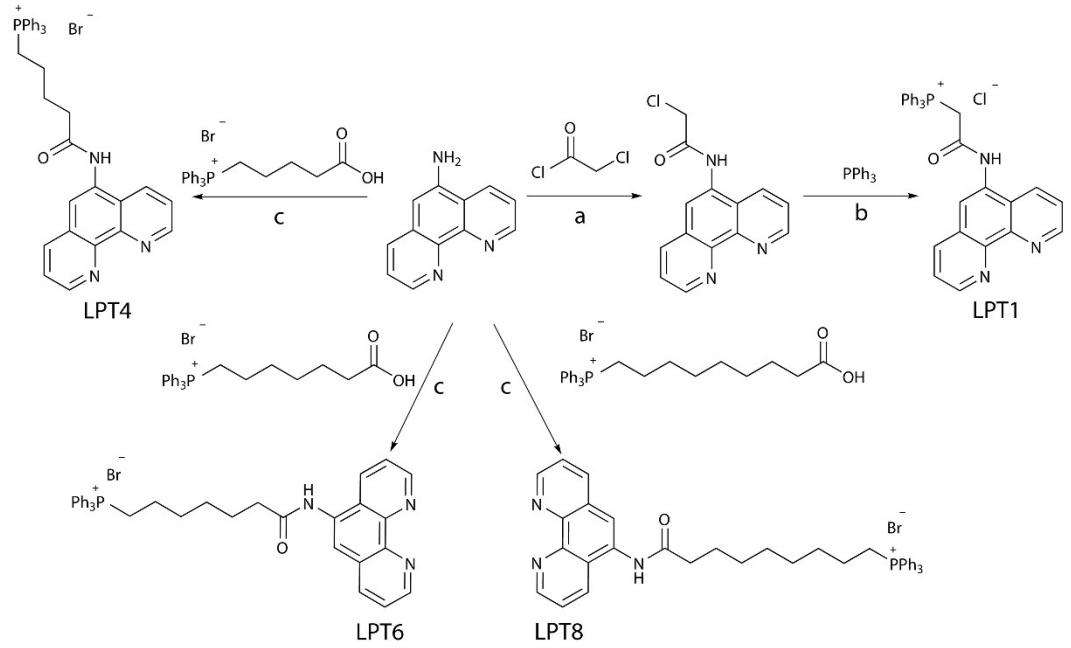
Electronic Supplementary Material (ESI) for Dalton Transactions.
This journal is © The Royal Society of Chemistry 2018

Supporting Information

Hypotoxic copper complexes with potent anti-metastatic and anti-angiogenic activities against cancer cells

Xiangchao Shi, Zhongyan Chen, Yanjun Wang, Zijian Guo* and Xiaoyong Wang*



Scheme S1 Synthetic routes to ligands. (a) Et_3N, DCM, N_2, RT; (b) K_2CO_3, CH_3CN, N_2, 90 °C; (c) EDCI, DMAP, DCM, RT.

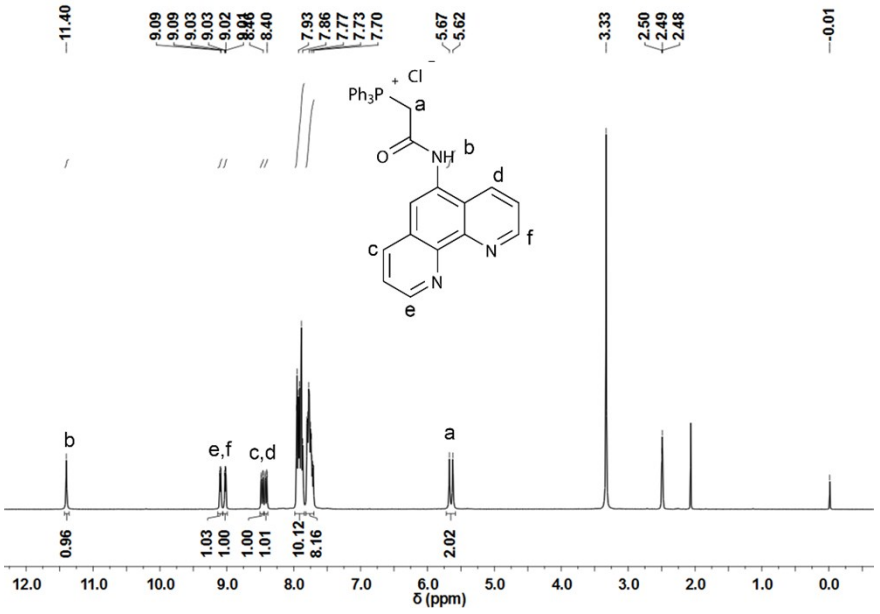


Fig. S1 ^{1}H-NMR spectrum of LPT1 in DMSO-d_6, 300 MHz.

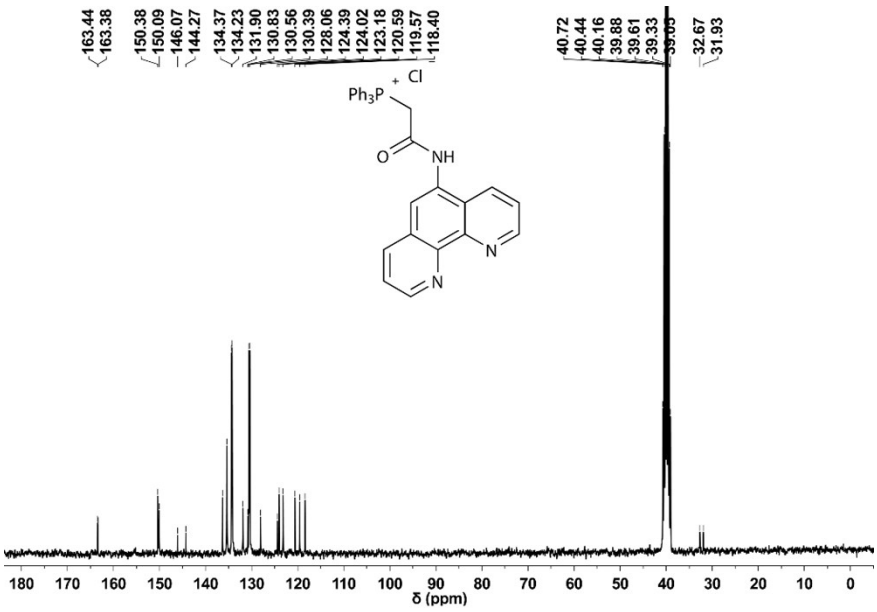


Fig. S2 ^{13}C-NMR spectrum of LPT1 in DMSO-d_6, 300 MHz.

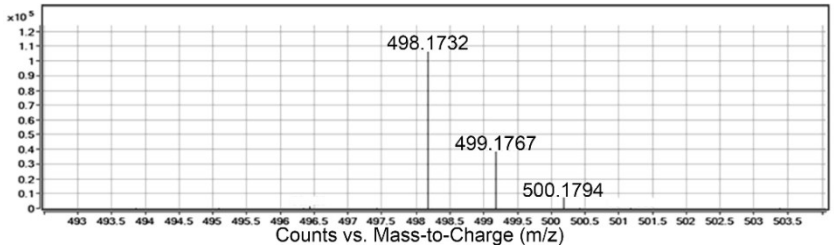


Fig. S3 High resolution ESI-MS of LPT1.

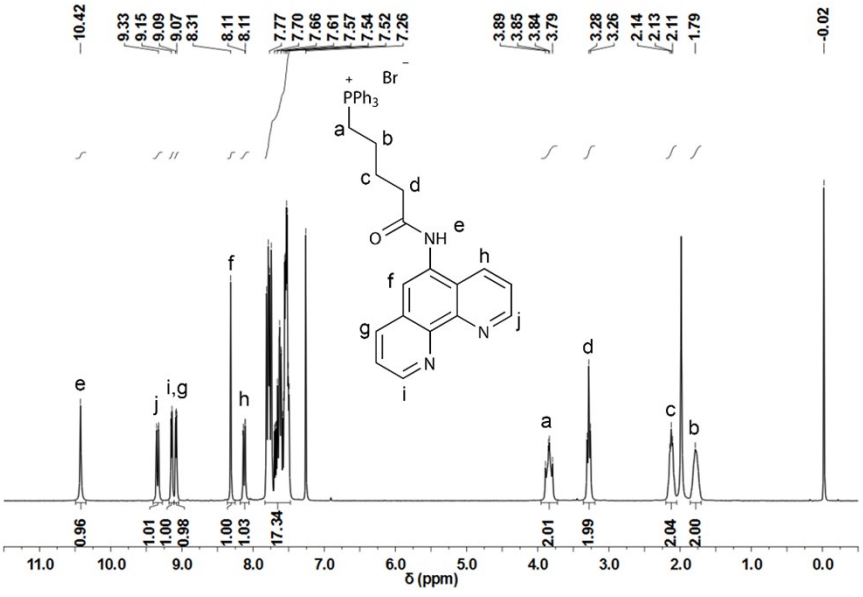


Fig. S4 ^{1}H-NMR spectrum of LPT4 in $CDCl_3$-d_6, 400 MHz.

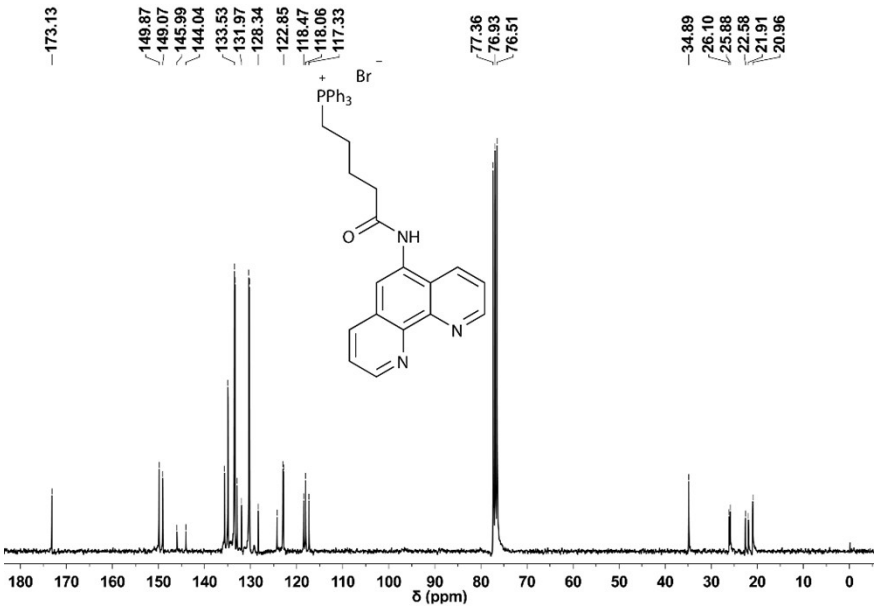


Fig. S5 ^{13}C-NMR spectrum of LPT4 in $CDCl_3$-d_6, 400 MHz.

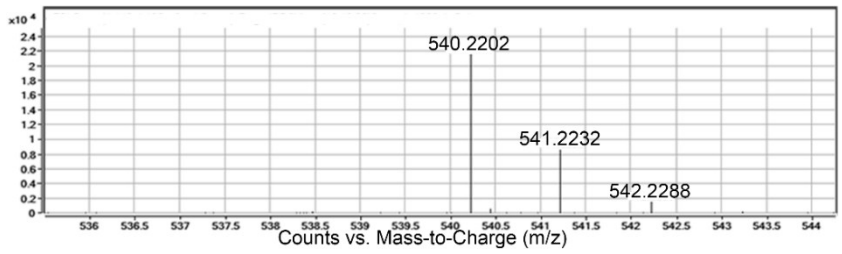


Fig. S6 High resolution ESI-MS of LPT4.

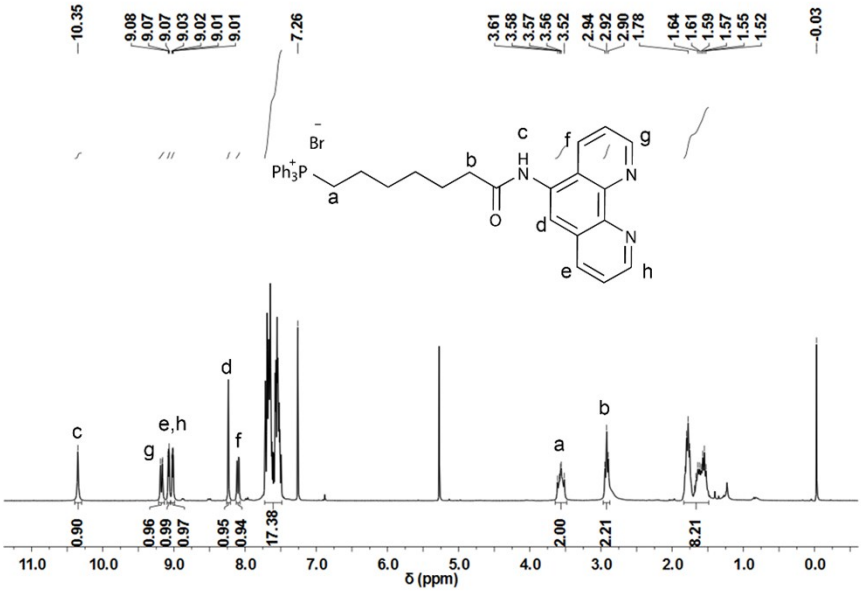


Fig. S7 ^{1}H-NMR spectrum of LPT6 in $CDCl_3$-d_6, 400 MHz.

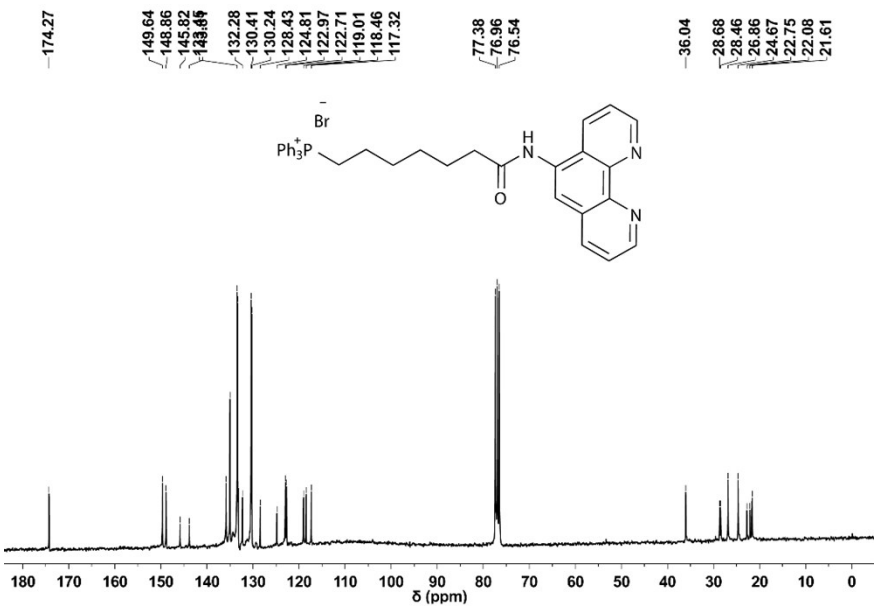


Fig. S8 ^{13}C-NMR spectrum of LPT6 in $CDCl_3$-d_6, 400 MHz.

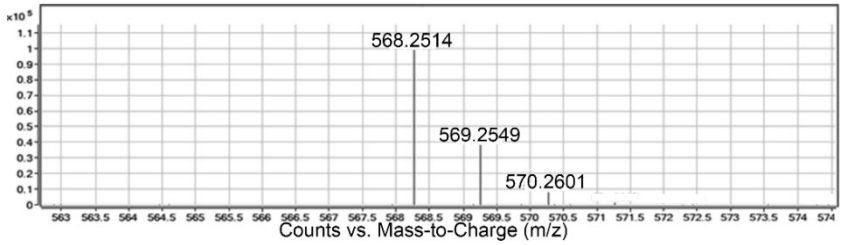


Fig. S9 High resolution ESI-MS of LPT6.

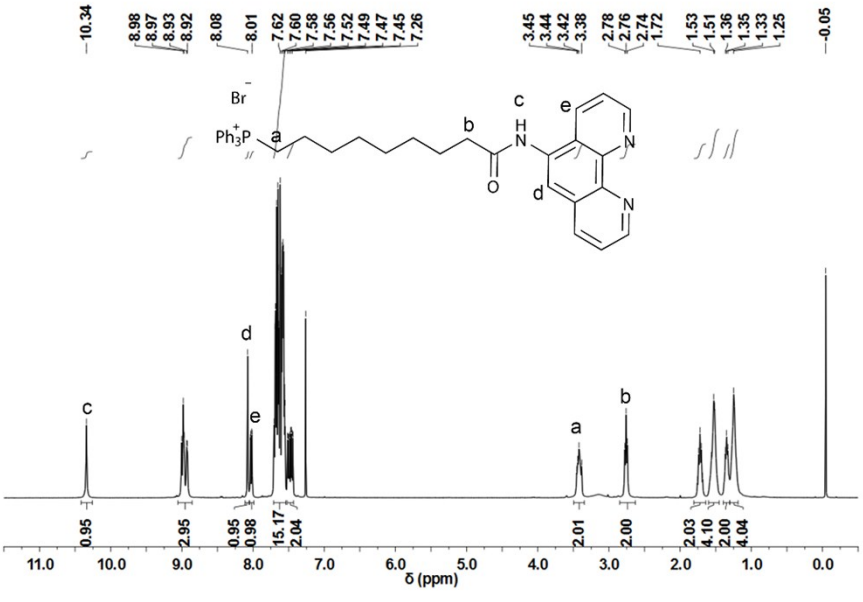


Fig. S10 ^{1}H-NMR spectrum of LPT8 in $CDCl_3$-d_6, 400 MHz.

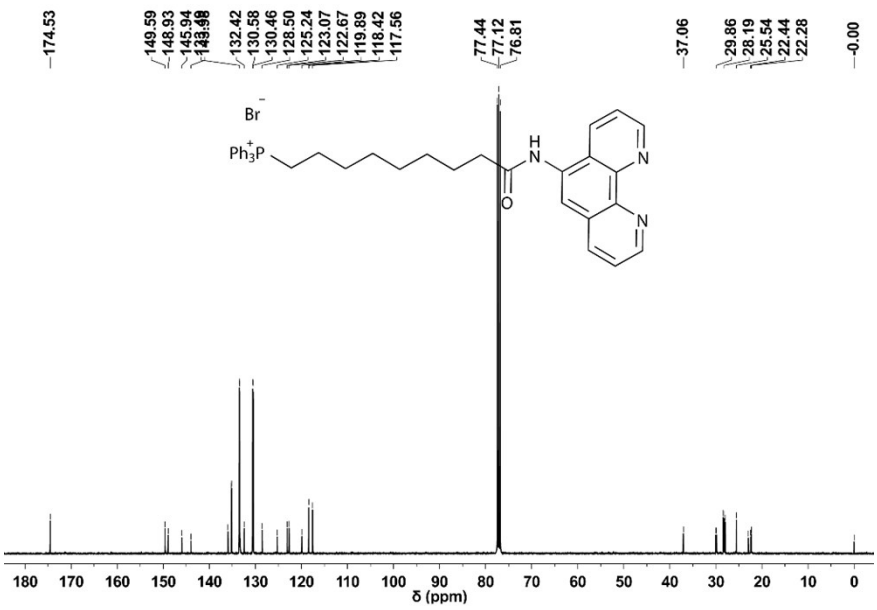


Fig. S11 ^{13}C-NMR spectrum of LPT8 in $CDCl_3$-d_6, 400 MHz.

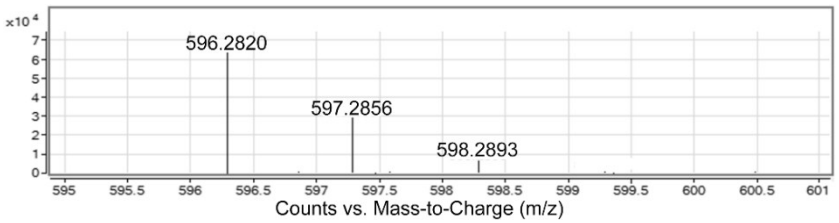


Fig. S12 High resolution ESI-MS of LPT8.

Table S1 Crystallographic data and structure refinement parameters of CPT1.

Formula	$C_{64}H_{50}Cl_2Cu_2N_{10}O_{14}P_2$
Formula weight	1443.06
Temperature/K	296(2)
Crystal system	Triclinic
Space group	P1
a/Å	9.4397(14)
b/Å	10.9574(17)
c/Å	15.288(2)
α/°	97.779(2)
β/°	104.651(2)
γ/°	91.253(2)
Volume/Å^3	1513.3(4)
Z	1
F(000)	738
Dx/g cm^{-3}	1.584
Goodness-of-fit on F^2	1.079
R_1, wR_2	0.0509, 0.1625

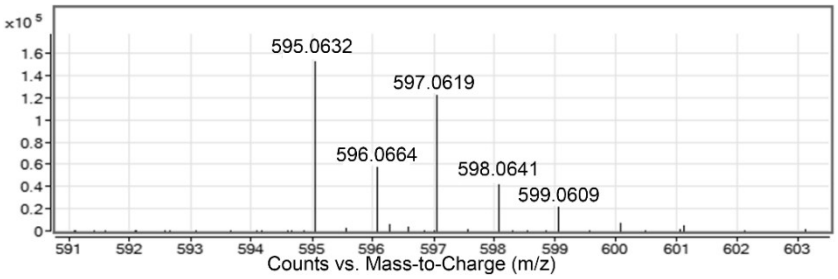


Fig. S13 High resolution ESI mass spectrum of CPT1.

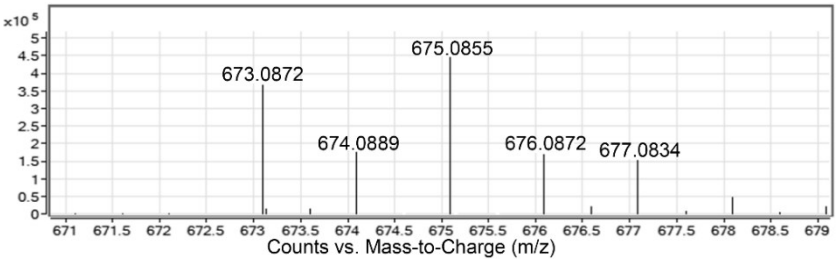


Fig. S14 High resolution ESI mass spectrum of CPT4.

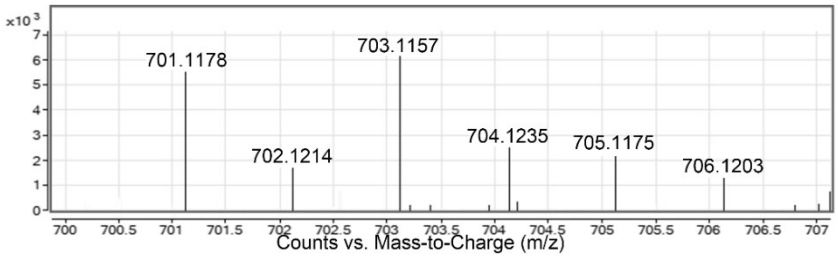


Fig. S15 High resolution ESI mass spectrum of CPT6.

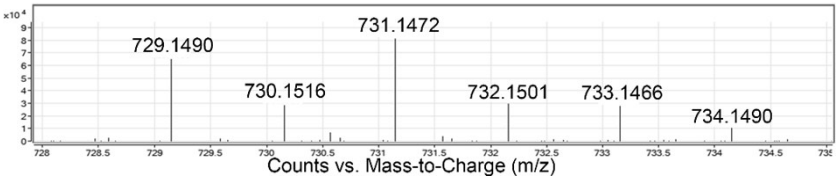


Fig. S16 High resolution ESI mass spectrum of CPT8.

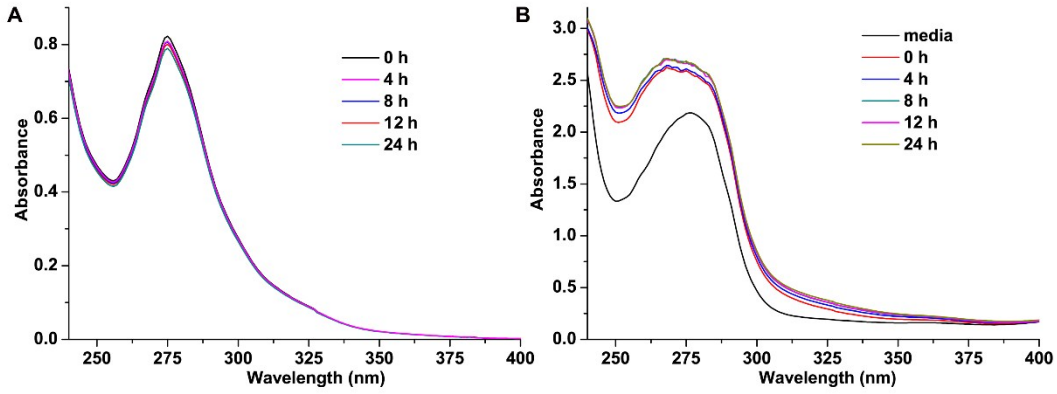


Fig. S17 UV-Vis spectra of CPT8 (30 μM) in PBS over 24 h at 37 °C (A), and those of CPT8 (30 μM) in 1640 cell culture media (containing 10% FBS) over 24 h at 37 °C (B).

Table S2 IC_{50} values (μM) of ligands against different cell lines at 24 h.

Ligands	HeLa	SKOV-3	HK-2
LPT1	＞100	＞100	＞100
LPT4	＞100	86.43 ± 2.56	＞100
LPT6	＞100	51.20 ± 2.34	＞100
LPT8	58.95 ± 2.87	24.07 ± 1.47	69.45 ± 2.14
CPT1	87.42 ± 3.46	74.25 ± 3.54	> 100
CPT4	58.63 ± 1.87	45.69 ± 1.59	> 100
CPT6	27.50 ± 0.98	23.93 ± 2.17	65.20 ± 3.58
CPT8	12.73 ± 1.76	13.50 ± 1.59	38.20 ± 2.25

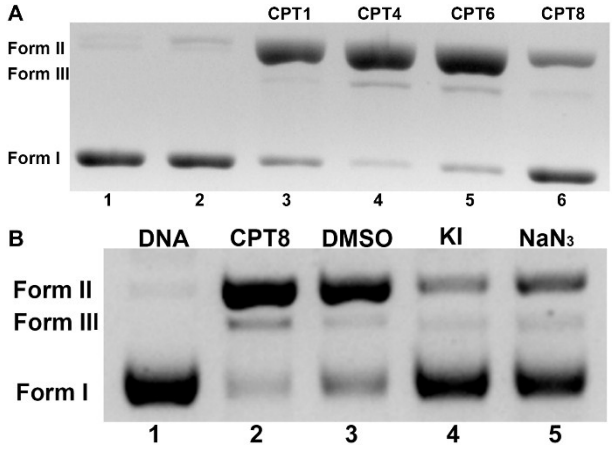


Fig. S18 (A) Cleavage patterns of pUC19 DNA (0.2 μg) by CPTn (5 μM) in the agarose gel electrophoresis in the presence of ascorbic acid (0.1 mM) in buffer (50 mM Tris–HCl/50 mM NaCl, pH 7.4) after reaction at 37 °C for 90 min. Lane 1: DNA; Lane 2: DNA+Vc; Lane 3: DNA+CPT1+Vc; Lane 4: DNA+CPT4+Vc; Lane 5: DNA+CPT6+Vc; Lane 6: DNA+CPT8+Vc. (B) Cleavage patterns of pUC19 DNA (0.2 μg) by CPT8 (10 μM) in the agarose gel electrophoresis in the presence of ascorbic acid (0.1 mM) after incubation without or with different ROS scavengers (10% DMSO, 10 mM KI, 10 mM NaN_3) at 37 °C for 90 min. Lane 1: DNA; Lane 2: DNA+CPT8+Vc; Lane 3: DNA+CPT8+Vc+DMSO; Lane 4: DNA+CPT8+Vc+KI; Lane 5: DNA+CPT8+Vc+NaN_3. DMSO: hydroxyl radical scavenger; KI: hydrogen peroxide scavenger; NaN3: singlet oxygen scavenger.[1]

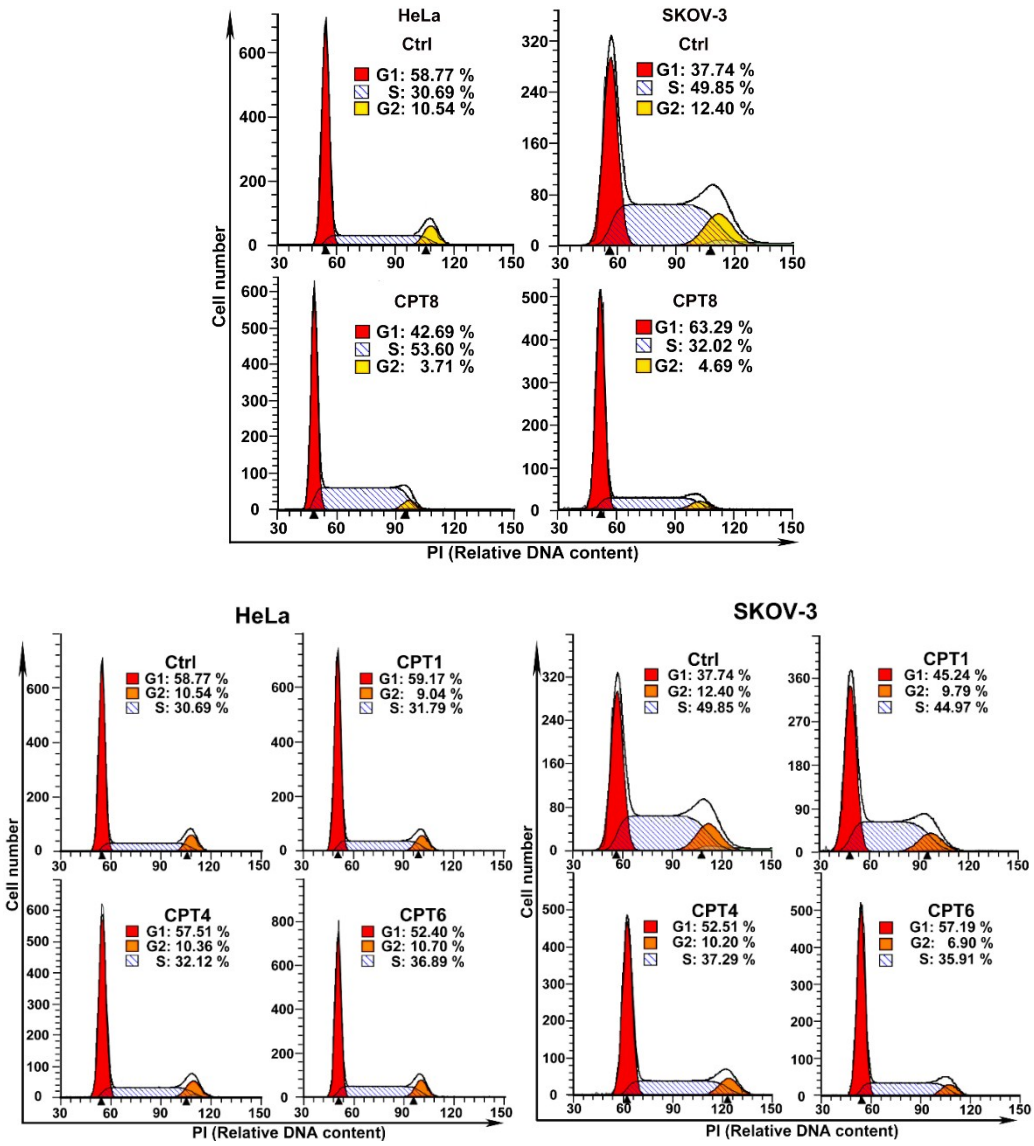


Fig. S19 Cell cycle analysis of HeLa and SKOV-3 cells after treatment with CPTn (5 μM) for 24 h.

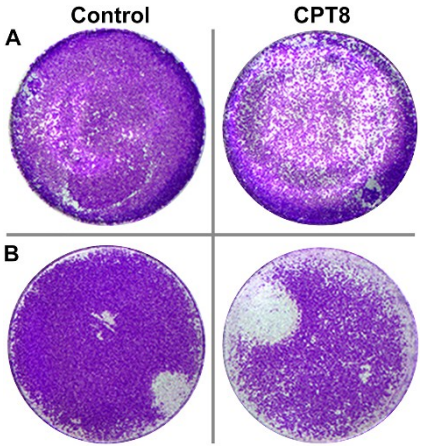


Fig. S20 Migratory (A) and invasive (B) ability of SKOV-3 cells after treatment with CPT8 (5 μM) for 24 h.

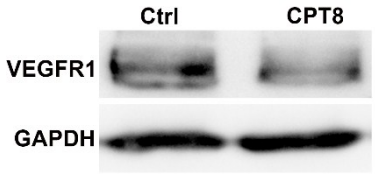


Fig. S21 VEGFR1 expression in HUV-EC-C treated with CPT8 (5 μM) for 24 h.

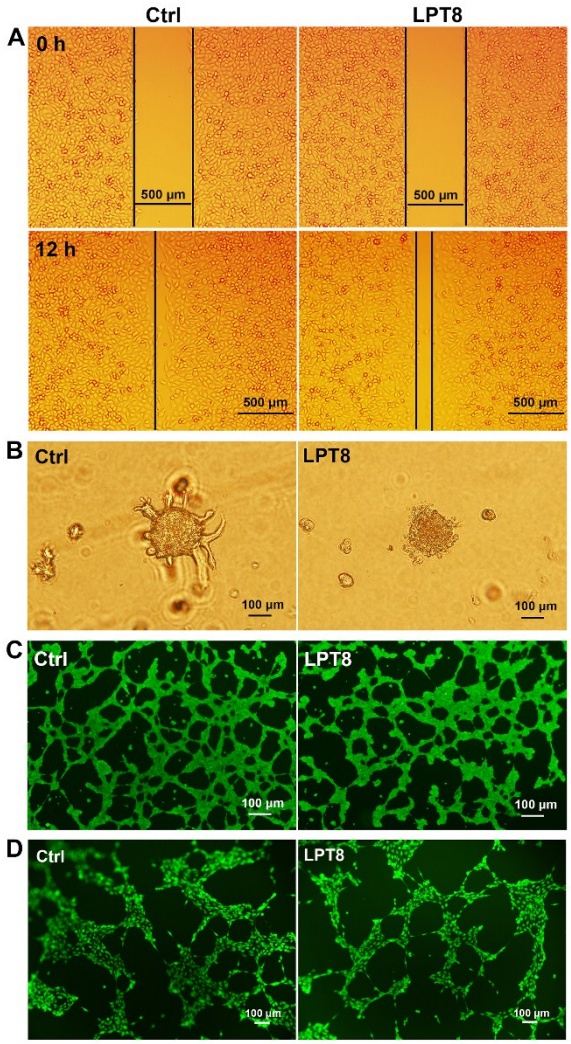


Fig. S22 Images of wound-healing of SKOV-3 cells after treatment with LPT8 (5 μM) for 0 and 12 h (A), HUV-EC-C spheroids after incubation with LPT8 (5 μM) for 3 days (B), tube formation of HUV-EC-C after incubation with LPT8 (5 μM) for 12 h (C), and vascular channel formation by melanoma B16F10 cells after incubation with LPT8 (5 μM) for 12 h (D).

Experimental Procedures

Materials and Measurements

All chemicals were received and used without further purification unless otherwise noted. 1,10-phenanthrolin-5-amine, 2-chloroacetyl chloride, triphenylphosphine and (4-carboxybutyl)triphenylphosphonium bromide, 1-Ethyl-3-(3-dimethylaminopropyl)-

carbodiimide (EDCI), 4-dimethylaminopyridine (DMAP) were purchased from Energy Chemical. 2-chloro-N-(1,10-phenanthrolin-5-yl)acetamide was prepared according to the literature.[2] 7-bromoheptanoic acid and 9-bromononanoic acid were purchased from Innochem. (6-carboxyhexyl)triphenylphosphonium bromide and (8-carboxyoctyl)triphenylphosphonium bromide were prepared according to the literatures.[3]

^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker Avance DRX-400 with TMS as the internal reference. High resolution mass spectrometric data were determined using an Agilent 6540Q-TOF HPLC-MS spectrometer. The isotopic distribution patterns of the observed species were simulated using the Isopro 3.0 program. X-ray crystallographic data were collected on a Bruker SMART APEX CCD area-detector diffractometer operating in the φ-ω scan mode with graphite-monochromated Mo-Ka radiation (λ = 0.71073 Å) at 298 K. The SMART software was used for data acquisition and the SAINT software for data extraction.[4] The inductively coupled plasma mass spectrometry (ICP-MS) data were obtained on ELAN9000 ICP-MS (PerkinElmer). The images of wound healing, tube formation, sprouting and vasculogenic mimicry assay were taken by OLYMPUS IX71 inverted microscope (external power TH4-200; fluorescent source X-120Q). Distilled water was purified by passage through a Millipore water purification system (18.2 MΩ).

Synthesis of Ligands

LPT1 (2-((1,10-phenanthrolin-5-yl)amino)-2-oxoethyl)triphenylphosphonium chloride.

2-Chloro-N-1,10-phenanthrolin-5-yl-acetamide (1.0 g, 3.69 mmol), triphenylphosphine (1.06 g, 4.06 mmol), and K_2CO_3 (1.02 g, 7.38 mmol) were refluxed in 100 mL dry acetonitrile for 10 h under N_2 atmosphere. The solution was filtered and then evaporated under reduced pressure. The light yellow solid was purified by silica column chromatography using gradient elution of CH_2Cl_2/MeOH (0 to 10%). Yield: 0.93 g (46%). ^{1}H NMR (300 MHz, DMSO-d_6) δ: 11.40 (s, 1H), 9.10 (dd, *J* = 4.2, 1.4 Hz, 1H), 9.02 (dd, *J* = 4.2, 1.6 Hz, 1H), 8.48 (dd, *J* = 8.4, 1.4 Hz, 1H), 8.42 (dd, *J* = 8.4, 1.6 Hz, 1H), 7.96-7.86 (m, 10H), 7.80-7.70 (m, 8H), 5.65 (d, *J* = 15.2 Hz, 2H). ^{13}C NMR (75 MHz, DMSO-d_6), 163.44, 163.38, 150.38, 150.09, 146.07, 144.27, 136.31, 135.37, 134.37, 134.23, 131.90, 130.83, 130.56, 130.39, 128.06, 124.39, 124.02, 123.18, 120.59, 119.57, 118.40, 32.67, 31.93. HRMS m/z Calcd for $[LPT1–Cl]^+$ ($C_{32}H_{25}N_3OP^+$) 498.1730, Found 498.1732.

LPT4 (5-((1,10-phenanthrolin-5-yl)amino)-5-oxopentyl)triphenylphosphonium bromide.

1,10-Phenanthrolin-5-amine (0.5 g, 2.56 mmol) was dissolved in 100 mL distilled DCM before the solution was cooled to 0 °C. (4-carboxybutyl)triphenylphosphonium bromide (0.6 g, 2.56 mmol), 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI) (1.23 g, 6.4 mmol) and 4-dimethylaminopyridine (DMAP) (0.31 g, 2.56 mmol) were added to the solution sequentially before the reaction mixture was stirred at 0 °C for 1 h. The mixture was allowed to reach room temperature before stirring for further 24 h. The solvent was removed under reduced pressure. The resulting orange oil was purified by silica gel column chromatography using gradient elution of CH_2Cl_2/MeOH (0 to 10%). Yield: 0.48 g (44%). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 10.42 (s, 1H), 9.34 (dd, *J* = 8.4, 1.3 Hz, 1H), 9.14 (dd, *J* = 4.3, 1.4 Hz, 1H), 9.08 (dd, *J* = 4.3, 1.6 Hz, 1H), 8.31 (s, 1H), 8.12 (dd, *J* = 8.1, 1.6 Hz, 1H), 7.81-7.50 (m, 17H), 3.89-3.79 (m, 2H), 3.28 (t, *J* = 6.9 Hz, 2H), 2.16-2.07 (m, 2H), 1.83-2.73 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$), 173.13, 149.87, 149.07, 145.99, 144.04, 135.65, 134.94, 133.53, 133.40, 132.95, 131.97, 130.40, 130.23, 128.34, 124.26, 123.00, 122.85, 118.47, 118.06, 117.33, 34.89, 26.10, 25.88, 22.58,

21.91, 20.96. HRMS m/z Calcd for $[LPT4–Br]^+$ ($C_{35}H_{31}N_3OP^+$) 540.2199, Found 540.2202.

LPT6 (7-((1,10-phenanthrolin-5-yl)amino)-7-oxoheptyl)triphenylphosphonium bromide.

LPT6 was synthesized using the similar procedure for LPT4 except (4-carboxybutyl)triphenylphosphonium bromide was replaced with (6-carboxyhexyl)triphenylphosphonium bromide. Yield: 0.45 g (40%). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 10.35 (s, 1H), 9.19 (dd, *J* =8.4, 1.1 Hz, 1H), 9.08 (d, *J* =4.3, 1.4 Hz, 1H), 9.02 (dd, *J* =4.3, 1.6 Hz, 1H), 8.24 (s, 1H), 8.10 (dd, *J* =8.1, 1.6 Hz, 1H), 7.65-7.49 (m, 17H), 3.61-3.52(m, 2H), 2.92 (t, *J* =7.1 Hz, 2H), 1.82-1.52 (m, 8H). ^{13}C NMR (100 MHz, $CDCl_3$), 174.27, 149.64, 148.86, 145.82, 143.81, 135.82, 135.00, 133.45, 133.32, 133.21, 132.28, 130.41, 130.24, 128.43, 124.81, 122.97, 122.71, 119.01, 118.46, 117.32, 36.04, 28.68, 28.46, 26.86, 24.67, 22.75, 22.08, 21.61. HRMS m/z Calcd for $[LPT6–Br]^+$ ($C_{37}H_{35}N_3OP^+$) 568.2512, Found 568.2514.

LPT8 (9-((1,10-phenanthrolin-5-yl)amino)-9-oxononyl)triphenylphosphonium bromide.

LPT8 was synthesized using the similar procedure for LPT4 except (4-carboxybutyl)triphenylphosphonium bromide was replaced with (8-carboxyhexyl)triphenylphosphonium bromide. Yield: 0.42 g (37%). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 10.34 (s, 1H), 9.00-8.92 (m, 3H), 8.08 (s, 1H), 8.03 (dd, *J* =8.1, 1.3 Hz, 1H), 7.71-7.55 (m, 15H), 7.52-7.44 (m, 2H), 3.45-3.38(m, 2H), 2.76 (t, *J* =7.4 Hz, 2H), 1.76-1.68 (m, 2H), 1.56-1.54 (m, 4H), 1.36-1.33 (m, 2H), 1.28-1.20 (m, 4H). ^{13}C NMR (100 MHz, $CDCl_3$)174.53, 149.59, 148.93, 145.94, 143.98, 135.93, 135.18, 135.15, 133.49, 133.39, 132.42, 130.58, 130.46, 128.50, 125.24, 123.07, 122.67, 119.89, 118.42, 117.56, 37.06, 30.01, 29.86, 28.40, 28.19, 27.99, 25.54, 22.94, 22.44, 22.32, 22.28. HRMS m/z Calcd for $[LPT8–Br]^+$ ($C_{39}H_{39}N_3OP^+$) 596.2825, Found 596.2820.

Synthesis of Copper(II) Complexes

CPT1 was prepared by reacting $Cu(NO_3)_2{\cdot}3H_2O$ (0.12 mmol, 28 mg) with LPT1 (0.1 mmol, 53 mg) in 10 mL CH_3CN under stirring for 4 h at room temperature. The formed solid was isolated, washed with cold CH_3OH and CH_3CN, dried in vaccuo. HRMS m/z Calcd for $[CPT1–2NO_3–H]^+$ ($C_{32}H_{24}ClCuN_3OP^+$) 595.0642, Found 595.0632.

CPT4 was prepared by reacting $CuCl_2{\cdot}2H_2O$ (0.12 mmol, 20 mg) with LPT4 (0.1 mmol, 62 mg) in 10 mL CH_3CN under stirring for 4 h at room temperature. The solid formed was isolated, washed with cold CH_3OH and CH_3CN, dried in vaccuo. HRMS m/z Calcd for $[CPT4–Br]^+$ ($C_{35}H_{31}Cl_2CuN_3OP^+$) 673.0872, Found 673.0872.

CPT6 and CPT8 were prepared under the similar procedure for CPT4, except the ligand was replaced with LPT6 and LPT8. HRMS m/z Calcd for $[CPT6–Br]^+$ ($C_{37}H_{35}Cl_2CuN_3OP^+$) 701.1185, Found 701.1178. HRMS m/z Calcd for $[CPT8–Br]^+$ ($C_{39}H_{39}Cl_2CuN_3OP^+$) 729.1498, Found 729.1490.

Cell Lines Culture

HeLa, SKOV-3, HK-2 and B16F10 cells were obtained from the American type culture collection. HeLa and HK-2 cells were cultured respectively in MEM and DMEM/F12 (KeyGEN BioTECH); SKOV-3 and B16F10 cells were cultured in RPMI-1640 (KeyGEN BioTECH) with 10% fetal bovine serum (Hyclone) at 37 ^{o}C in a humidified incubator with 5% CO_2. HUV-EC-C were purchased from KeyGEN BioTECH was grown in F-12K (Gibco) medium supplemented with heparin (0.1 mg/mL), endothelial cell growth supplement

(0.03–0.05 mg/mL), 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin (Gibco) at 37 ^{o}C in a humidified incubator with 5% CO_2.

Cell Cytotoxicity Assay

HeLa, SKOV-3, HK-2 and HUV-EC-C cells (5000 cells/well) were seeded on a 96-well plate (Corning) in 100 μL of desired medium and incubated at 37 ^{o}C in humidified incubator with 5% CO_2 over night. The cells were treated with compounds at various concentrations. At the required time point, 20 μL 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT, 5 mg/mL in PBS) (KeyGEN BioTECH) was added into each well and incubated for 4 h at 37 ^{o}C. After that, the medium in each well was replaced with 200 μL DMSO for solubilization of formazan. The plate was shaken gently for homogeneous mixture of the solution. The absorbance of solution in each well was measured at 570 nm by microplate reader (Thermo Scientific Varioskan Flash). Each concentration was performed in triplicate. Cytotoxicity data was fitted to a sigmoidal curve. IC_{50} values were interpolated from the dose dependent curves. The reported IC_{50} values are the average from at three independent experiments.

Cellular Copper Uptake Assay

HeLa and SKOV-3 cells were seeded in 6-well plate (Corning) at a density of 10^5 cells/mL and incubated overnight to 60–70% confluence under standard growth condition. The culture media for the cells were replaced with fresh growth media. The cells were treated with CPTn (5 μM) for 24 h. After incubation, the cells were harvested with 0.25% trypsin (Gibco), washed with PBS, and digested by concentrated nitric acid (100 μL) at 95 ^{o}C for 2 h, hydrogen peroxide (30%, 50 μL) at 95 ^{o}C for 1.5 h and concentrated hydrochloric acid (50 μL) at 37 ^{o}C until total volume is less than 50 μL. Distilled water was added to each sample to adjust the solution volume to 1 mL. All samples were tested on ICP-MS to detect the copper content. The average of three parallel experimental data was reported as the final result.

Measurement of Lipophilicity

Log P values were obtained using the shake-flask method and UV spectroscopy. Octanol used in this experiment was pre-saturated with water. An aqueous solution of CPTn (50 μM, 500 μL) was incubated with the pre-saturated octanol (500 μL) in a 2 mL tube. The tube was shaken at room temperature for 24 h. The two phases were separated by centrifugation (Eppendorf 5415) and the content of CPTn in each phase was determined by UV spectroscopy (λ_{max}= 275 nm) (Thermo Scientific Varioskan Flash). The octanol/water partition coefficient $P_{o/w}$ ($P_{o/w} = C_o/C_w = A_o/A_w$, A stands for absorbance) were calculated. The average of three parallel experimental data was reported as the final result.

DNA Cleavage Assay

Plasmid DNA (pUC19) was purchased from Invitrogen. To probe the effect of CPTn on cleavage, the solution containing DNA (0.2 μg), CPTn (10 μM) and ascorbic acid (0.1 mM) with a total reaction volume of 10 μL were incubated at 37 ^{o}C for 90 min in buffer (50 mM Tris-HCl/50 mM NaCl buffer, pH 7.4). To determine the cleavage mechanism, solution containing DNA (0.2 μg), CPTn (10 μM), ascorbic acid (0.1 mM) and various radical scavengers (10% DMSO, 10 mM KI, 10 mM NaN_3) with a total reaction volume of 10 μL was

incubated at 37 °C for 90 min. After incubation, 10× loading buffer (1.1 μL) (Invitrogen) was added and the reaction mixtures were immediately loaded onto a 1% agarose gel. Electrophoresis was completed at 100 V for 1 h in 1× TAE buffer and the gel was visualized by ethidium bromide staining on Bio-Rad Gel-Doc XR imaging system.

Cellular ROS Determination

The production of cellular ROS was measured using the fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) (KeyGEN BioTECH). HeLa and SKOV-3 cells were seeded in 6-well plate (Corning) at a density of 10^5 cells/mL and incubated overnight to 60–70% confluence. After 24 h incubation with CPTn (5 μM), cancer cells were washed and incubated with 1 μM probe (diluted with incomplete growth medium) at 37 °C for 30 min in situ. Cancer cells were harvested with 0.25% trypsin (Gibco), washed with PBS and then resuspended in 500 μL PBS. Analysis was performed via BD FACSCalibur flow cytometry within 1 h.

Cell Cycle Analysis

HeLa and SKOV-3 cells were seeded in 6-well plate (Corning) at a density of 10^5 cells/mL and incubated overnight to 60–70% confluence. After incubation for 24 h with CPTn (5 μM), cancer cells were harvested with 0.25% trypsin (Gibco) and washed with PBS. The cells were fixed in 70% ethanol and kept at 4 °C for 12 h at least. After that, the cells were washed with PBS and incubated with 100 μL RNAnase (KeyGEN BioTECH) in 37 °C water bath for 30 min. The cells were washed with PBS and stained with 400 μL PI (KeyGEN BioTECH) solution and incubated for 30 min at room temperature in the dark. Each sample was washed with PBS and resuspended in 500 μL PBS. Analysis was performed via BD FACSCalibur flow cytometry within 1 h.

Wound Healing Assay

Culture-inserts (ibidi) were placed in a 6-well plate (Corning). SKOV-3 cell suspension (3–7×10^5 cells/mL) was prepared. Cell suspension (70 μL) was seeded into each well of culture insert and incubated at 37 °C for 12 h at least. After appropriate cell attachment, the culture-inserts were gently removed by using sterile tweezers, and then a cell-free gap of 500 μm was created. The wells were filled with recommended volume of medium in the absence or presence of CPT8. Images of the wound were photographed immediately and 12 h later.

Cell Migration and Invasion Assay

The migration assay was performed with a 24-well Transwell system (8 μm pore size, BD Biosciences). SKOV-3 cells were resuspended in serum-free medium containing CPT8 and added into the chambers (5×10^4 cells/well). The lower plate was filled with 600 μL medium containing 10% fetal bovine serum as a chemoattractant. After 24 h incubation, the cells on the upper side of the chambers were removed using a cotton swab. At room temperature, the cells on the lower side of the chambers were fixed in 4% paraformaldehyde (KeyGEN BioTECH) for 10 min, stained with crystal violet (KeyGEN BioTECH) for 10 min, washed with distilled water and dried off. The images of chamber bottom were taken by Mshot digital microscope camera. For the invasion assay, the chambers were coated with a mixture (8:1) of serum-free medium and Matrigel (BD Biosciences), incubated at 37 °C for 2 h. The remaining liquid was

carefully removed without disturbing the matrix layer. The invasion assay was performed with coated chambers under the same procedure of migration assay.

Western Blot Analysis

After treatment for 24 h with CPT8, SKOV-3 cells in 10 cm dishes (Corning) were harvested with 0.25% trypsin (Gibco) and washed with PBS. Cell lysis buffer (100 μL, KeyGEN BioTECH) was added to each sample on the ice. The lysate (100 ng) were electrophoresed on a 10% SDS-PAGE gel by Bio-Rad Mini-PROTEAN Tetra System (80 V, 20 min; 100 V, 1 h). Proteins were transferred to nitrocellulose membrane (90 V, 1.5 h) and probed with monoclonal MMP-2 and GAPDH antibody (abcam), followed by incubation with peroxidase-labeled secondary antibody (abcam). The western blot results were visualized by HRP substrate (Millipore) on chemiluminescence detection system (LAS500).

Tube Formation Assay

Tube formation assay was performed with μ-Slide Angiogenesis (ibidi) according to the manufacturer's instruction. BD Matrigel (Growth Factor Reduced, 356231) was thawed on ice. μ-Slide and pipet tips were precooled in the refrigerator. After applying 10 μL of Matrigel to each inner well, μ-Slide was placed in a humid petri dish prepared with water soaked paper towels. The whole assembly was placed into the incubator for polymerization (30 min). In the meantime, HUV-EC-C cell suspension (2×10^5 cells/mL) was prepared. Cell suspension (50 μL) was applied to each well. After 30 min, the medium in the well was replaced with medium containing certain amount of CPT8. After 12 h incubation, the supernatant was discarded, and 50 μL calcein (KeyGEN BioTECH) (6.25 μg/μL) diluted with serum-free medium was added into each well. μ-Slide was incubated in the dark for 30 min at room temperature, then washed with PBS for three times. The pictures were taken at 485 nm/529 nm by OLYMPUS IX71 inverted microscope.

Sprouting Assay

Poly-HEMA (5 mL, 12 mg/mL, 95% ethanol) (Sigma) was added to 25 cm^2 culture flask (Thermo Fisher). The flask was placed in an oven at 40 ^oC and left for 48 h, ensuring that the flask remained sterile. HUV-EC-C were passaged to the flask. The spheroids were formed after 4~5 days at standard growth condition. μ-Slide Angiogenesis (ibidi) with BD matrigel was prepared as tube formation assay. The spheroids were collected and resuspended in 1 mL F12K medium. The spheroids suspension was added to the μ-Slide at 50 μL/well. After 30 min, supernatant was replaced with flash F12K medium containing CPT8. Photographs were taken by OLYMPUS IX71 inverted microscope after 3 days to assess the spheroids sprouting.

Vasculogenic Mimicry Assay

μ-Slide Angiogenesis (ibidi) with BD matrigel was prepared as described in the tube formation assay. B16F10 cell suspension was added (5000 cells/well) into each well and incubated with serum-free medium in the presence or absence of CPT8 for 12 h. The culture method, staining process and picture taking are same as described in the tube formation assay.

References

1. W. Zhou, X. Y. Wang, M. Hu, and Z. J. Guo, *J. Inorg. Biochem.* 2013, **121**, 114–120.

2. T. Gunnlaugsson, J. P. Leonard, K. Sénéchal, and A. J. Harte, *J. Am. Chem. Soc.* 2003, **125**, 12062–12063.
3. (a) N. M. Carballeira; H. Cruz, C. A. Hill, J. J. De Voss and M. Garson, *J. Nat. Prod.* **2001**, *64*, 1426–1429; (b) S. Thurnhofer and W. Vetter, *Tetrahedron* **2007**, *63*, 1140–1145.
4. (a) SMART, Version 5.625, Bruker AXS, Inc., Madison, WI, 2000; (b) SAINT, Version 6.01, Bruker AXS, Inc., Madison, WI, 2000.