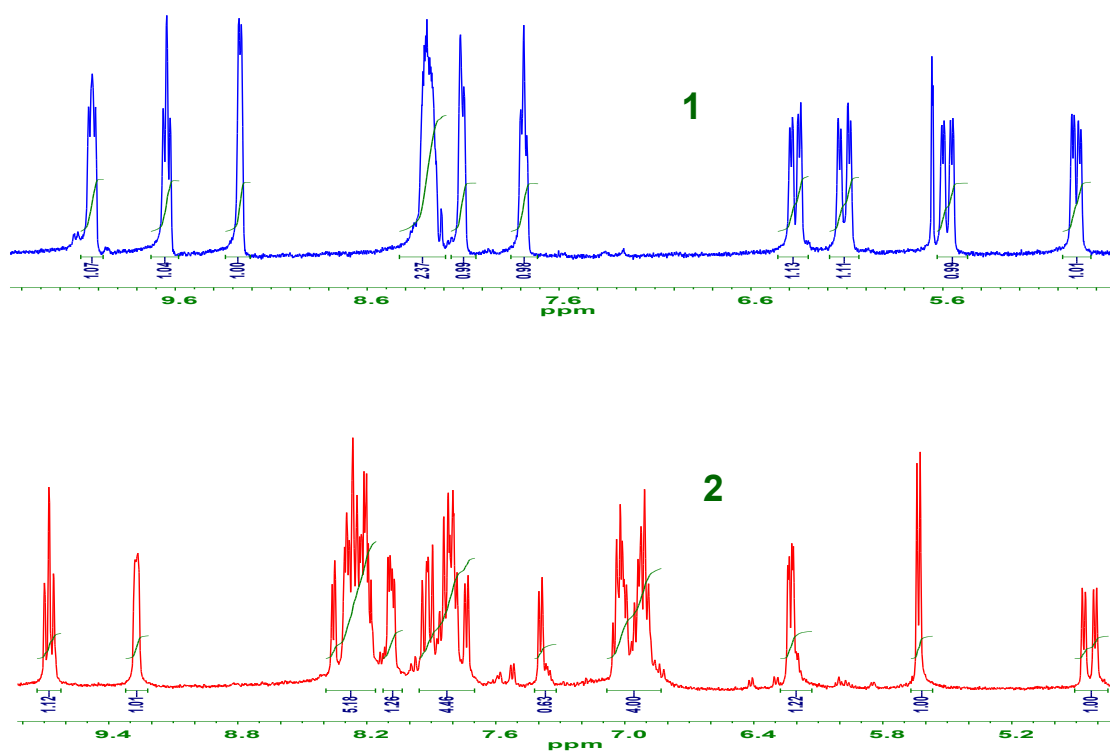


Fig. S3 (a) UV-Vis spectra of **1** and **2** and (b) excitation and emission spectra of **2** recorded in CH₃CN at room temperature.

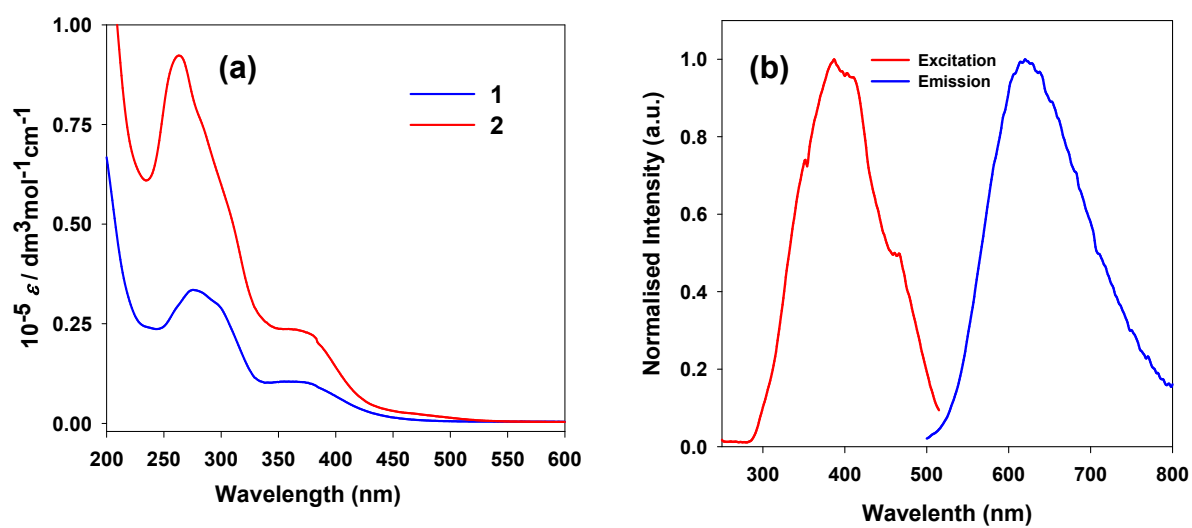


Fig. S4 Cyclic voltammograms of complexes **1** and **2** recorded in $\text{CH}_3\text{CN}/0.1 \text{mol dm}^{-3}$ Et_4NClO_4 versus calomel electrode (scan rate 50 mV s^{-1}).

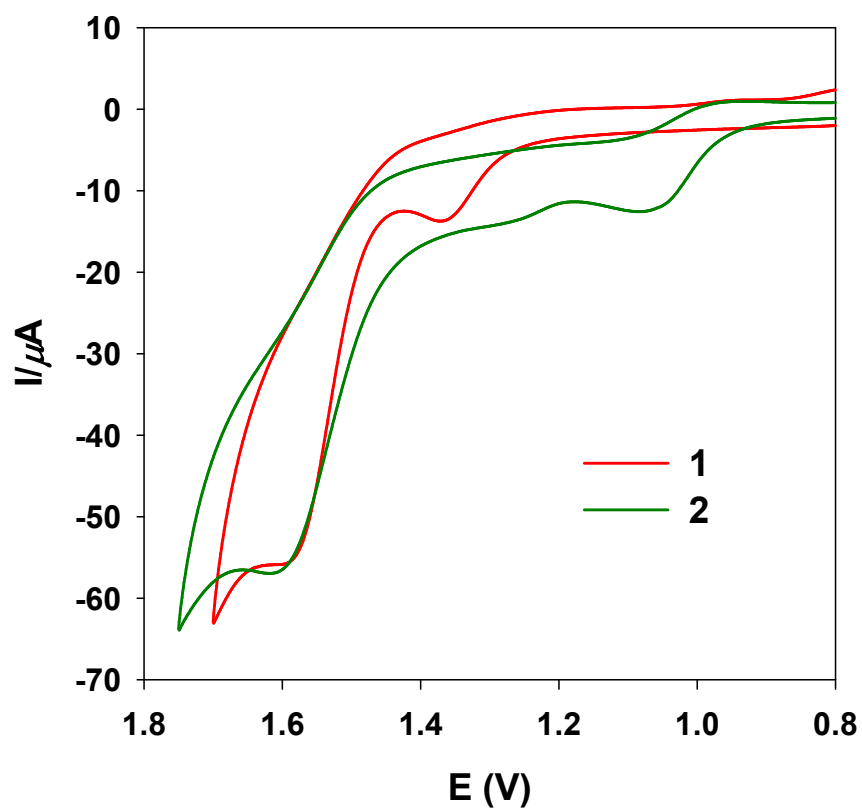


Fig. S5 Dose dependent suppression of cell viability of complex **1** towards human breast (MCF 7), Ovarian (SKOV3), prostate (PC3) and endometrial (Ishikawa) cancer cell lines.

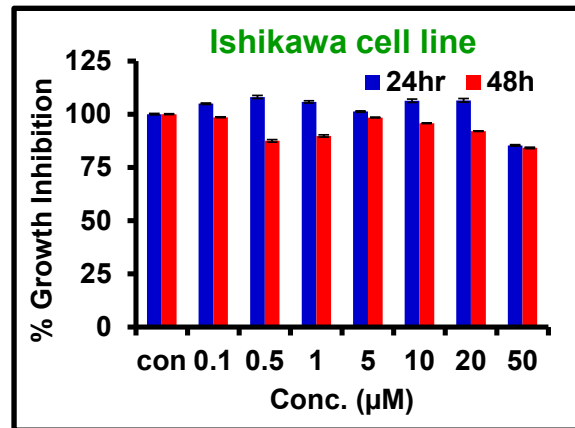
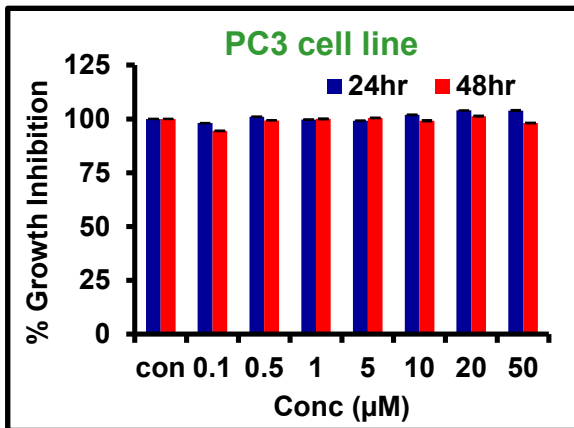
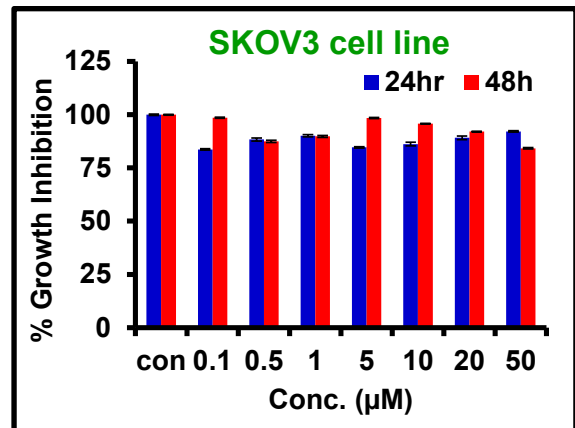
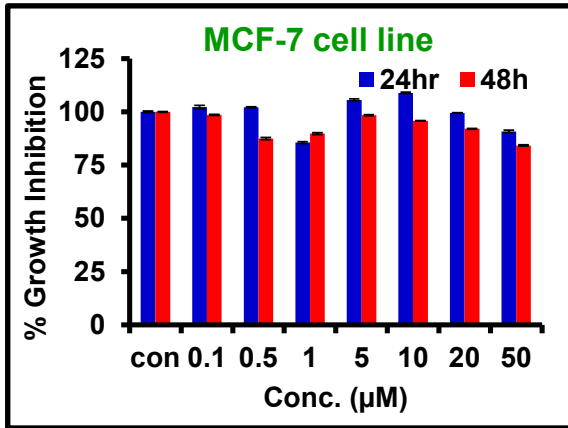


Fig. S6 Dose dependent suppression of cell viability of complex 2 towards human breast (MCF 7), Ovarian (SKOV3), prostate (PC3) and endometrial (Ishikawa) cancer cell lines.

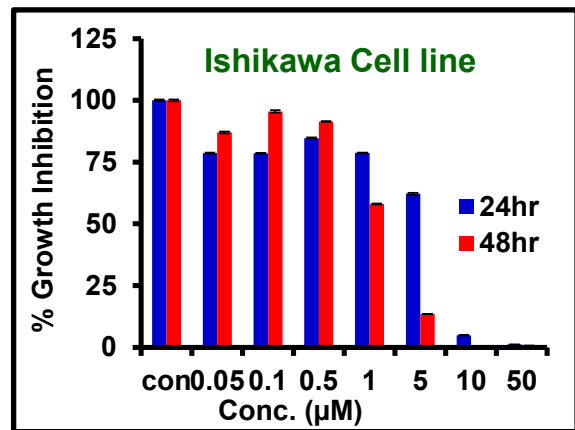
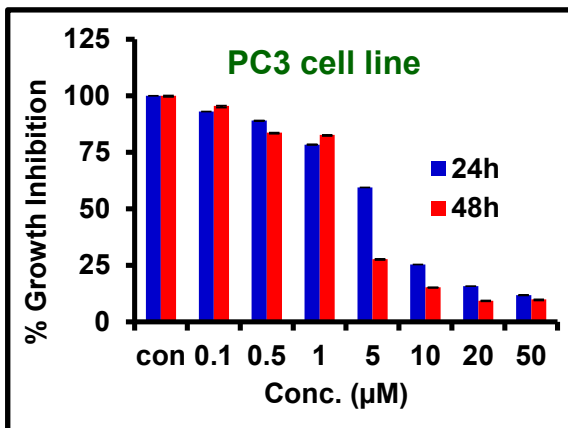
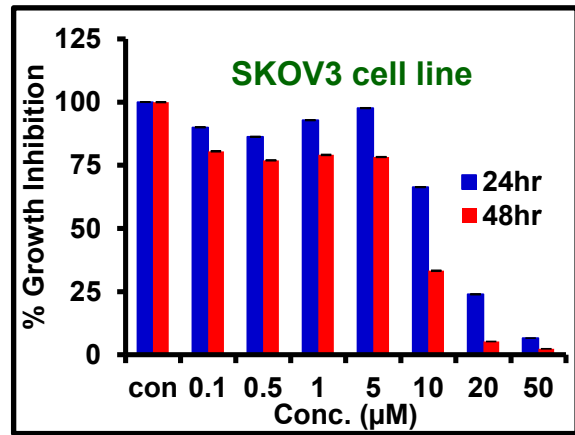
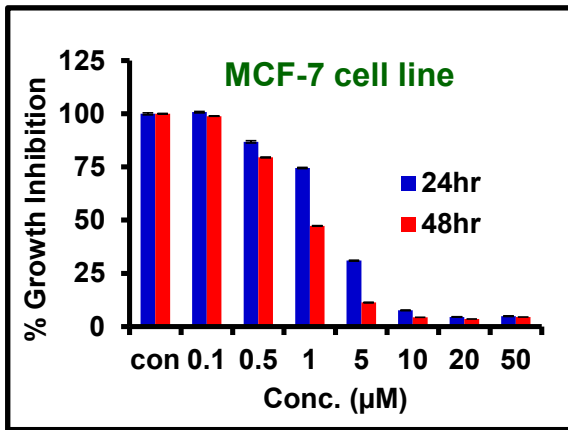


Fig. S7 Fluorescence microscopic images (400x) of human breast (MCF7) cancer cell line with or without treatment of complex 2 (5 μM) at 37°C.

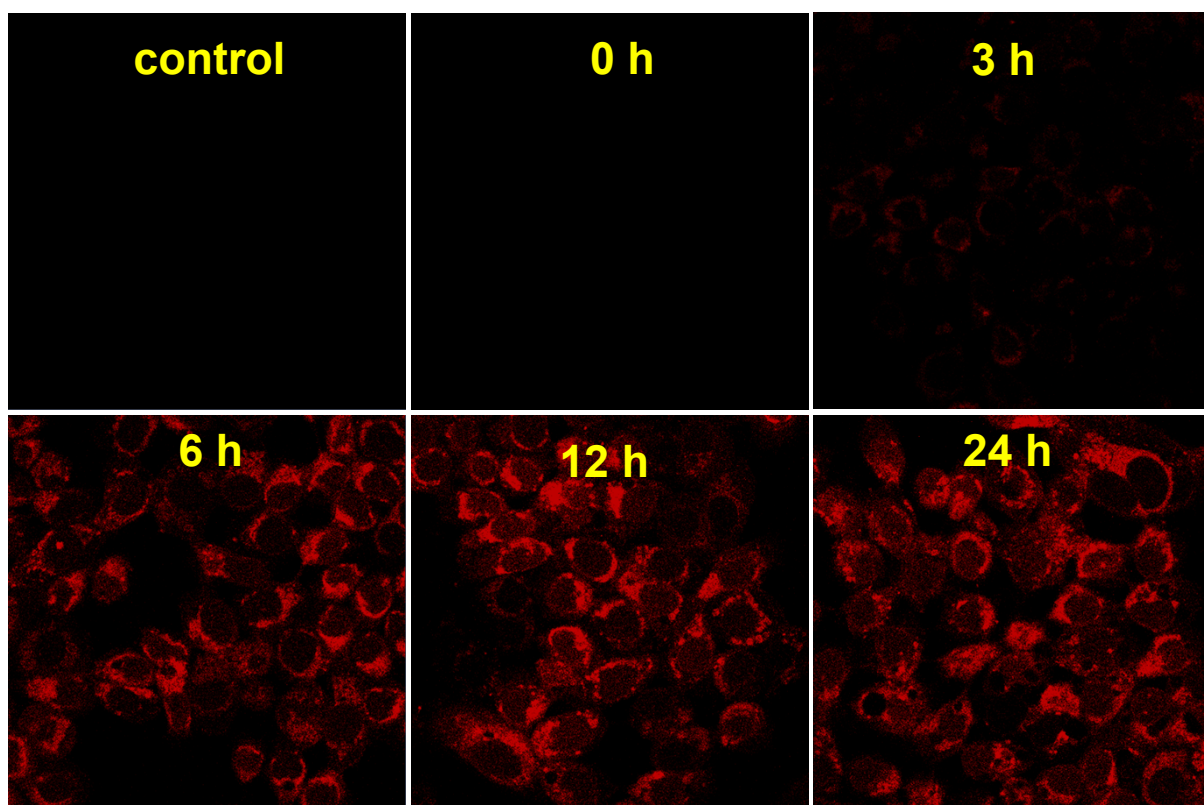


Fig. S8 Cell cycle analysis of human breast (MCF7) cancer cell line with or without treatment of complex **2** at indicated concentrations after 24h.

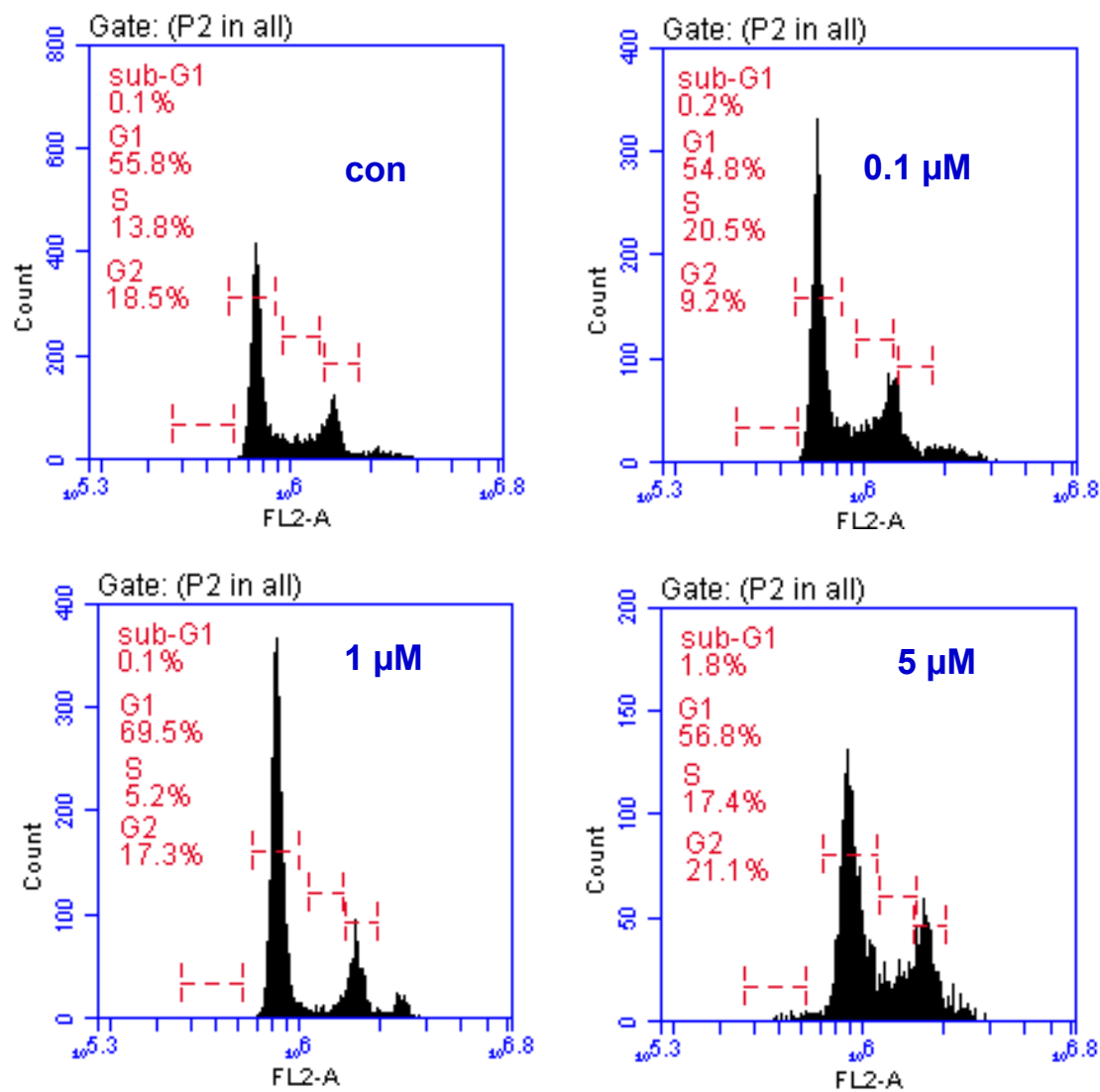
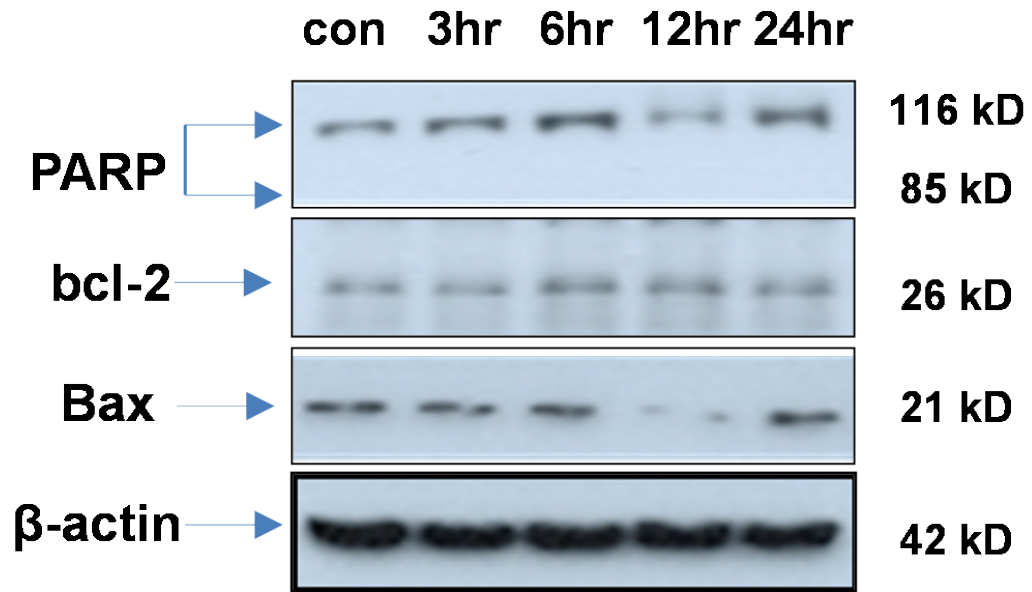


Fig. S8 Western blot analysis of the expressions of apoptosis related proteins of human breast (MCF7) cancer cell line with or without treatment of complex 2 at indicated concentrations in different time.



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