Supplementary Material

for

A tridentate Cu(II) complex with a 2-(4'-aminophenyl)benzothiazole derivative: crystal structure and biological evaluation for anticancer activity

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Materials and methods

General

All chemicals (reagent grade) were obtained from Sigma-Aldrich, Alfa Aesar, and Acros Organics and used without further purification. Copper nitrate trihydrate ($Cu(NO_3)_2 \cdot 3H_2O$) was used as metal ion source and purchased from Sigma-Aldrich (Germany). Calf thymus (CT) DNA and ethidium bromide (EtBr) were obtained from Sigma Corp (St. Louis, MO, USA) and used as received. The media/agents for cell lines were purchased from Biowest (France). The MTT reagent (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) and phosphate-buffered saline (PBS) solution (pH 7.4) were bought from AppliChem (Germany) whereas 2′,7′-dichlorofluorescin diacetate (DCFH-DA), RNase, propidium iodide (PI) solution were bought from Sigma-Aldrich (Germany).

The infrared spectra of all complexes were recorded using a Perkin-Elmer 883 double beam infrared spectrophotometer in the range of 200 – 4000 cm-1. UV-visible (UV-vis) measurements were carried out on a Hitachi U2001 spectrophotometer in the range from 190 to 700 nm. Steady state fluorescence emission and excitation spectra were recorded on a Hitachi F-700 fluorescence spectrophotometer from Hitachi High-Technologies Corporation. The employed slit width was 5.0 nm and the scan speed was 240 $nm \cdot min^{-1}$. The entire system was supported by the FL Solutions 2.1 computer software, running on Windows XP. The Electrospray mass (ESI-MS) spectra were recorded in the range of 250 to 1400 m/z, on a TSQ 7000 Finnigan MAT. For the mass spectrometric studies the sample was dissolved in DMSO and the resulting solution was supplied to the electrospray capillary through a syringe pump. A ThermoFinnigan Flash EA 1112 CHNS elemental analyzer was used for the simultaneous determination of carbon, hydrogen, and nitrogen (%). The pH measurements were performed using a digital Xenon pH meter and a Russel CMAWL/3.7/180 combined electrode. Standardization was done at 25 °C with Russel buffers. Continuous-wave (cw) EPR measurements at X-band were performed on a Bruker ESP 380E spectrometer equipped with an EN 4118X-MD4 Bruker resonator. Experimental conditions: microwave (mw) frequency, 9.612 GHz; mw power incident to the cavity, 200 μW; modulation frequency, 100 kHz; modulation amplitude, 0.13 mT; temperature, 120 K. Measurements at cryogenic temperatures were performed using a helium cryostat from Oxford Inc. The microwave frequency was measured using a HP 5350B microwave frequency counter and the temperature was stabilized using an Oxford ITC4 temperature controller. The cw EPR spectrum of the diluted sample in DMSO (Cu(II) concentration was about 3 mM) was recorded in frozen solution at 120 K. Cw EPR spectral simulations were performed with the EasySpin package [1] assuming the following spin Hamiltonian for the Cu(II) ion (electronic configuration $3d^9$, S=1/2) interacting with one I=3/2 nuclear spin (^{63}Cu or ^{65}Cu):

$$H = \beta_e \mathbf{B_0 g S}/h + \mathbf{SAI} - \beta_n g_n \mathbf{B_0 I}/h$$

where the terms describe the electron Zeeman interaction, the hyperfine interaction, and the nuclear Zeeman interaction. The electron Zeeman interaction is characterized by the g = [gx, gy, gz] tensor that is essentially determined by the metal ion and the

directly coordinated ligand atoms. The principal g values observed in the EPR spectrum together with the metal hyperfine coupling A = [Ax, Ay, Az] provide information on the symmetry of the paramagnetic center.

All DNA experiments were performed in phosphate buffer (0.05 M, pH = 7.2) consisting of $Na_2HPO_4 \cdot 7H_2O$ and K_2HPO_4. The solution of CT-DNA found to be free of protein as depicted by the ratio of its UV absorbance at 260 and 280 nm (approx. 1.9:1). The CT-DNA concentration per nucleotide was determined at 260 nm using a UV-vis spectrophotometer (molar absorption coefficient of $\varepsilon = 6600\ M^{-1} \cdot cm^{-1}$ per nucleotide). Stock solutions of CT-DNA (in buffer) and compounds (10^{-3} M, in DMSO) were stored in the dark at 4 °C. Before measurement, DNA and compound solutions were pre-incubated for 24h at 25 °C to reach the equilibrium state. Each reported measurement value was the average of three independent experiments. Electronic absorption titration studies were performed using a Varian Cary 300E spectrometer at 25 °C using quartz cuvettes with 1.0 cm optical path length. Melting temperature measurements were carried out using a Varian Cary 300 spectrophotometer equipped with a heating multiple cell block apparatus and the increase temperature rate was 0.5 °C/min. Cuvettes (1.0 cm capped quartz) were mounted in a thermal block and a thermistor monitored the solution temperatures in the reference cuvette. Solutions were allowed to equilibrate for 1 min at each temperature and three repetitions for each sample were performed. Circular dichroism (CD) spectra of CT-DNA were recorded in the range 180 - 600 nm at 25 °C on a Jasco J-715 spectropolarimeter equipped with Peltier heating system. CD spectra were collected after averaging over three accumulations using quartz cuvettes with 1.0 optical path length, a scan speed of 100 nm/min and a 1 s response time. Data were analyzed through the standard Jasco software package. Fluorescence spectra were recorded using a HITACHI F-2500 spectrofluorometer at 25 °C in the wavelength range 550 – 800 nm. The excitation wavelength was 526 nm and the emitted fluorescence intensity was observed at 600 nm at room temperature, scan speed was 300 nm/min, and both excitation and emission slit widths were 2.5 nm. Viscosity measurements were carried out using SI Analytics CK 300 viscometer. The viscometer was thermostated at 25.0 ± 0.1 °C in a constant temperature bath. Flow time of solutions was measured with a digital stop watch and each sample was measured three times (values did not differ by more than 0.2 s from each other) and an average flowtime was calculated. All solutions were filtered through 1 μm filters (Millipore Acrodisc) before the measurements.

MCF-7 and MDA-MB-231 (human breast adenocarcinoma), U-87 MG (human glioblastoma-astrocytoma), PC-3 (human prostate cancer), A-549 (human lung adenocarcinoma), and DSF (healthy human skin fibroblasts) cell lines came from the cell bank of the Institute of Biosciences & Applications, NCSR "Demokritos". The cell lines were free of mycoplasma contamination, as judged visually under microscopic observation and by regular 4′,6'-diamidino-2-phenylindole (DAPI) staining of cell cultures. Fluorescence was measured on a FACS Calibur (Becton Dickinson, San Jose, CA, USA) flow cytometer, and DNA content was estimated using the ModFit software (Verity Software House, Topsham, ME, USA). Fluorescent staining observation was performed with a Leica TCS SP8 MP (Wetzlar, Germany)

confocal microscope equipped with an IR MaiTai DeepSee Ti:Sapphire laser (Spectra-Physics, Santa Clara, CA) for multiphoton applications. Images were acquired with LAS X software (Leica Microsystems CMS GmbH, Wetzlar, Germany) and are presented without any further processing. For the MTT and DCFH-DA experiments were conducted by TECAN Infinite 200 PRO plate reader (USA). Human cancer cells and healthy fibroblasts were grown in DMEM growth medium of pH 7.4 supplemented with 10% FBS, 100 U/mL of penicillin, 2 mM glutamine and 100 lg/mL of streptomycin. The cell cultures were maintained in flasks and were grown at 37 °C in a humidified atmosphere of 5% CO_2 in air. The sub-confluent cells were detached using a 0.05% (w/v) trypsin – 0.25% (w/v) ethylenediaminetetraacetic acid (EDTA) solution and the subcultivation ratio was 1:3 to 1:5.

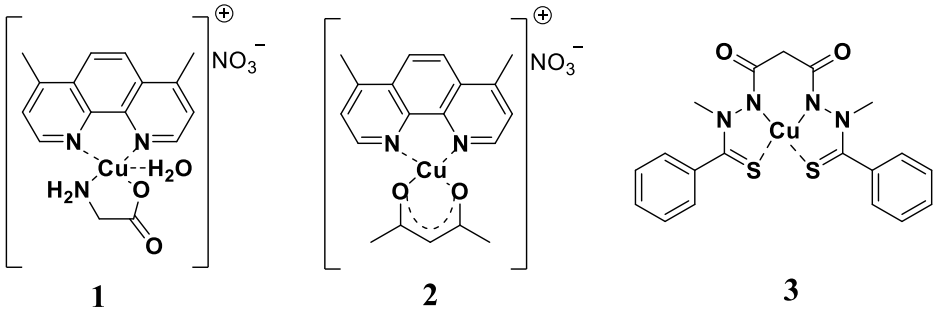

Figure S1. The structures of highly promising anticancer Cu complexes

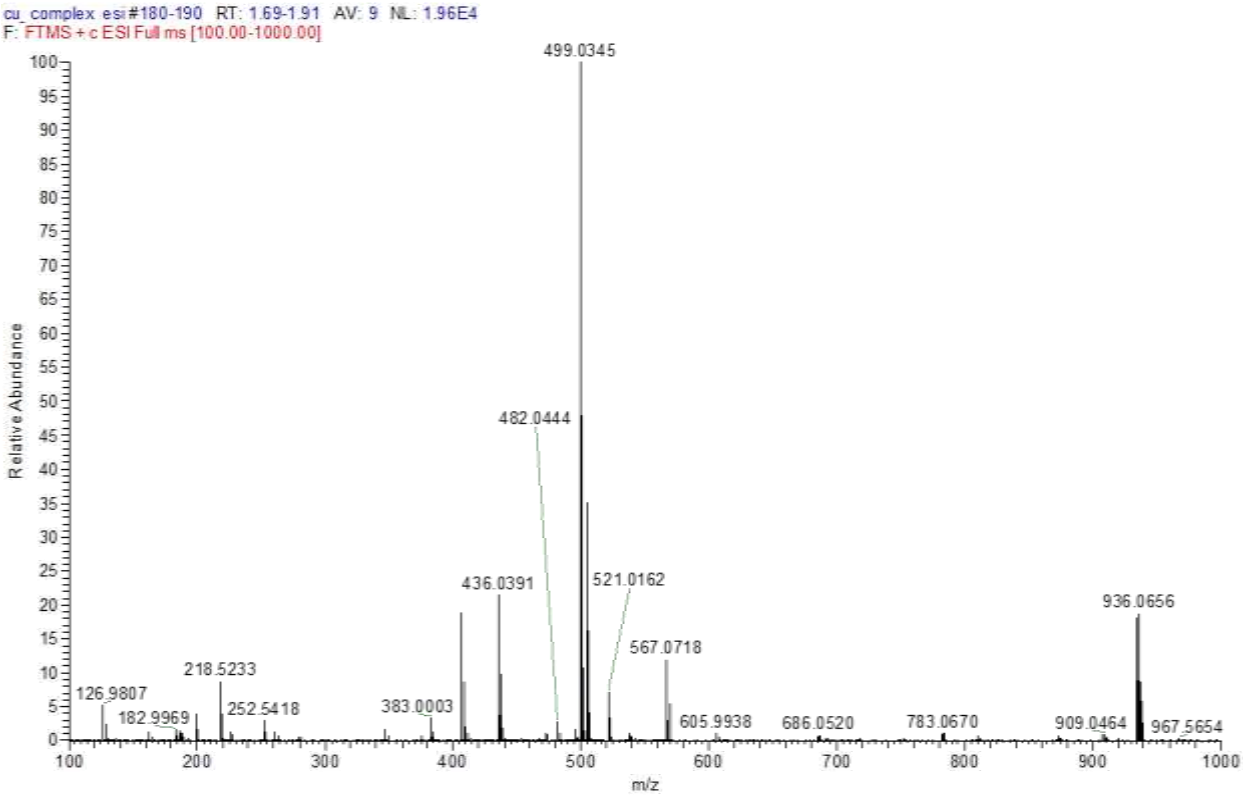

Figure S2. ESI-MS spectrum of complex **Cu-1**

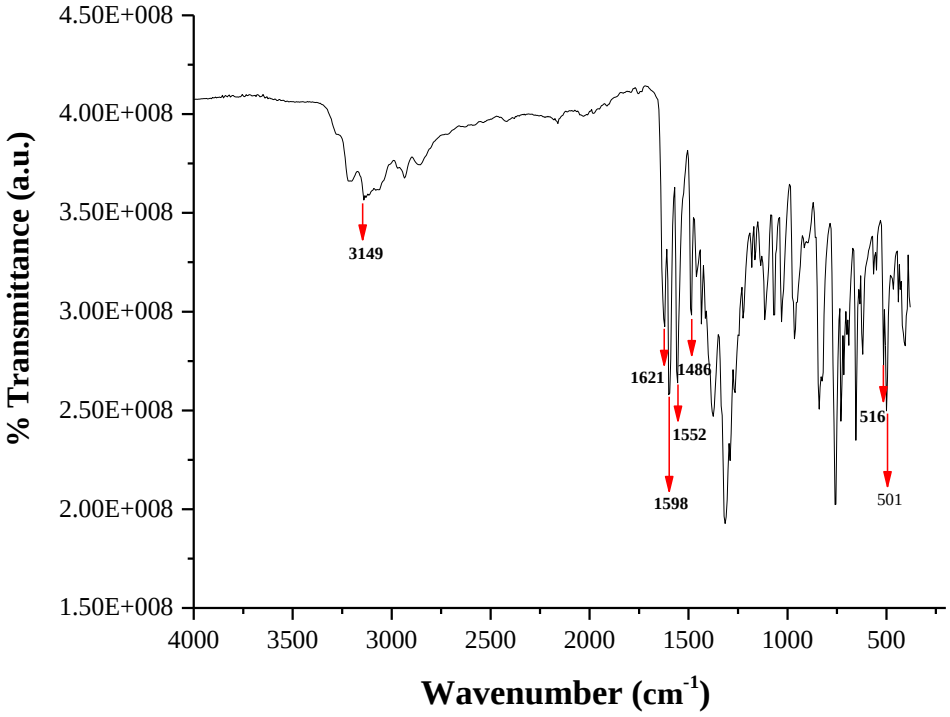

Figure S3. IR spectrum of complex **Cu-1**

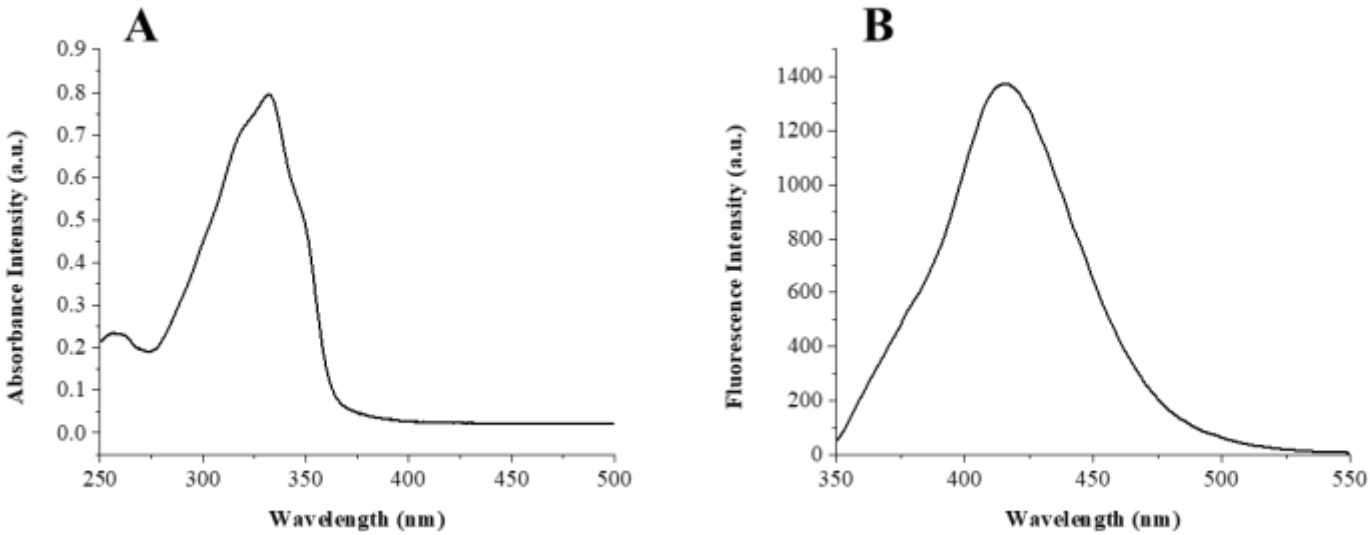

Figure S4. (**A**) Absorbance (250 – 500 nm) and (**B**) fluorescence spectra (350 - 550 nm, after excitation at 340 nm) of complex **Cu-1** (50 μM) in DMSO

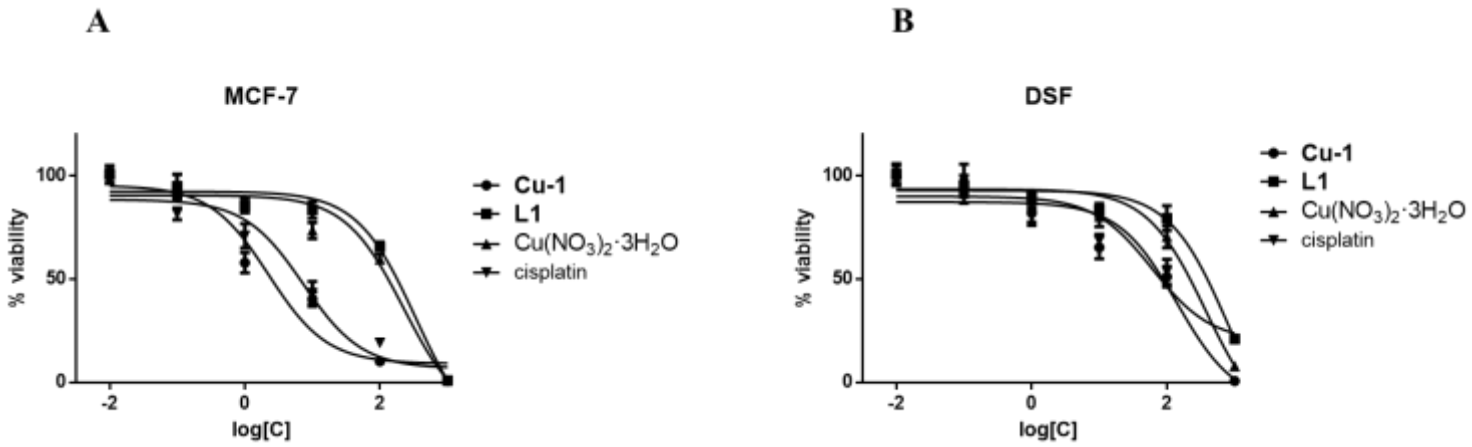

Figure S5. Indicative dose response curves for MCF-7 (**A**) and DSF (**B**) cells cultured with **Cu-1, L1,** $Cu(NO_3)_2 \cdot 3H_2O$ and cisplatin for 72 hours. All data are representative of at least three independent experiments.

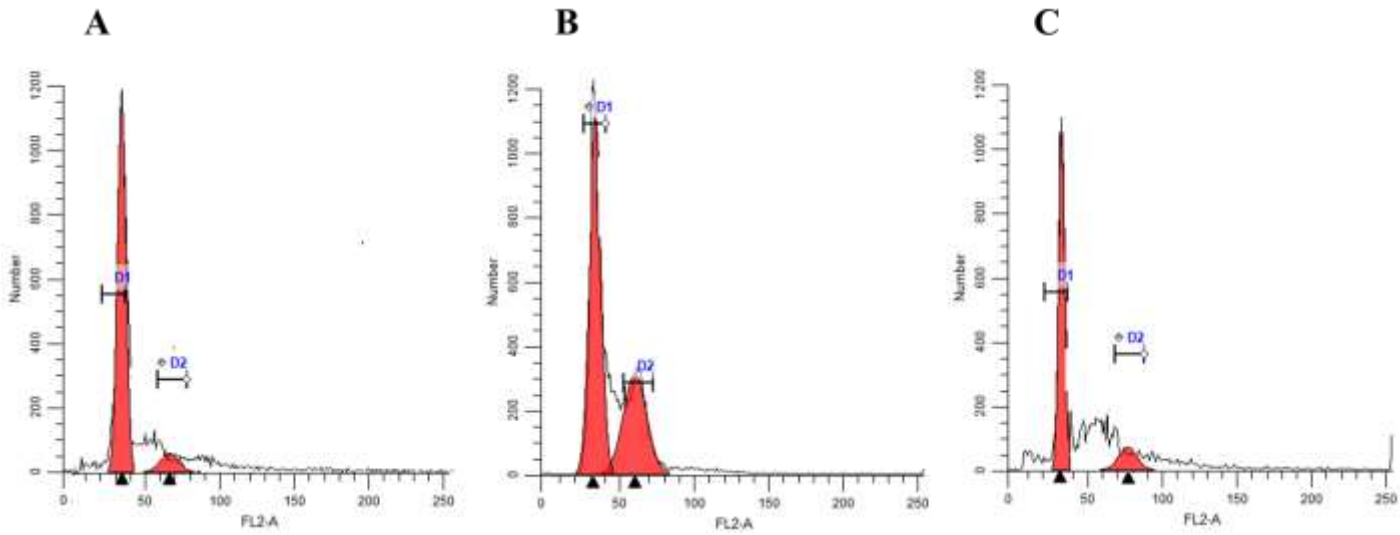

Figure S6. Representative histograms showing cell cycle arrest following treatment with DMSO (**A**), **Cu-1** (**B**) and **L1** (**C**) in MCF-7 cells.

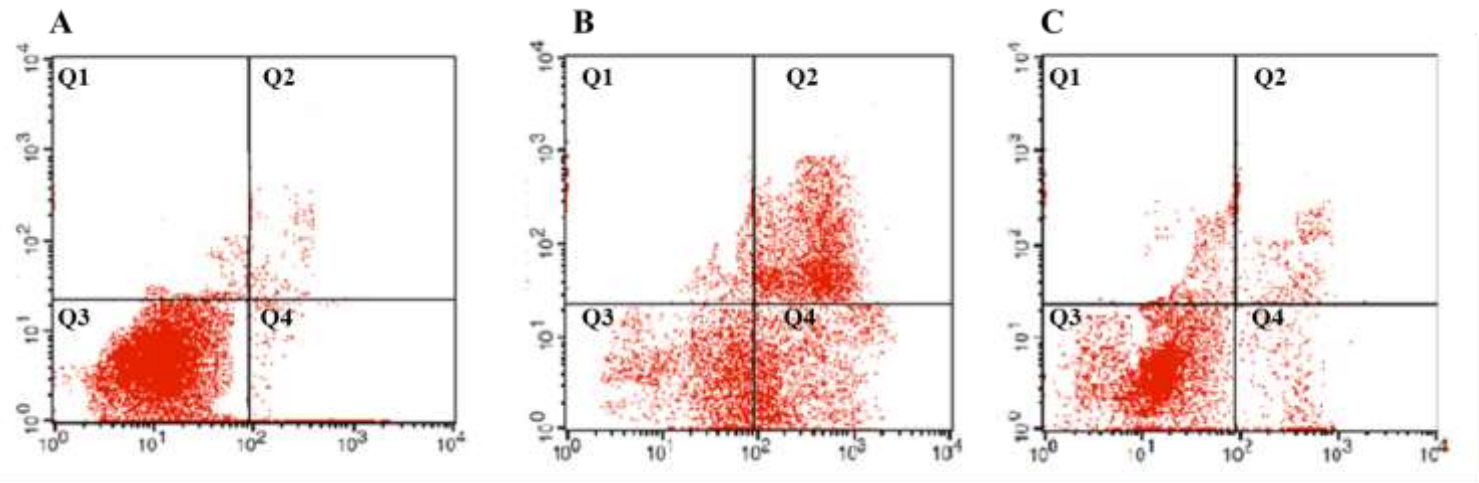

Figure S7. Representative dot plots from flow cytometric analysis of the Annexin V-FITC/PI Assay in MCF-7 cells after treatment with either DMSO (**A**) or the IC50 concentration of **Cu-1** (**B**) and **L1** (**D**) for 72 h. Displayed on x-axis: fluorescence intensity of propidium iodide (PI). Displayed on y-axis: fluorescence intensity of fluorescein isothiocyanate (FITC). Analysis quadrants: Q1. Necrotic cells (PI positive/FITC negative), Q2. Late apoptotic cells (FITC/PI positive), Q3. Viable cells (FITC/PI negative), Q4. Early apoptotic cells (FITC positive/PI negative).

Table S1. Hydrogen bonds in complex **Cu-1**.

D—H···*A*	*D*—H	H···*A*	*D*···*A*	*D*—H···*A*
N2—H21···O4^{i}	0.86	2.30	2.941 (4)	132
N2—H21···O5	0.86	2.45	3.155 (4)	139
N3—H31···O7^{ii}	0.83	2.16	2.904 (4)	149
N6—H61···O5^{iii}	0.87	1.94	2.784 (4)	162
N6—H61···O7^{iii}	0.87	2.45	3.129 (4)	135
N6—H61···N8^{iii}	0.87	2.48	3.287 (4)	154

Symmetry codes: (i) $x+1, y, z$; (ii) $x-1, y, z$; (iii) $x-1/2, -y+1/2, z+1/2$.

References

1. Stoll, S.; Schweiger, A. EasySpin, a comprehensive software package for spectral simulation and analysis in EPR. *J Magn Reson* **2006**, *178*, 42-55