Synthesis, DNA-binding and in vitro antitumor activity of water-soluble copper porphyrin and its' Schiff base complexes

Qi-Meige Hasi[a*, #], Yu-Ping Guo[a#], Jia-Qi Yang[a], Xiao-Tong Mu[a], Li-Hua Chen[a], Shan-Shan Wang[a], Chao-Hu Xiao[b], Yu-Han Zhang[a] and Zhi-Chao Han[a]

[a]*Key Laboratory of Environment-Friendly Composite Materials of the State Ethnic Affairs Commission, Key Laboratory for Utility of Environment-Friendly Composite Materials and Biomass in University of Gansu Province, Gansu Provincial Biomass Function Composites Engineering Research Center, College of Chemical Engineering, Northwest Minzu University, Lanzhou, Gansu 730030, P. R. China*

[b]*Center of Experiment, Northwest Minzu University, Lanzhou 730030, PR China*

[#]*Contributed equally to this work.*

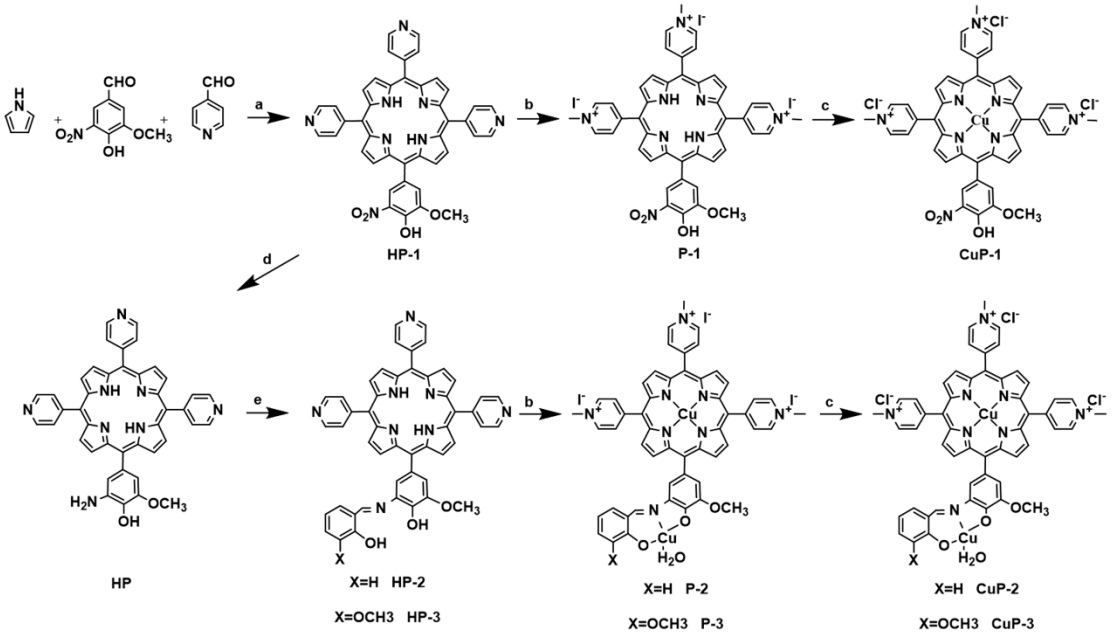
*Corresponding authors.

Prof. Dr. Qi-Meige Hasi. E-mail address: ha2qimei@163.com (Q. -M. Hasi).

Experimental

1.Synthesis

All solvents and starting chemicals were of commercially analytical grade and used without further purification unless otherwise noted. All pyrrole in this work was distilled before use. The special synthesis routes are illustrated in **Scheme S1**.



Scheme. S1. The synthetic route of complex CuP-1, CuP-2, CuP-3**.** (a) propionic acid, propionic anhydride, 140 °C, reflux 2 h, (b) CH_3I, DMF, 45 °C, 5 h; (c) $CuCl_2{\cdot}2H_2O/CH_3OH$, 65 °C, 5 h, (d) $SnCl_2{\cdot}2H_2O$, 6M HCl, 65 ℃, 18 h, (e) salicylaldehyde /o-Vanillin, DMF, $CHCl_3$, CH_3OH, Acetic acid, 72 ℃, 36 h.

***Synthesis of* 5, 10, 15-*tris*-(4-pyridyl)-20-(3-methoxyl-4-hydroxyl-5-nitro)Pheny-l-prophyrin**

To a refluxing mixture of 150 ml propionic acid and 15 ml propionic anhydride, 1.97 g (0.01 mol, 1 eq) 3-methoxyl-4-hydroxyl-5-nitro-benzaldehyde and 2.7 ml (0.03 mol, 3 eq) pyridine-4-aldehyde were added successively. After the 3-methoxyl-4-hydr -oxyl-5-nitro-benzaldehyde was dissolved, 2.6 ml (0.04 mol, 4 eq) freshly distilled pyrrole was added dropwise (ca. 5-7 min) to the mixture. This reaction mixture was then refluxed at 140 °C for 120 min. The solution was evaporated under reduced pressure to ca.20 ml and afterwards stored overnight at −18 °C after the addition of methanol (ca.75 ml, 1:3.5).[1] The purple precipitate was obtained by filtration, thoroughly washed with cold methanol, and dried in vacuo at room temperature. The sediment including six porphyrin isomers was separated on silica gel (MeOH:$CHCl_3$=2:98) and then the fifth eluted isomer, was obtained in 3.5 % yield. ^{1}H NMR (400 MHz，$CDCl_3$) δ 9.15-9.00 (m，6H)，8.92 (dd，9H)，8.47 (d，6H)，8.17 (d，3H)，8.07 (d，1H)，7.25 (s，1H)，4.01 (s，3H)，-2.91 (s，2H). Anal. Calcd. For $C_{42}H_{28}N_8O_4$: C, 71.18 ; H, 3.98 ; N, 15.81 %； Found: C, 71.14; H, 3.96; N, 15.79 %.

Synthesis of P-1

under an inert atmosphere, an excess of iodomethane (0.5 mL) was added to a stirred solution of 5, 10, 15-*tris*-(4-pyridyl)-20-(3-methoxyl-4-hydroxyl-5-nitro)Pheny-l-prophyrin (0.1 g, 0.14 mmol) in anhydrous DMF (3 mL). The mixture was stirred at 45 °C for 5 h in the dark.[2] At reaction completion, the resulting solution was poured into $CHCl_3$ (50 mL). The resulting precipitate was washed with $CHCl_3$ and dried under vacuum, and then received the target product 5,10,15-*tris*-(4-methylpyridyl)-20-(3-methoxyl-4-hydroxyl-5-nitro)Phenyl-prophyrin in 85 % yield.

Synthesis of CuP-1

A solution of $CuCl_2 \cdot 6H_2O$ (135 mg, 1.0 mmol) in methanol (10 mL) was added to a solution of 5, 10, 15-*tris*-(4-methylpyridyl)-20-(3-methoxyl-4-hydroxyl-5-

nitro)Phenyl-Prophyrin (100 mg, 0.1 mmol) in DMF(3 mL). This reaction mixture was stirred at 65 °C for 5 h. At reaction completion, the product was isolated by centrifugation and gathered liquor, then the solution was poured into $CHCl_3$ (30 mL), filtered, washed with $CHCl_3$ and dried under vacuum to give the corresponding porphyrins as amaranthine solid.

CuP-1: ^{1}H NMR (400 MHz，DMSO) δ 9.28 (s, 6H)，8.00 (s, 6H)，4.58 (s，4H)，3.34 (s，9H). ESI-MS(m/z):814 (M^{3+}, Calcd for $C_{45}H_{35}CuN_8O_4^{3+}$:814). Anal. Calc. for $C_{45}H_{35}Cl_3CuN_8O_4$: C, 58.64 %; H, 3.83 %; N, 12.16 %. Found: C, 58.61 %; H, 3.86 %; N 12.19 %.

Synthesis of 5, 10, 15-tris-(4-pyridyl)-20-(3-methoxyl-4-hydroxyl-5-amino)Phenyl-prophyrin

5, 10, 15-*tris*-(4-pyridyl)-20-(3-methoxyl-4-hydroxyl-5-nitro)Phenyl-prophyrin 0.1 g (0.14 mmol) was dissolved in a solution of 6 N HCl (60 mL) followed by addition of $SnCl_2$ 0.16 g (0.7 mmol) and the mixture was stirred at 65 °C for 18 h under an inert atmosphere. After that, the reaction mixture was eutralized with a solution of 5 N NaOH on ice, filtered and washed with a solution of 5 N NaOH (3×). The filter cake was dissovled in MeOH/CH_2Cl_2 (V/V=1/5) and extracted with deionized water, the organic phase was dried over Na_2SO_4 and the solvent was evaporated under reduced pressure affording of purple solid in 75 % yield. ^{1}H NMR (600 MHz， DMSO) δ 9.15 – 8.75 (m, 6H), 8.55 (dd, 6H), 8.33 – 8.06 (m, 3H), 7.94 (d, 1H), 7.23 – 6.82 (m, 2H), 4.12 (s, 2H), 3.84 (d, 2H), 3.56 (d, 3H), -3.00 (s, 2H). ESI-MS (m/z): 679.1 (M^+, Calcd for $C_{42}H_{30}N_8O_2$: 678).

Synthesis of *HP-2*

A 15.6 μL amount of salicylaldehyde (0.15mmol) was added dropwise to a solution of 5, 10, 15-*tris*-(4-pyridyl)-20-(3-methoxyl-4-hydro-xyl-5-nitro)Phenyl-prophyrin (100 mg, 0.15 mmol) in a DMF/MeOH mixture ((1/2, v/v, 60 mL) plus three drops of glacial acetic acid, and then the resulting mixture was refluxed at 72 °C for 36 h. After the reaction completion, the resulting reaction solution was evaporated under reduced pressure and then putted distilled water into residual liquid until large amounts of precipitates appeared, then filtered and washed with the water, MeOH successively

in two times, dried in vacuum to give aubergine solid products **HP-2.**

^{1}H NMR (600 MHz，$CDCl_3$) δ 13.58 (s，1H)，9.90 (s，1H)， 9.52 – 7.42 (m，14H)， 6.99 (ddd，2H)， 5.30 (s，2H)， 4.71 – 3.39 (m，4H)， -2.86 (s，2H)。ESI-MS (m/z): 783.2 (M^+, Calcd for $C_{49}H_{34}N_8O_3$:782).

Synthesis of *HP-3*

The preparation of **HP-3** was similar to that of **HP-2,** except that o-Vanillin (22.9 mg, 0.15 mmol) was used instead of salicylaldehyde. ^{1}H NMR (600 MHz，$CDCl_3$) δ 13.58 (s，1H)，9.90 (s，2H)，9.05 – 8.85 (m，12H)，6.99 (d, 2H)，5.30 (s，2H)，4.71 – 3.39 (m，4H)，-3.00 (s，2H)。ESI-MS (m/z): 813.2 (M^+, Calcd for $C_{50}H_{36}N_8O_4$:812).

Synthesis of *P-2* or ***P-3***

Porphyrin **HP-2** (100 mg, 0.15 mmol) or **HP-3** (100 mg, 0.12 mmol) was dissolved in anhydrous DMF (4 mL). Subsequently a large excess of CH_3I (0.5 mL, 8 mmol) was added and the mixture was heated at 45 °C for 5 h. At reaction completion, the resulting porphyrin was precipitated by addition of acetone, filtered and washed with $CHCl_3$. And the resulting precipitate dried under vacuum, and then received the mulberry target product **P-2** or **P-3**.

Synthesis of Cu*P-2* or **Cu*P-3***

The synthetic method of **CuP-2** or **CuP-3** was similar to that of **CuP-1**, except that **P-2** (100 mg, 0.082 mmol) or **P-3** (100 mg, 0.081 mmol) was used instead of 5, 10, 15-*tris*-(4-methylpyridyl)-20-(3-methoxyl-4-hydroxyl-5-nitro)Phenyl-Prophyrin.

CuP-2: ^{1}H NMR (600 MHz, DMSO) δ 9.19 (s, 6H), 8.79 (s, 6H)， 7.96 (s, 6H), 4.52(s, 4H), 3.26(s, 9H). ESI-MS(m/z):1044(M^+, Calcd for $C_{52}H_{43}Cl_2Cu_2N_8O_4^+$:1044). Anal. Calc. for $C_{52}H_{41}Cl_3CuN_8O_4$: C, 58.08 %; H, 3.84 %; N, 10.42 %. Found: C, 58.11 %; H, 3.86 %; N 10.42 %.

CuP-3: ^{1}H NMR (600 MHz，DMSO) δ 9.18 (s, 6H), 8.84(s, 6H), 7.95 (s, 6H), 4.52 (s, 2H), 3.28 (s, 9H). ESI-MS (m/z): 1071(M^+, Calcd for $C_{53}H_{45}Cl_2Cu_2N_8O_5^+$: 1071). Anal. Calc. For $C_{53}H_{43}Cl_3Cu_2N_8O_5$: C, 57.59 %; H, 3.92 %; N, 10.14 %. Found: C, 57.61 %; H, 3.89 %; N 10.19 %.

2.DNA binding studied

All the experiments involving the interaction of the test compounds with ct-DNA were performed in the Tris–HCl buffer (pH=7.20). A solution of ct-DNA in the Tris–HCl buffer (PH=7.20) gave ratios of UV absorbance at 260 and 280 nm of about 1.8–1.9:1, indicating that the ct-DNA was sufficiently free of protein.[3] The concentration of ct-DNA per nucleotide was determined by absorption spectroscopy using the molar absorption coefficient of 6600 M^{-1} cm^{-1} at 260 nm.[4] The test compounds were dissolved in a solvent mixture of 1% DMF and 99% Tris–HCl buffer (pH 7.20) at the concentration 1.0×10^{-5} M.

Electronic absorption spectroscopy

Electronic absorption titration experiments were carried out using fixed concentration of test compounds (10 μM), while gradually increasing the concentration of ct-DNA with range from 0 to 25 μM. Absorbance values were recorded after each successive addition of ct-DNA solution, followed by an equilibration for 5 min. Each sample solution was scanned in the range of 190–800 nm. The intrinsic binding constant K_b was calculated by using following equation:

$$[DNA] / (\varepsilon_a - \varepsilon_f) = [DNA] / (\varepsilon_b - \varepsilon_f) + 1 / [K_b (\varepsilon_b - \varepsilon_f)]$$

Where ε_a, ε_f and ε_b correspond to A_{obsd}/[Por], the extinction coefficient for the free porphyrin compound and the extinction coefficient for the porphyrin comound in the fully bound form, respectively. [DNA] is the concentration of DNA in base pairs. In plots of [DNA]/(ε_a -ε_f) versus [DNA], K_b is given by the ratio of slope to the intercept.

Competitive studies with EB

The Ethidium bromide (EB) displacement experiments were studied according to the following procedure. At constant room temputerature, the test complexes solutions were incrementally added to the ct-DNA (4 μM) solutions containing 0.32 mM EB, until the drop of fluorescence intensity (λ_{ex}=537 nm) attained a saturation. The quenching constants K_{sv} were calculated using the given equation:

$$F_0 / F = 1 + K_{sv} [Q]$$

Where F_0 and F are the fluorescence intensity in the absence and presence of the test compound at [Q] concentration respectively; K_{sv} is the quenching constant and [Q]

is the quencher concentration. In plots of F_0/F versus [Q], K_{sv} is given by the ratio of slope to the intercept.

ICD studies

The ICD spectra of the test compounds were recorded in presence of the ct-DNA. Mixing the test compounds with ct-DNA at a ratio of [DNA]/[CuP-X] =0.1 in the Tris buffer, and then the mixture was incubated at 4 °C overnight. Each measurement was the average of three repeated scans recorded.[5]

Viscosity measurements

An Ubbelodhe viscometer was used in viscosity measurements experiments, whose temperature was controlled by an external thermostat (25 ± 0.1 °C). Titrations were carried out for the test compounds, and the stock solution of each test compound was then introduced into a ct-DNA solution (0.5 mM) present in the viscometer. The viscosity data were analyzed by the expression $L/L_0 = (\eta/\eta_0)^{1/3}$, where η is the viscosity of DNA in the presence of the test compound and η_0 is the viscosity of DNA alone. The relative viscosities for DNA in either the presence or absence of the test compounds were calculated using the given equation:

$$\eta/\eta_0 = (t_c - t_0)/(t_c - t_0)$$

Where t_c is the observed flow time of the DNA solution containing the test compound, t_0 is the flow time of Tris-HCl buffer solution and t_D is the flow time of the ct-DNA in buffer.

3.In Vitro Antitumor Activity

3.1.Cytotoxicity Assays

To investigate the growth inhibitory effect toward tumor cell lines of these derivatives, we employed MTT assay in three different cancer cell lines. Hela cell, MDA cell and TCA8113 cell respectively were seeded into 96-well plates cultured in 100 μL growth medium and then maintained at 37 °C in humidified atmosphere containing 5 % CO_2 for 24 h, before the treatment with the tested compounds. After 24 h, the growth medium was removed and replaced with a fresh one containing the different concentrations of the target compounds were added to cells cultivated for

different time periods at the same circumstance. Triplicate cultures were established for each treatment. At the end of this incubation, each well was treated with 20 μL 5mg/mL MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide) saline solution and incubated for an additional 4 h. After that, the growth inhibition of the cell induced by the target compounds was determined by measuring the absorbance of each well at 570 nm using a microplate reader (Xmark, BioRad). Mean absorbance for each drug dose was expressed as a percentage of the control untreated well absorbance and plotted versus drug concentration. IC_{50} values represent the drug concentrations that reduced the mean absorbance at 570 nm to 50 % of those in the untreated control wells.

3.2.Cell Cycle Analysis.

The cell cycle analysis of control and treated cancer cells was investigated by using standard methods. Hela cell, MDA cell and TCA8113 cell respectively were treated with the different drug doses of **CuP-1~3** for 48 h, 72 h, harvested by centrifugation at 1000 ×g for 5 min. And then the collected cells were fixed with cold 70 % ethanol overnight, washed twice with cold PBS, followed by stained with PI solution consisting of PI (50 μg/mL), RNase (10 mg/mL) and TritonX-100 (2 % v/v) were added to cells and incubated for 25 min at room temperature. Finally, samples were measured by flow cytometry using a 488 nm laser.

3.3. AnnexinV-FITC/PI Assay of Apoptotic Cells

For cell apotosis studies, Hela cell, MDA cell and TCA8113 cell respectively were treated with the various drug doses of **CuP-1~3** for 48 h, 72 h. After the treatment, cells were harvested and washed twice with cold PBS (4 °C) and then resuspended cells in binding buffer at a concentration of 1×10^6 cells/mL, followed 5 μL each of Annexin V and propidium iodide were added to each sample and incubated for 15min at room temperature. At last, samples were analyzed by flow cytometry.

3.4. Fluorescence Microscopy of Apoptosis Assays.

In this studies, Hela cell, MDA cell and TCA8113 cell respectively were exposed to **CuP-1~3** for 48 h, and then washed with PBS for twice, followed by stained with Hoechst 33342 solution (10 μg/mL) at 37 ℃ for 30 min. Finally, the cells were observed under the fluorescence microscope.

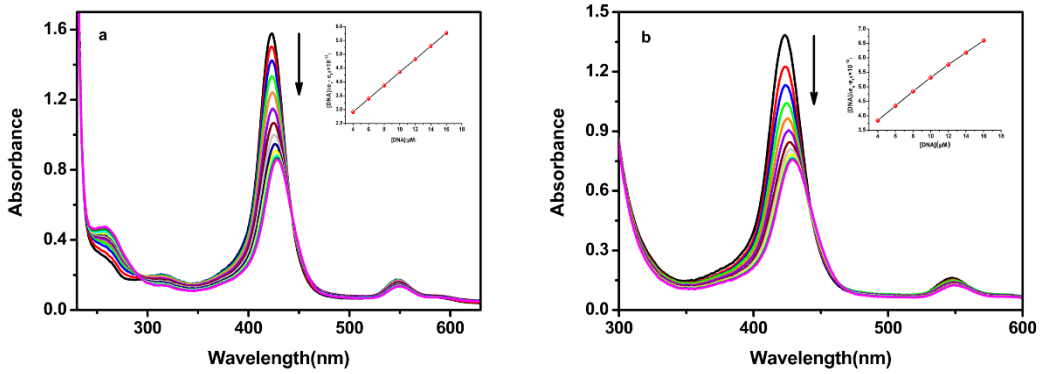


Fig. S1. Absorption spectra of CuP-2 (a), CuP-3 (b) in buffer at 25 °C in the presence of increasing amounts of ct- DNA. Black arrow indicate the change in absorbance upon increasing the DNA concentration. Insets: Plot of [DNA] vs [DNA]/(ε_a - ε_f).

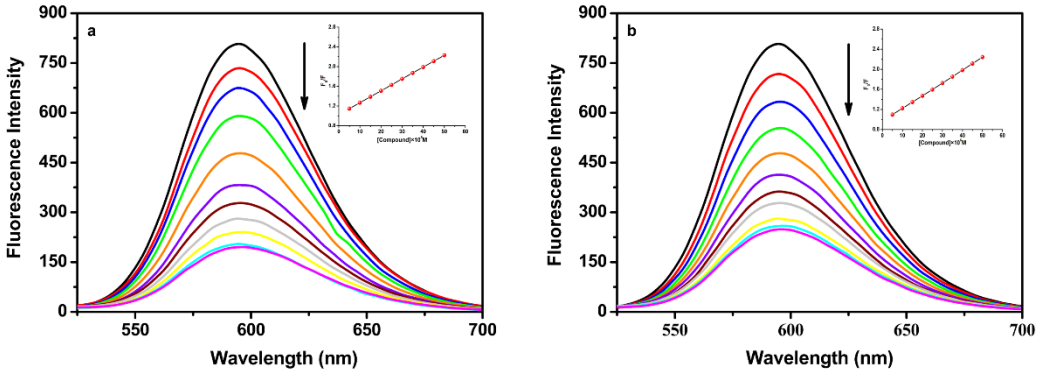


Fig. S2. Emission spectra of EB-DNA system in absence and presence (other colored lines) of CuP-2 (a), CuP-3 (b). Insets: Stern-Volmer plots of the EtBr-DNA fluorescence titration.

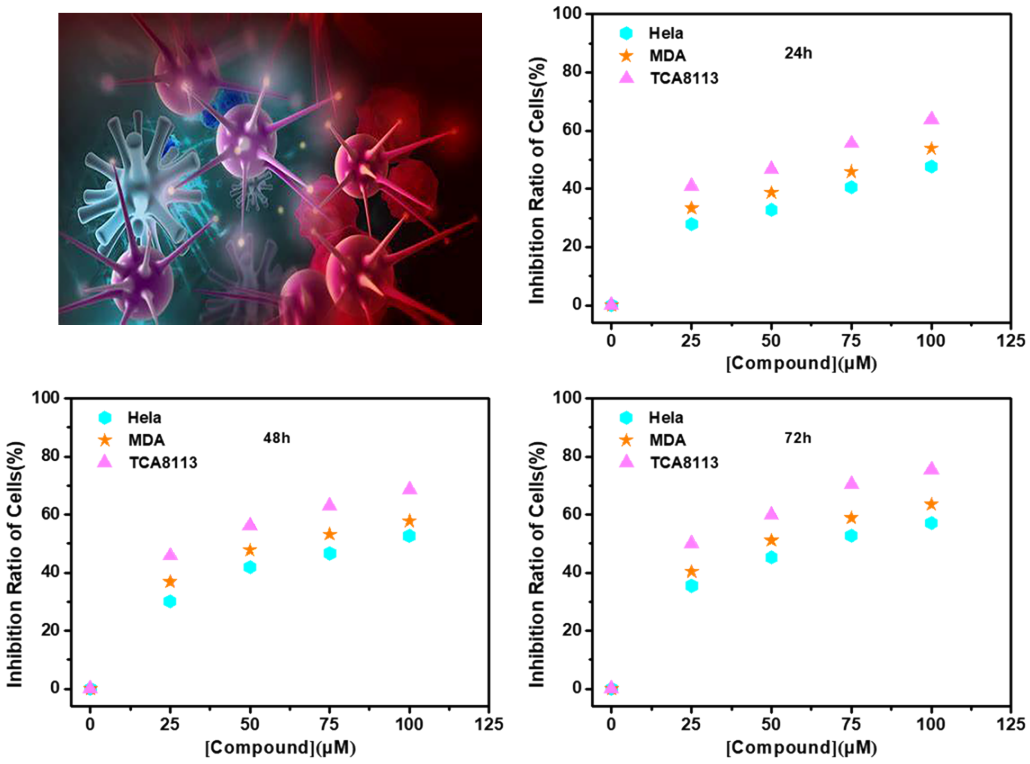


Fig.S3. The Comparison of the inhibition ratio of cell of Complex CuP-2 towards Hela, MDA, TCA8113 cell lines in different cultivated time and diverse doses.

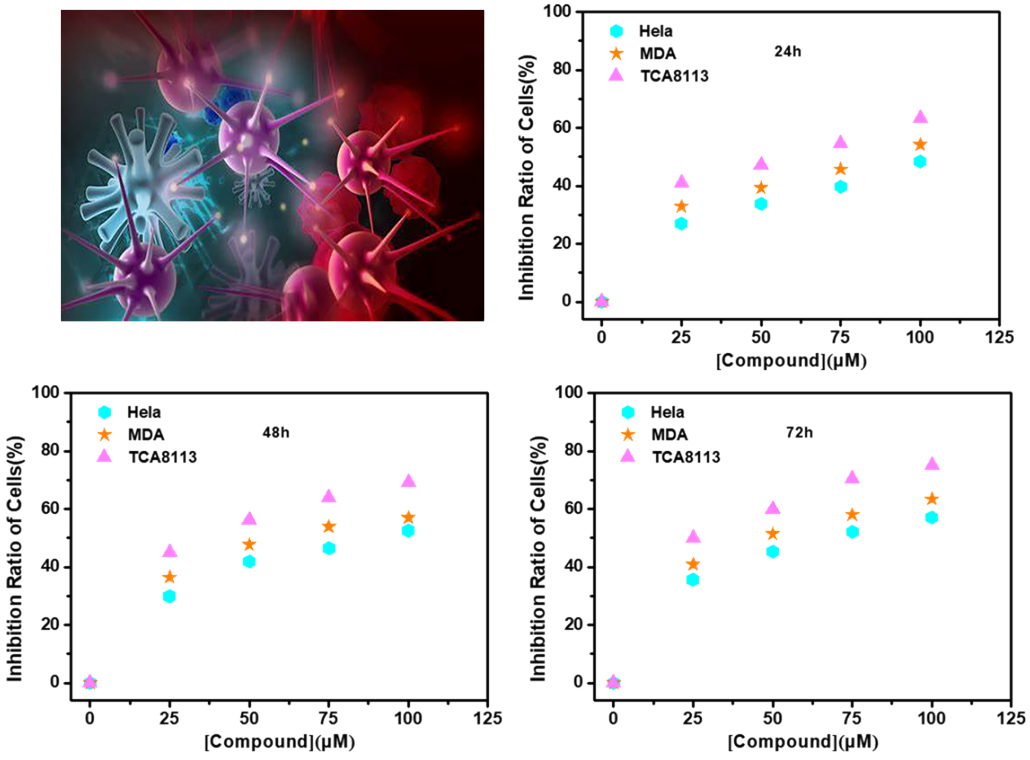


Fig.S4. The Comparison of the inhibition ratio of cell of Complex CuP-3 towards Hela, MDA,

TCA8113 cell lines in different cultivated time and diverse doses.

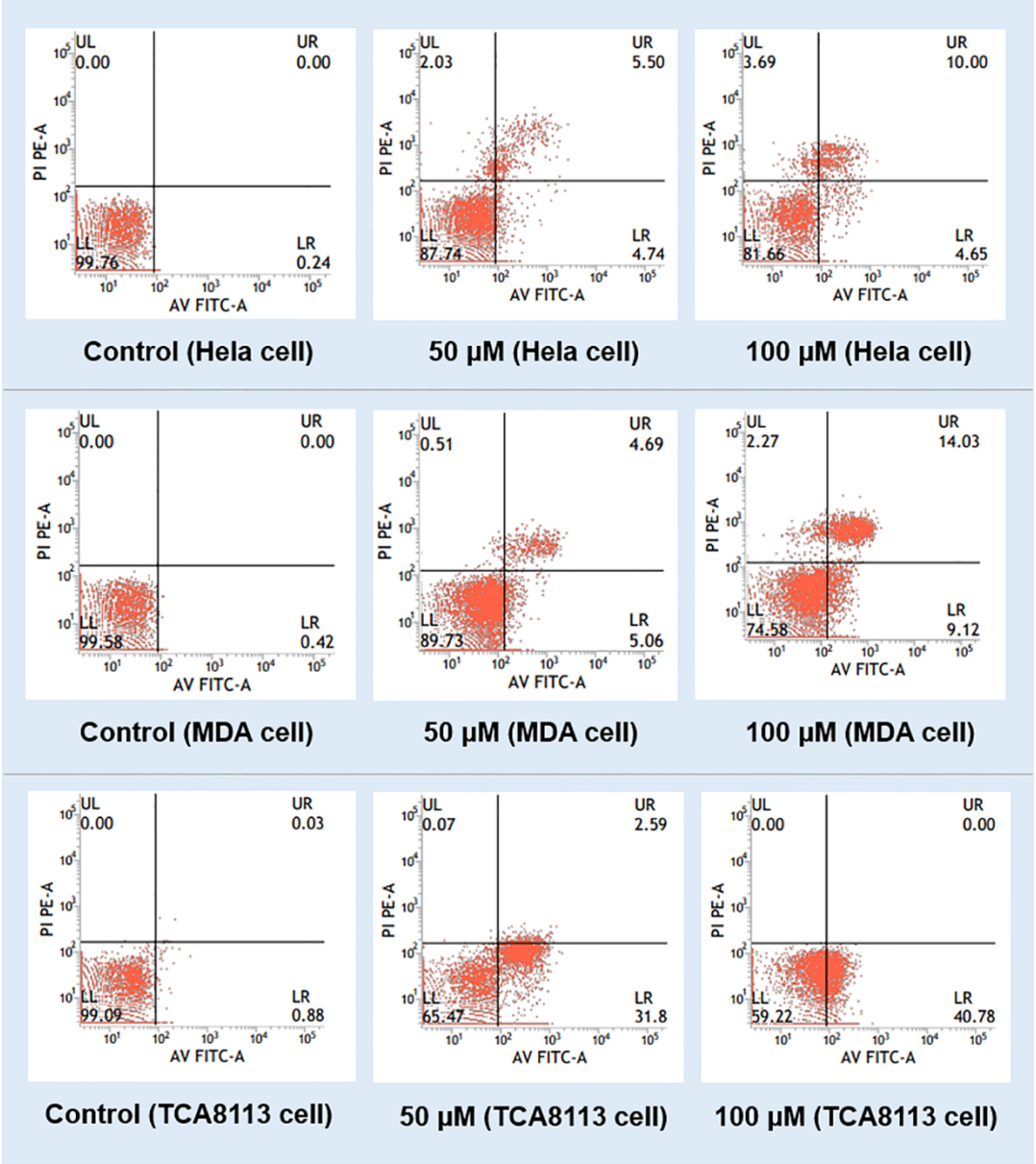


Fig. S5. Flow cytometric analysis of the cell death mechanism induced by the CuP-2 with multiple doses. Data are obtained from three independent experiments. The cell populations at different phases of cell death, namely, viable (AnnexinV−FITC$^-$/PI$^-$, lower left quadrant), early apoptotic (Annexin V−FITC$^+$/PI$^-$, lower right quadrant), and necrotic (Annexin V−FITC$^-$/PI$^+$, upper left quadrant) or late-stage apoptotic (Annexin V−FITC$^+$/PI$^+$, upper right quadrant).

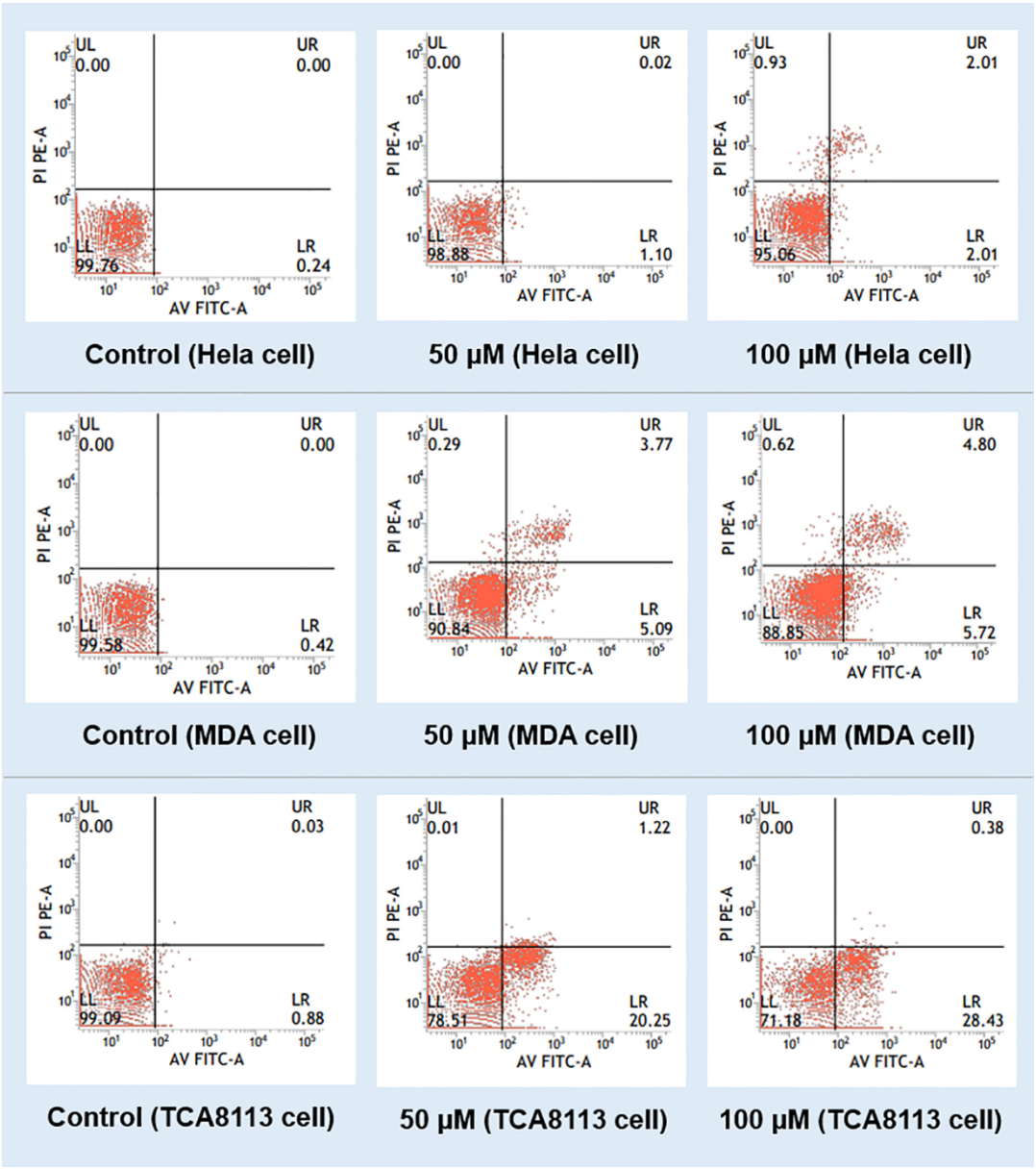


Fig. S6. Flow cytometric analysis of the cell death mechanism induced by the CuP-3 with multiple doses. Data are obtained from three independent experiments. The cell populations at different phases of cell death, namely, viable (AnnexinV−FITC$^-$/PI$^-$, lower left quadrant), early apoptotic (Annexin V−FITC$^+$/PI$^-$, lower right quadrant), and necrotic (Annexin V−FITC$^-$/PI$^+$, upper left quadrant) or late-stage apoptotic (Annexin V−FITC$^+$/PI$^+$, upper right quadrant).

Reference

1. C. Spagnul, R. Alberto, G. Gasser, S. Ferrari, V. Pierroz, A. Bergamo, T. Gianferrara, E. Alessio, *J Inorg Biochem*, 2013, **122**, 57-65.
2. M. Perrée-Fauvet, C. Verchère-Béaur, E. Tarnaud, G. Anneheim-Herbelin, N. Bône, A. Gaudemer, *Tetrahedron*, 1996, **52**, 13569-13588.
3. R. Eshkourfu, B. Obelji, M. Vuji, I. Turel, A. Pevec, K. Sepi, M. Zec, S. Radulovi, Tatjana. Srdi-Radi, D. Miti, *J Inorg Biochem*, 2011, **105**, 1196-1203.
4. G. Felsenfeld, S. Z. Hirschman, *J Mol Biol*, 1965, **13**, 407-427.
5. S. Yellappa, J. Seetharamappa, L. M. Rogers, R. Chitta, R. P. Singhal, F. D'Souza, *Bioconjug Chem*, 2006, **17**, 1418-1425.