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Electronic Supplementary Information

Novel water-soluble Cu (II) complexes based on acylhydrazone porphyrin ligands for DNA binding and in vitro anticancer activity as potential therapeutic targeted candidates

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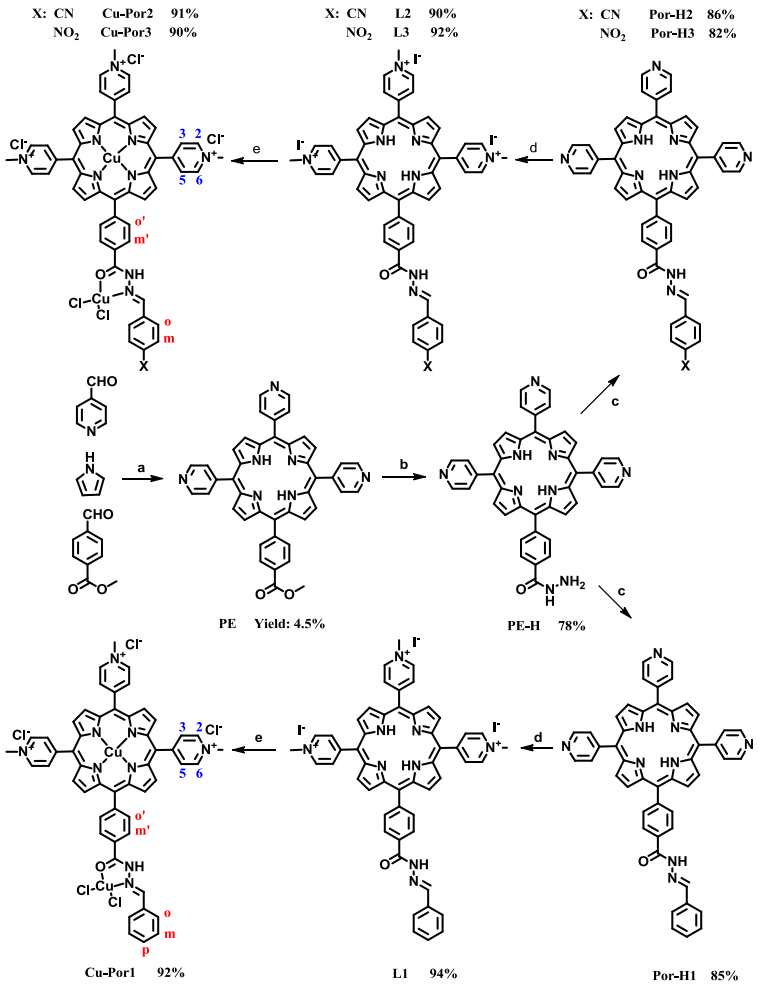
Experimental Section

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 synthesis routes is illustrated in Scheme. S1.



Scheme. S1 The synthetic pathway of **Cu-Por1, Cu-Por2 and Cu-Por3**. (a) propionic acid, reflux 1.5h; (b) NH_2-NH_2 ·H_2O, CH_2C1_2, EtOH, 70°C; (c) benzaldehyde/p-cyanobenzaldehyde/ p-nitrobenzaldehyd, $CHCl_3$, EtOH, Acetic acid; (d) CH_3I, DMF, 50°C, 3h; (e) $CuCl_2$·$2H_2O$, DMF, EtOH, 60 °C, 3 h.

1.1. Synthesis of porphyrin Por-H1.

PE and PE-H were synthesized according to the procedure reported by our group[1]. The special synthesis routes are illustrated in Scheme. S1.

A 100 mg amount of PE-H (0.15mmol) was dissolved in a 10 mL $CHCl_3$,a solution of benzaldehyde (15.10 μL, 0.15 mmol) in ethanol (20 mL) and acetic acid were added drop wise were added, later the mixture warmed under reflux for 24hunder the argon atmosphere[2]. After reaction completed, the solution was evaporated under reduced pressure and then putted diethyl ether (ca.20mL) into residual liquid; the colature was acquired by filtration and washed twice with the mixture of EtOH/Et_2O to further depurate by thoroughly, dried in vacuum to give aubergine solid products Por-H1. ^{1}H NMR (600 MHz, DMSO-d_6) : δ (ppm) -3.02 (2H, s; NH-H), 7.49 (3H, m; mPh-H+pPh-H), 7.82 (2H, d; J=6.6 Hz, o'Ph-H), 8.25 (6H, m; 3,5-Py-H), 8.36 (4H, d; J=4.2 Hz, m'Ph-H+oPh-H), 8.59 (1H, s; N=CH-H), 8.89 (8H, s; β-H), 9.02 (6H, s; 2,6-Py-H), 12.23 (1H, s; 25 CONH-H). HRMS (m/z): $[M + H]^+$, Calcd for $C_{49}H_{33}N_9O$:764.28808, found: 763.28751.

1.2. Synthesis of porphyrin Por-H2.

The synthetic procedure of Por-H2 was analogous to that of Por-H1, except that p-cyanobenzaldehyde (19.40mg, 0.15 mmol) was used instead of benzaldehyde. ^{1}H NMR (600 MHz, $CDCl_3$) : δ (ppm) -2.88 (2H, s; NH-H), 7.76 (2H, s; mPh-H), 7.84 (1H, d; *J*=5.4 Hz, oPh-H), 7.99 (1H, d; *J*=8.4 Hz, oPh-H), 8.16 (6H, s; 3,5-Py-H), 8.36 (5H, m; o'Ph-H+m'Ph-H+N=CH-H), 8.87 (8H, s; β-H), 9.06 (6H, s; 2,6-Py-H), 10.08 (1H, s; CONH-H). HRMS (m/z): $[M + H]^+$, Calcd for $C_{50}H_{32}N_{10}O$:789.28333, found: 789.28259.

1.3. Synthesis of porphyrin Por-H3.

The synthetic procedure of Por-H3 was analogous to that of Por-H1, except that p-nitrobenzaldehyd (22.36mg, 0.15 mmol) was used instead of benzaldehyde.^{1}H NMR (600 MHz, DMSO-d6): δ (ppm) -3.03 (2H, s; NH-H), 8.07 (2H, s; o'Ph-H), 8.26 (6H, s; oPh-H+mPh-H+o'Ph-H+m'Ph-H), 8.37 (6H, m; 3,5-Py-H), 8.68 (1H, s; N=CH-H), 8.90 (8H, s; β-H), 9.04 (6H, s; 2,6-Py-H), 12.54 (1H, s; CONH-H). HRMS (m/z): $[M + H]^+$, Calcd for $C_{49}H_{32}N_{10}O_3$:809.27316, found: 809.27275.

1.4. Synthesis of porphyrin L1, L2 and L3.

A total of 100mg of Por-H1 (0.13 mmol) or Por-H2 (0.13 mmol) or Por-H3 (0.12

mmol) was dissolved in arid DMF (3 mL). Subsequently CH3I (0.5 mL, 8 mmol) was added to the solution and the mixture was heated at 50 °C for 3 h[3]. At reaction completion, the solution was poured into acetone (50 mL), the Centrifuged precipitates was washed with $CHCl_3$ and dried under vacuum, and then received the aubergine target product **L1, L2, and L3**.

L1: ^{1}H NMR (600 MHz, DMSO-d_6) : δ (ppm) -3.02 (2H, s; NH-H), 4.71 (9H, s; N-CH3-H), 7.51 (3H, m; mPh-H+pPh-H), 7.81~7.93 (2H, m; o'Ph-H), 8.40 (4H, d; J=9.6 Hz oPh-H+m'Ph-H), 8.62 (1H, s, N=CH-H), 9.00~9.17 (14H, m; β-H+3,5-Py-H), 9.47 (6H, d; J=4.8 Hz 2,6-Py-H), 12.25(1H, s; CONH-H). HRMS (m/z): M^{3+}, Calcd for $[C_{52}H_{42}N_9O]^{3+}$: 269.44986, found: 269.44980.

L2: ^{1}H NMR (600 MHz, DMSO-d_6) : δ (ppm) δ -3.03 (2H, s; NH-H), 4.71 (9H, s, N-CH3-H), 7.96 (4H, d; J=13.8 Hz, oPh-H+mPh-H), 8.42 (4H, d; J=6.6 Hz, o'Ph-H+m'Ph-H), 8.66 (1H, s, N=CH-H), 8.99~9.17 (14H, m; β-H+3,5-Py-H), 9.47 (6H, s; 2,6-Py-H), 12.49 (1H, s; CONH-H). HRMS (m/z): M^{3+}, Calcd for $[C_{53}H_{41}N_{10}O]^{3+}$: 277.78161, found: 277.78152.

L3: ^{1}H NMR (600 MHz, DMSO-d_6) : δ (ppm) -3.03 (2H, s; NH-H), 4.71 (9H, s, N-CH3-H), 7.96 (4H, d; J=13.8 Hz, oPh-H+mPh-H), 8.42 (4H, d; J=6.6 Hz, o'Ph-H+m'Ph-H), 8.66 (1H, s, N=CH-H), 8.99~9.17 (14H, m; β-H+3,5-Py-H), 9.47 (6H, s; 2,6-Py-H), 12.49 (1H, s; CONH-H). HRMS (m/z): M^{3+}, Calcd for $[C_{52}H_{41}N_{10}O_3]^{3+}$: 284.44489, found: 284.44472.

1.5. Synthesis of porphyrin Cu-Por1, Cu-Por2 and Cu-Por3.

A solution of $CuCl_2 \cdot 2H_2O$ (171.0 mg, 1.0 mmol) in methanol (10 mL) was added to a solution of L1, L2 or L3 (0.1 mmol) in DMF (3 mL) respectively. The mixture was gently heated at 60 °C for 6 h. The product was isolated by centrifugation and collected the supernatant, then the solution was poured into $CHCl_3$ (30 mL), filtered, washed with $CHCl_3$ and dried under vacuum to give the corresponding porphyrins **Cu-Por1**, **Cu-Por2** and **Cu-Por3** as amaranthine solid.

The IR of the three compounds displayed similar characteristics, with strong bands mainly appearing between 700 and 1700 cm^{-1} (**Fig. S1 ~ S3**). The spectrum of

the free ligands showed two characteristic bands at 3313 ~ 3319 and 963 ~ 965 cm^{-1}, which are caused by the N−H stretching vibration and in-plane bending vibration of the pyrrole rings. However, due to the deprotonation and metallization of the pyrrole rings, these two bands disappeared in the spectra of Cu-Por1, Cu-Por2, and Cu-Por3, and a new Cu-N absorption band appeared at 993 ~ 999cm^{-1}. This observation supports the coordination of the two nitrogen atoms of the pyrrole ring with the metal [4]. In addition, compared to the free ligand L1, the complex Cu-Por1 showed a weak characteristic peak at 435 cm^{-1} (428 cm^{-1} for Cu-Por2 and 429 cm^{-1} for Cu-Por3), which indicates the coordination of carbonyl O with Cu^{2+} [5]. This further proves that we have successfully synthesized the complexes Cu-Por1 ~ Cu-Por3.

Cu-Por1: HRMS (m/z): M^{3+}, Calcd for $[C_{52}H_{40}Cl_2Cu_2N_9O]^{3+}-CuCl_2$: 289.75451, found: 289.75409. Elemental analysis: Anal. Calc. for $C_{52}H_{40}Cl_5Cu_2N_9O$: C, 56.20%; H, 3.63%; N, 11.34%. Found: C, 56.52%; H, 3.46%; N, 11.12%. UV–vis (DMSO) λmax, nm (ε): 423(181000), 548(13100). S(25℃, mol/L H_2O): 8.85×10^{-3}.

Cu-Por2: HRMS (m/z): M^{3+}, Calcd for $[C_{53}H_{39}Cl_2Cu_2N_{10}O]^{3+}-CuCl_2$: 298.08626, found: 298.08620. Elemental analysis: Anal. Calc. for $C_{53}H_{39}Cl_5Cu_2N_{10}O$: C, 56.02%; H, 3.46%; N, 12.33%. Found: C, 56.68%; H, 3.75%; N, 12.23%. UV–vis (DMSO) λmax, nm (ε): 424(138000), 550(10500). S(25℃, mol/L H_2O): 8.40×10^{-3}.

Cu-Por3: HRMS (m/z): M^{3+}, Calcd for $[C_{52}H_{39}Cl_2Cu_2N_{10}O_3]^{3+}-CuCl_2$: 304.74954, found: 304.74933. Elemental analysis: Anal. Calc. for $C_{52}H_{39}Cl_5Cu_2N_{10}O_3$: C, 54.01%; H, 3.40%; N, 12.11%. Found: C, 54.46%; H, 3.89%; N, 12.63%. UV–vis (DMSO) λmax, nm (ε): 424(206000), 547(14900). S(25℃, mol/L H_2O): 9.53×10^{-3}.

2. DNA binding studies

Calf tymus DNA was supplied from Sigma (Saint Louis, USA) and the stock solutions of ct-DNA were prepared in 5 mM Tris buffer at pH = 7.2 (5 mM Tris–HCl, 50 mM NaCl, Tris = Tris(hydroxymethyl)-aminomethane). Stock solutions were stored at 4 °C and used within 7 days. Purity of DNA solutions were confirmed by ratio of UV absorbance at 260 and 280 nm (A260/A280 = 1.85~1.9), indicating that

the DNA was sufficiently free of protein[6]. The DNA concentration per nucleotide was determined using absorption intensity at 260 nm after adequate dilution with the buffer and using the reported molar absorptivity of 6600 $M^{-1}cm^{-1}$ in the Tris-HCl-NaCl buffer medium [7, 8]. The stock solutions of complexes were prepared by dissolving them in buffer contained 1% DMSO and diluting suitably with the corresponding buffer to the required concentrations for all of the experiments.

UV-Visible absorption titration experiments was carried out to prove the interaction between complexes and ct-DNA by using a fixed concentration of the complexes (10 μM) upon increasing concentration of ct-DNA following a certain ratio [9, 10] . First, measuring the absorption spectra of complexes (Cu-Por1-Cu-Por3) in absence of ct-DNA , and then ct-DNA solution were added stepwise after 5 min incubation at room temperature of each addition until a saturation state was achieved, the spectra were recorded in the range of 300–600 nm.

Ethidium bromide fluorescence quenching assays have been performed by monitoring changes in the fluorescence intensity at excitation (λex = 537 nm) after aliquot gradually addition of complexes (Cu-Por1~Cu-Por3,5×10^{-4} M)to an aqueous solution of the EtBr-DNA system[11]. The titration processes were repeated until there was no spectral change for at least three titrations indicating the binding or quenching had been achieved.

Induced Circular Dichroism spectra (ICD) spectra of DNA in the absence and presence of porphyrins were recorded in the range of 300-500 nm. Porphyrins mixed with ct-DNA at a ratio of [DNA]/[Cu-porx] = 0.1 (ct-DNA = 10^{-4} M, complexes = 10^{-5} M) in the Tris buffer, each measurement was the average of three repeated scans recorded[12].

Viscosity tests were measured on an Ubbelohde viscometer, immersed in a thermostatted water-bath maintained at 30 ± 0.1 °C [13, 14]. DNA concentration was kept constant (50 mM) and gradually increased the concentration of tested compounds. Titrations were performed by adding same volume of stock solutions of complexes (10^{-3} M) to a solution of ct-DNA in the viscometer. The flow time was measured three

times for each sample with a digital stopwatch and average flow time was calculated.

Cyclic voltammetry (CV) measurements were performed on an Autolab (CHI660E), a standard three-electrode system was used comprising a Glassy carbon (GC) working electrode, a platinum-gauze auxiliary electrode and a Ag/AgCl reference electrode, and Pt gauze as counter electrode, before every reading working electrode was polished with alumina powder and rinsed with distilled water. The supporting electrolyte was 50 mM NaCl, 5 mM Tris, pH = 7.2 [15]. The cyclic voltammetry curve was recorded after the extent concentration of DNA was added in 5 min ' balancing process.

3. In vitro anticancer activity

3.1. Cytotoxicity assay

Cytotoxicity of synthesized porphyrin complexes and their ligands was determined using the MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] (Solarbio) colorimetric assay. Briefly, cells were seeded in 96-well plates (A549, H-1975, HepG2, T47D, Hs 578Bst) at a density of 5×10^3 cells/well and allowed growing for 24 h at 37°C in 5% CO_2. After 24 h incubation, the cells were incubated with 200 µL fresh complete medium contained 20 µL various tested compounds which were dissolved in DMSO and serially diluted with culture medium (DMSO < 0.1%) for different time periods at the same circumstance. The control group only contained complete medium. After this incubation, 20 µL MTT (5 mg/mL) solution was added to each well and incubated for another 4 h. Next, the supernatant was removed, and the formazan crystals were dissolved in and the formazan crystals were dissolved in DMSO (150 µL). The absorbance at 490 nm was measured in each well using a microplate reader (Xmark, BioRad) and the percentage of cell survival was calculated using the given formula:

$$\%\ \text{cell survival} = \frac{\text{Absorbance of test sample}}{\text{Absorbance of control sample}} \times 100\%$$

The evaluation is based on means from at least three independent experiments, each comprising three microcultures per concentration level.

3.2. Cellular uptake

About exponentially grown 5.0 × 10^5/well cells in complete growth medium(2 mL) were seeded into 6-well plates and incubated overnight at 37 °C in wettish atmosphere including 5% CO_2. Afterwards removed medium, the cells were washed twice with PBS, and then the cells were incubated with a solution of complexes in the medium (10 μM, 2 mL) for 24 h under the same conditions. After removing the solution, the cells were rinsed with PBS (2 mL) and harvested by 0.25% trypsin-EDTA (200 μL/well), followed by quenching the trypsin with medium (500 μL/well) including 4% FBS. Upon the suspension was transferred to 1.5 mL centrifuge tube that was centrifuged at 2400 rpm for 3 min and then the pellet was slightly washed with PBS (1mL/tube) which was centrifuged again. Followed on getting rid of the PBS, the cells were lysed with DMF (1 mL/tube) and the hybrid was sonicated for 20 min as well as centrifuged once again. The supernatants were transferred to cuvette and the absorbance (At) at soret band for all complexes has been measured by UV−vis spectroscopic [16]. In addition, the absorption intensity (Ac) of 10 μM complexes DMF solution (2 mL) have been also detected. The percentage of uptake obtained from (At / Ac) ×100% and each experiment was at least repeated three times.

3.3. Fluorescence microscopy

H-1975 cells were seeded in a 6-well plate with the density of 1 × 10^5 cells/well, then the solutions of Cu-Por1 with different concentrations (0, 12.5, 25.0, 50.0 μM) were added to each well. After 24 h and 48 h incubation at 37 °C, all cells were fixed with 4% paraformaldehyde. The fixed cells were washed with PBS for three times and then strained with PI and Hoechst 33342 for 30 min according to the manufacture ’ s protocol and washed with PBS for three times. Imaging of cells was performed by a inverted fluorescent microscope in red and blue channel [17].

3.4. Cell cycle distribution experiments

Cell cycle distribution was analyzed by flow cytometry. Briefly, H1975 cells were seed on a 6-well plate with a density of 5 × 10^5 cells/well in 2.0 mL complete medium, and then treated with Cu-Por1 complex at the indicated concentrations (0,

12.5 and 25 μM), incubated at 37 °C under 5% CO_2 for 24 h and 48 h. After incubation, the cells were harvested in ice-cold PBS at 4 °C, centrifuged, and fixed with 200 μL of 70% ice-cold ethanol stayed overnight at -20 °C. After a further washing steps with cold PBS, then stained with PI solution containing 50 μg/mL PI, 10 mg/mL RNase (Solarbio, Beijing, China), 2% v/v of TritonX-100 and upon incubated for 25 min at 4 °C in the dark, following measured by flow cytometry using a 488 nm laser. For each specimen, 30000 events were recorded.

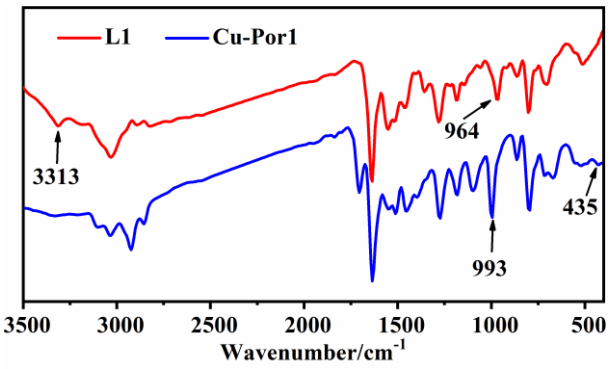


Fig. S1 IR spectra of L1 and Cu-Por1

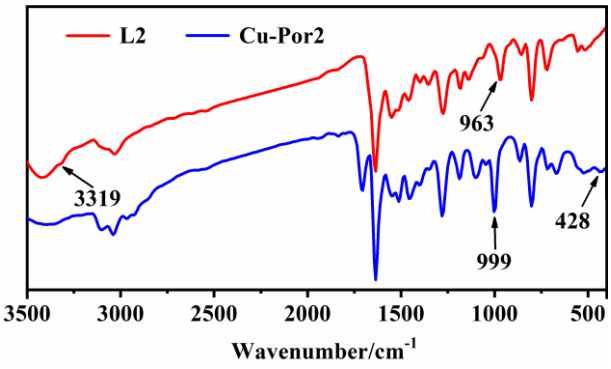


Fig. S2 IR spectra of L2 and Cu-Por2

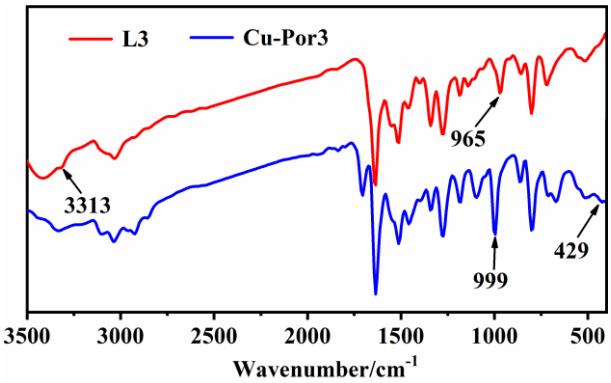


Fig. S3 IR spectra of L3 and Cu-Por3

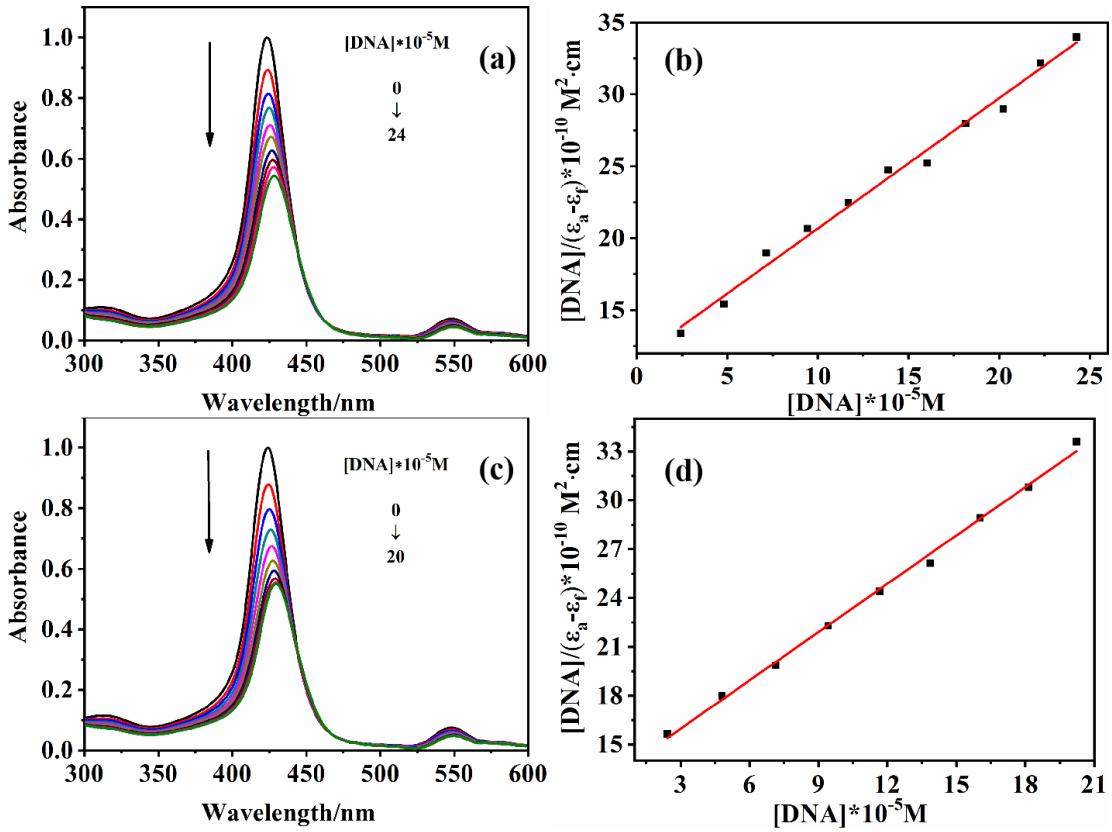


Fig. S4 Normalized absorption spectra of Cu-Por1 (a), Cu-Por2 (c) in buffer at 25℃ in the presence of increasing amounts of ct- DNA. [Cu-Porx] = 10 μM. Black arrow indicate the change in absorbance upon increasing the DNA concentration; Plot of [DNA] vs [DNA]/(ε_a - ε_f) of Cu-Por1 (b), Cu-Por2 (d).

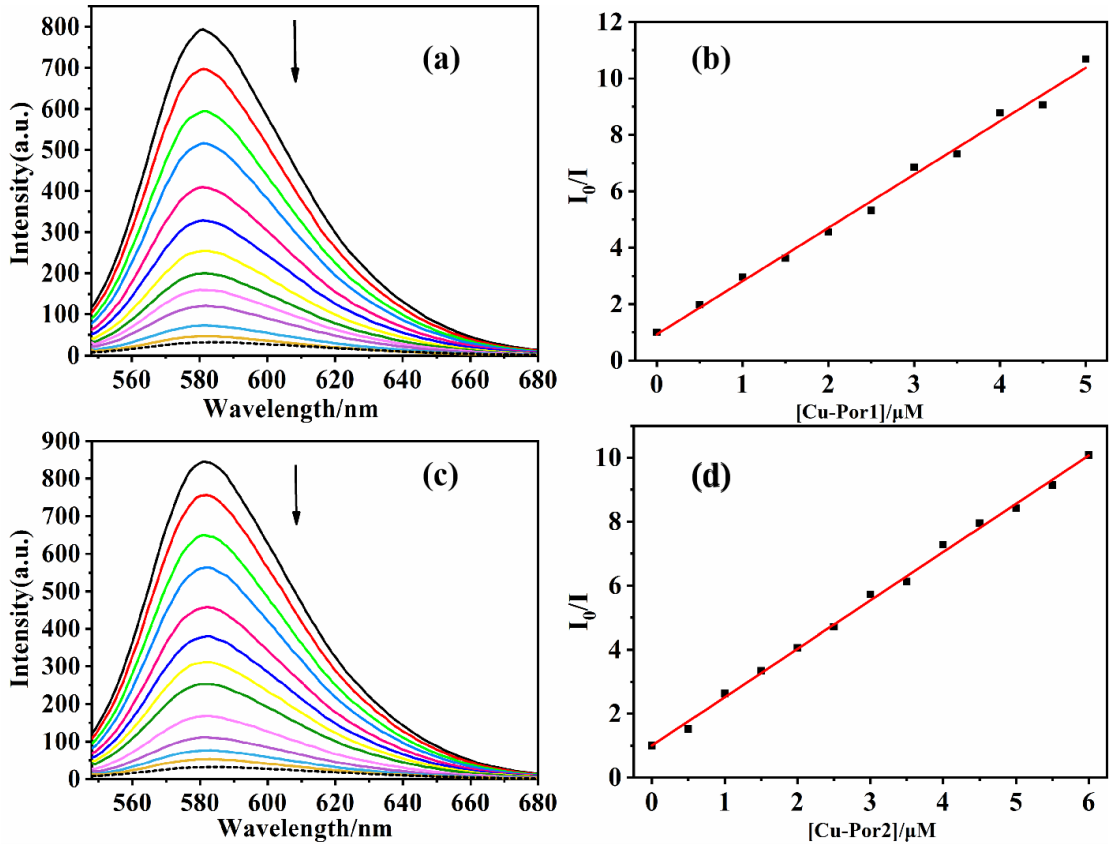


Fig. S5 Emission spectra of EtBr (black dotted line), EtBr bound to the ct-DNA (black solid line), and in the presence (other colored lines) of Cu-Por1 (a), Cu-Por2 (c) with increasing amounts 0-20 Mm. [EtBr] = 5 μM, [DNA] = 1 mM. Arrow indicates changes in the emission intensity upon addition of the Porphyrins concentration; Stern-Volmer plots of the EtBr-DNA fluorescence titration for complexes Cu-Por1 (b) and Cu-Por2 (d).

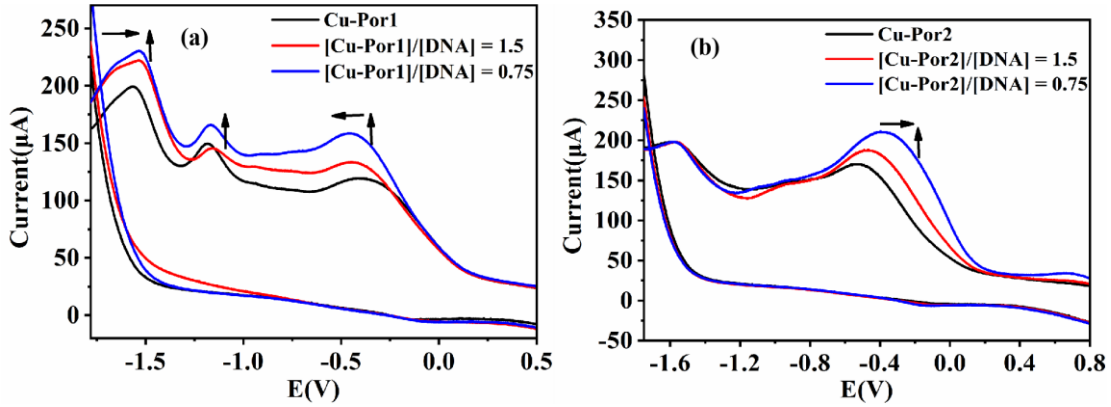


Fig. S6 Cyclic voltammogram of (a) Cu-Por1 (2×10^{-4} M), (b) Cu-Por2 (2×10^{-4} M) in the absence and presence of ct-DNA in 50 mM NaCl, 5 mM Tris, pH 7.2. [complex] / [DNA] = 0, 1.5, 0.75, scan rate, 100 mVs^{-1}.

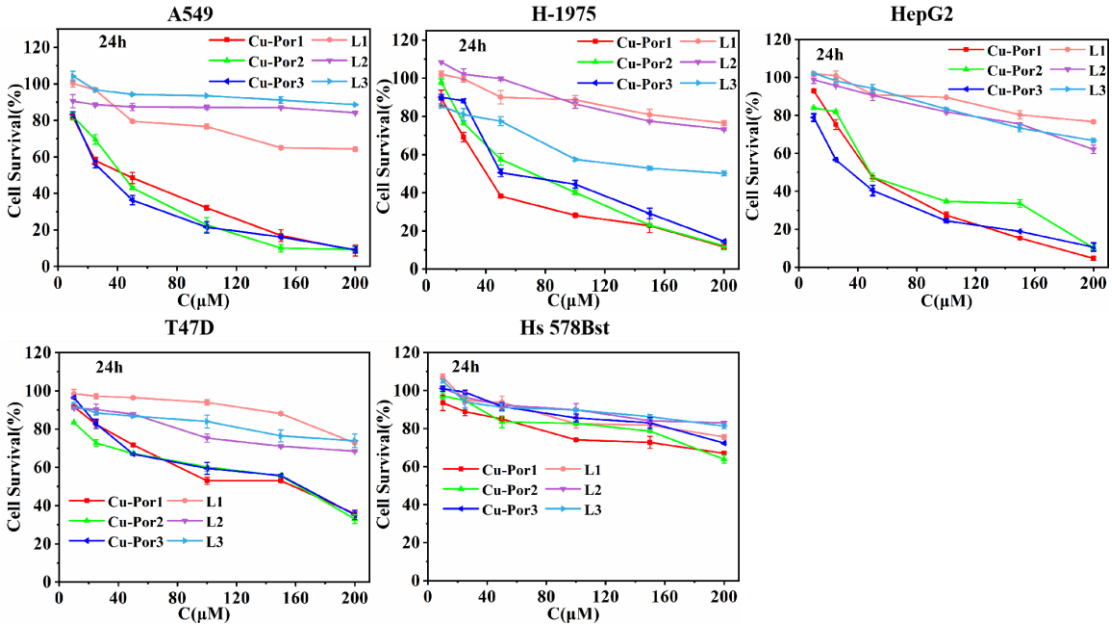


Fig. S7 The comparison of the cell survival of complexes and their ligands towards different cell lines and diverse doses for 24 h.

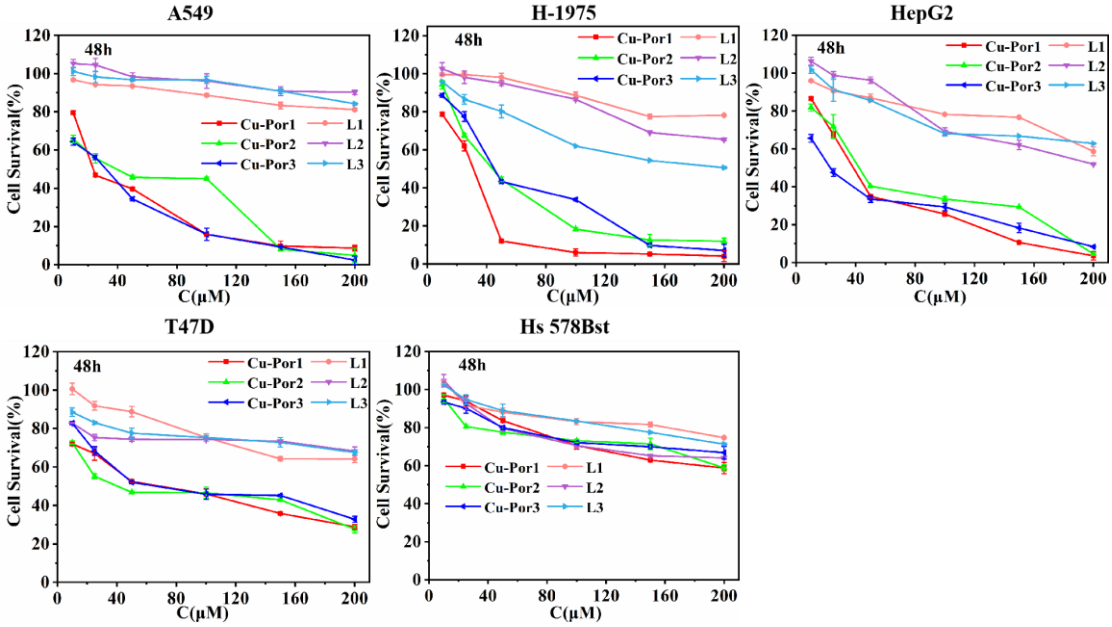


Fig. S8 The comparison of the cell survival of complexes and their ligands towards different cell lines and diverse doses for 48 h.

Table S1

IC_{50} Values of Complexes against different Cell Lines for 24 h and 48 h. The data are expressed at the mean from three independent experiments with three replicates per dose level.

Compds	IC_{50}(μM)									
	A549		H-1975		HepG2		T47D		Hs 578Bst	
	24h	48h	24h	48h	24h	48h	24h	48h	24h	48h
Cu-Por1	39.30	27.80	37.60	24.46	48.81	37.50	128.73	60.22	> 500	251.36
Cu-Por2	38.71	30.27	65.33	43.78	56.98	44.83	135.21	51.99	> 400	> 400
Cu-Por3	33.25	23.14	69.13	47.20	33.75	22.85	135.07	79.15	> 400	> 500

Table S2

IC_{50} Values of Ligands against different Cell Lines for 24 h and 48 h. The data are expressed at the mean from three independent experiments with three replicates per dose level.

Compds	IC_{50}(μM)									
	A549		H-1975		HepG2		T47D		Hs 578Bst	
	24h	48h	24h	48h	24h	48h	24h	48h	24h	48h
L1	> 300	> 500	> 500	> 300	> 500	> 400	> 500	> 300	> 500	> 500
L2	> 500	> 500	299.41	273.74	> 300	191.12	> 500	> 500	> 500	> 300
L3	> 500	> 500	194.91	185.19	> 300	> 300	> 500	> 500	> 500	> 500

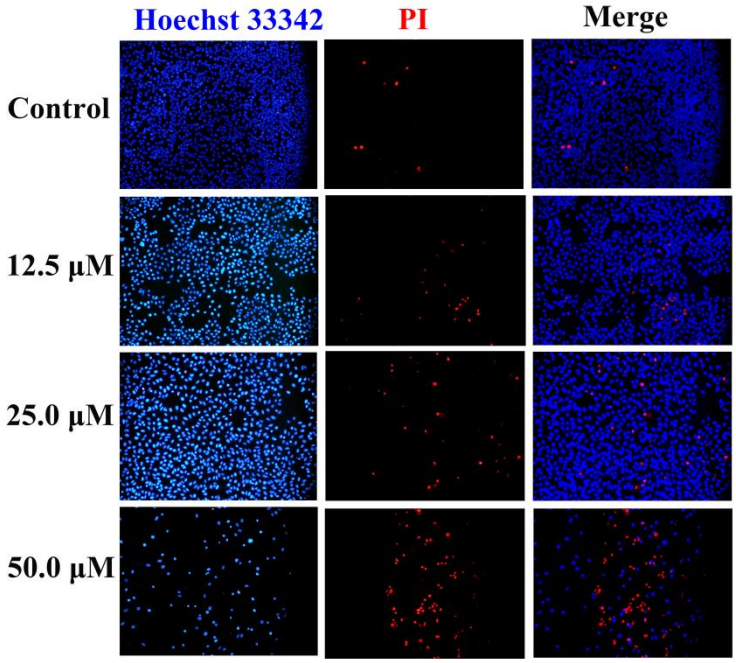


Fig. S9 Fluorescence images of H-1975 cells without treatment (control) and treated with Cu-Por1 for 24 h in different concentrations. Hoechst 33342 stained the live cells with intact plasma membrane (blue fluorescence, left panel); propidium iodide (PI) stained dead and apoptotic ones (red fluorescence, middle panel) with disrupted plasma membrane; and dying cells (pink fluorescence, right panel), magnification = 10 ×.

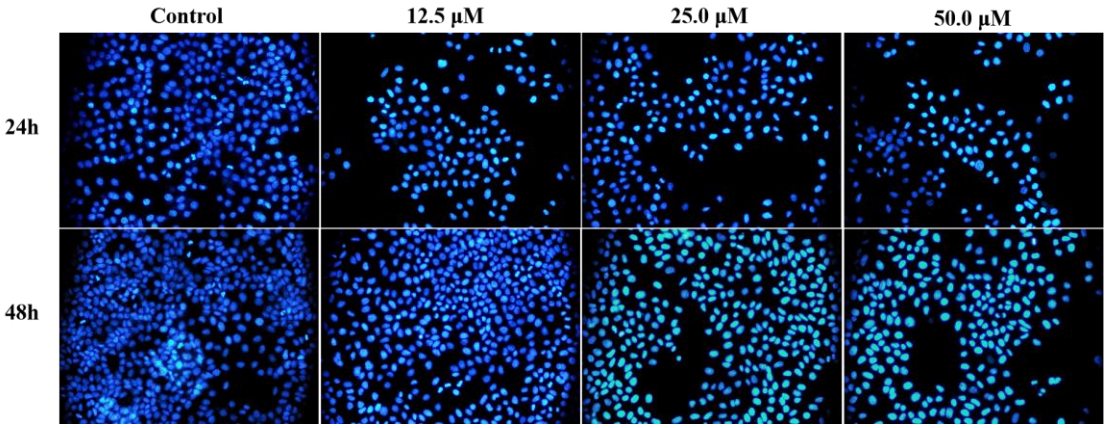


Fig. S10 Cell morphological observation for cell apoptosis induction on the HepG2 cells treated with Cu-Por3 for 24 h and 48 h, respectively, Cells were stained by Hoechst 3342 (magnification = 20×).

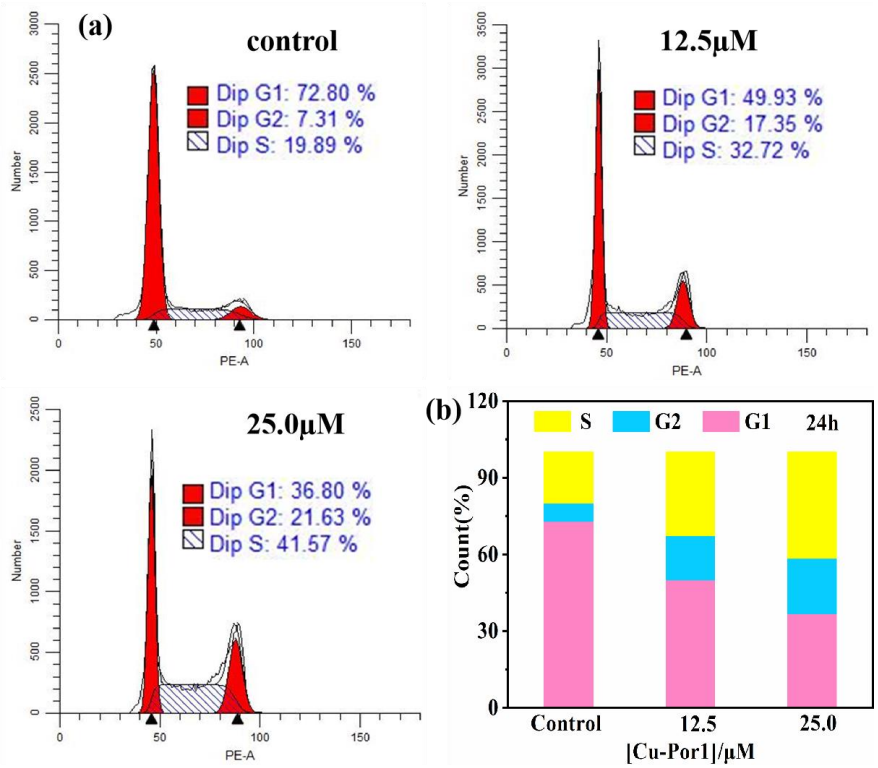


Fig. S11 (a) Effect of cell cycle of H-1975 treated with various concentrations of the Cu-Por1 compound for 24 h comparing with untreated cells. (b) Graphical representation of the cell cycle data. The percentage of cells in each phase of the cell cycle was determined at least in triplicate.

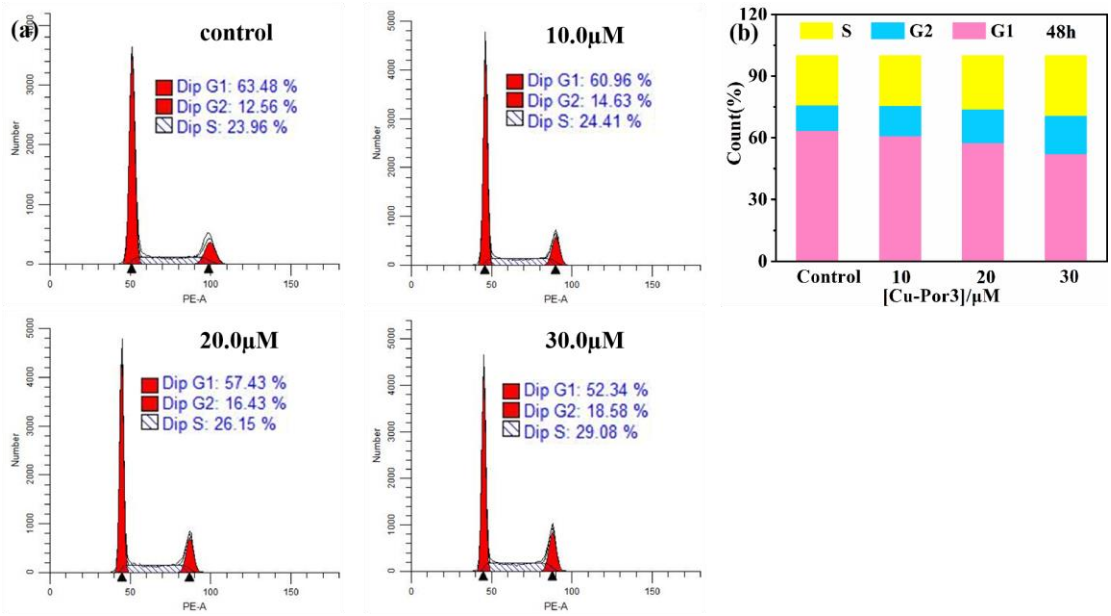


Fig. S12 (a) Effect of cell cycle of HepG2 treated with various concentrations of the Cu-Por3 compound for 48 h comparing with untreated cells. (b) Graphical representation of the cell-cycle data. The percentage of cells in each phase of the cell cycle was determined at least in triplicate.

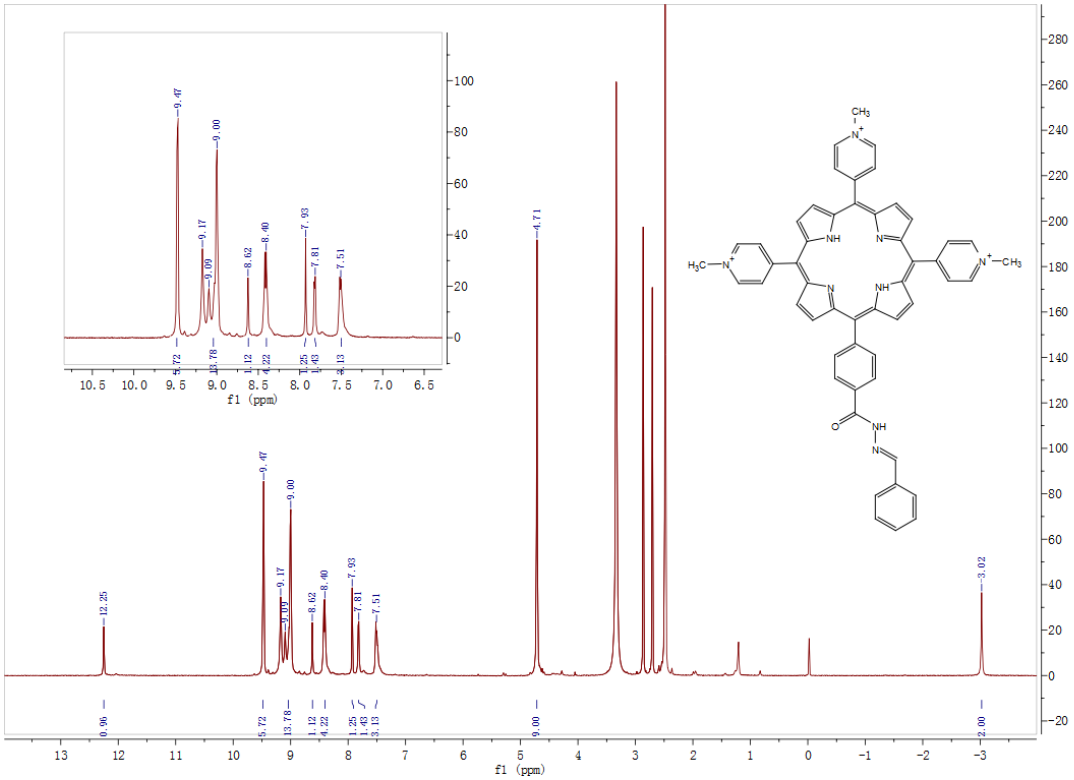


Fig. S13 ^{1}H NMR spectrum of L1 in DMSO-d_6.

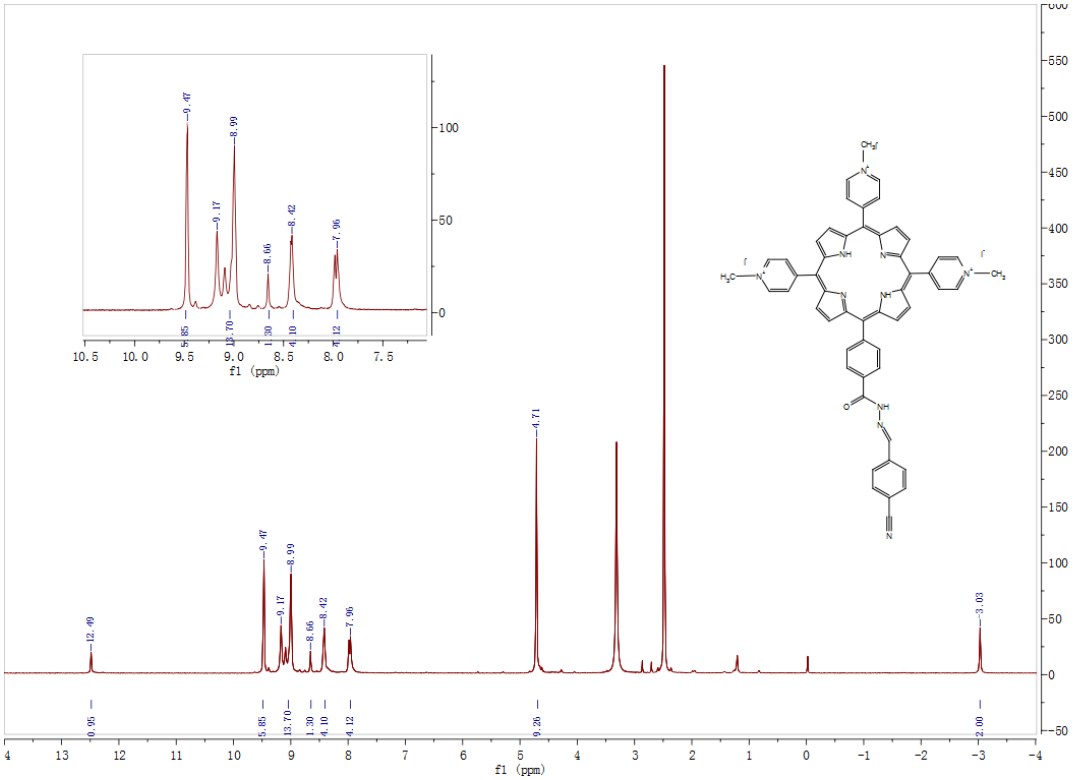


Fig. S14 ^{1}H NMR spectrum of L2 in DMSO-d_6.

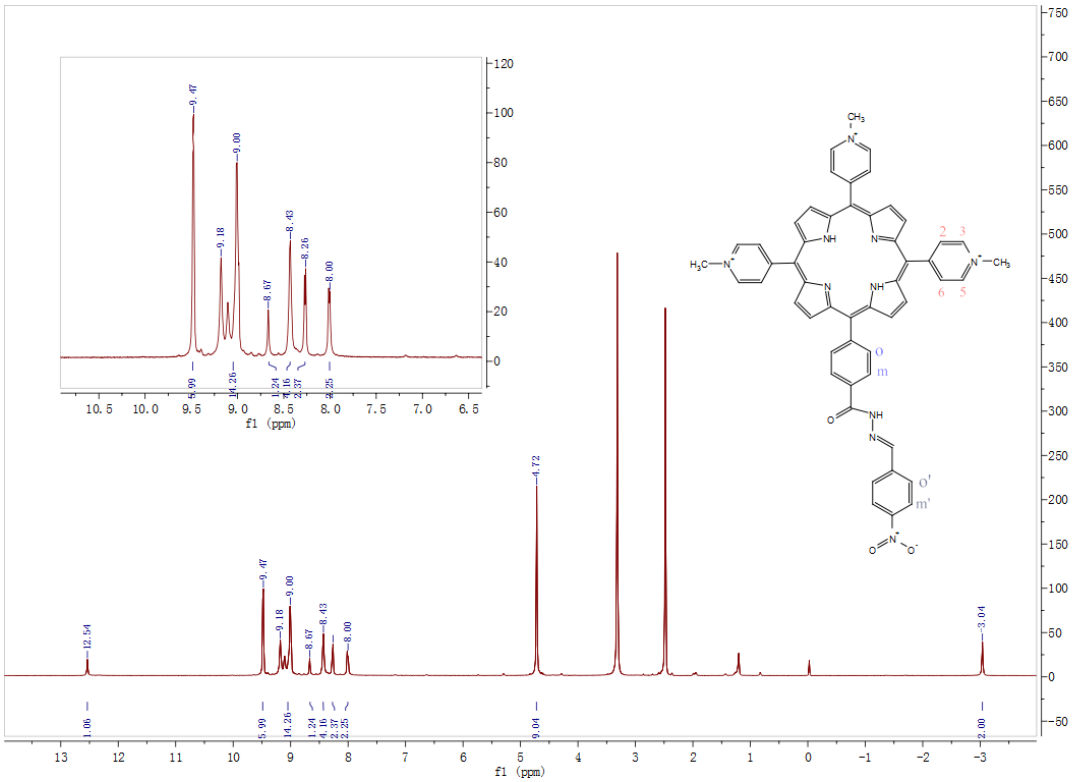


Fig. S15 ^{1}H NMR spectrum of L3 in DMSO-d_6.

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