Supporting Information

A biotinylated ruthenium(II) photosensitizer for tumor-targeted two-photon photodynamic therapy

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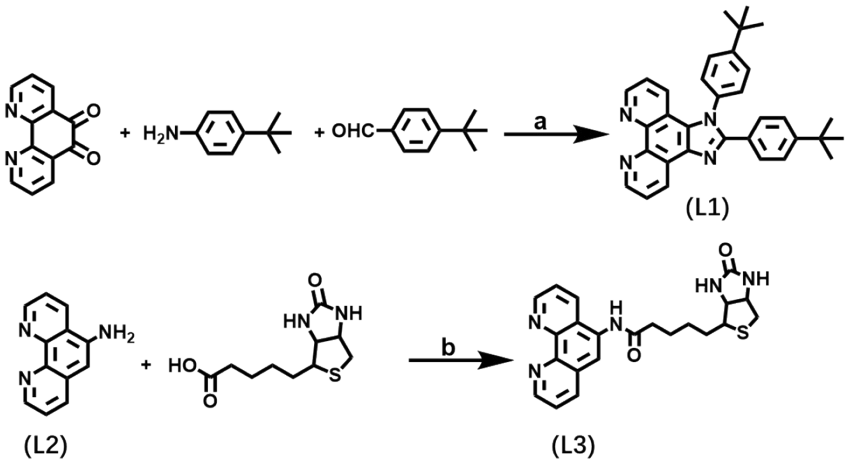
Experimental Section:

Materials

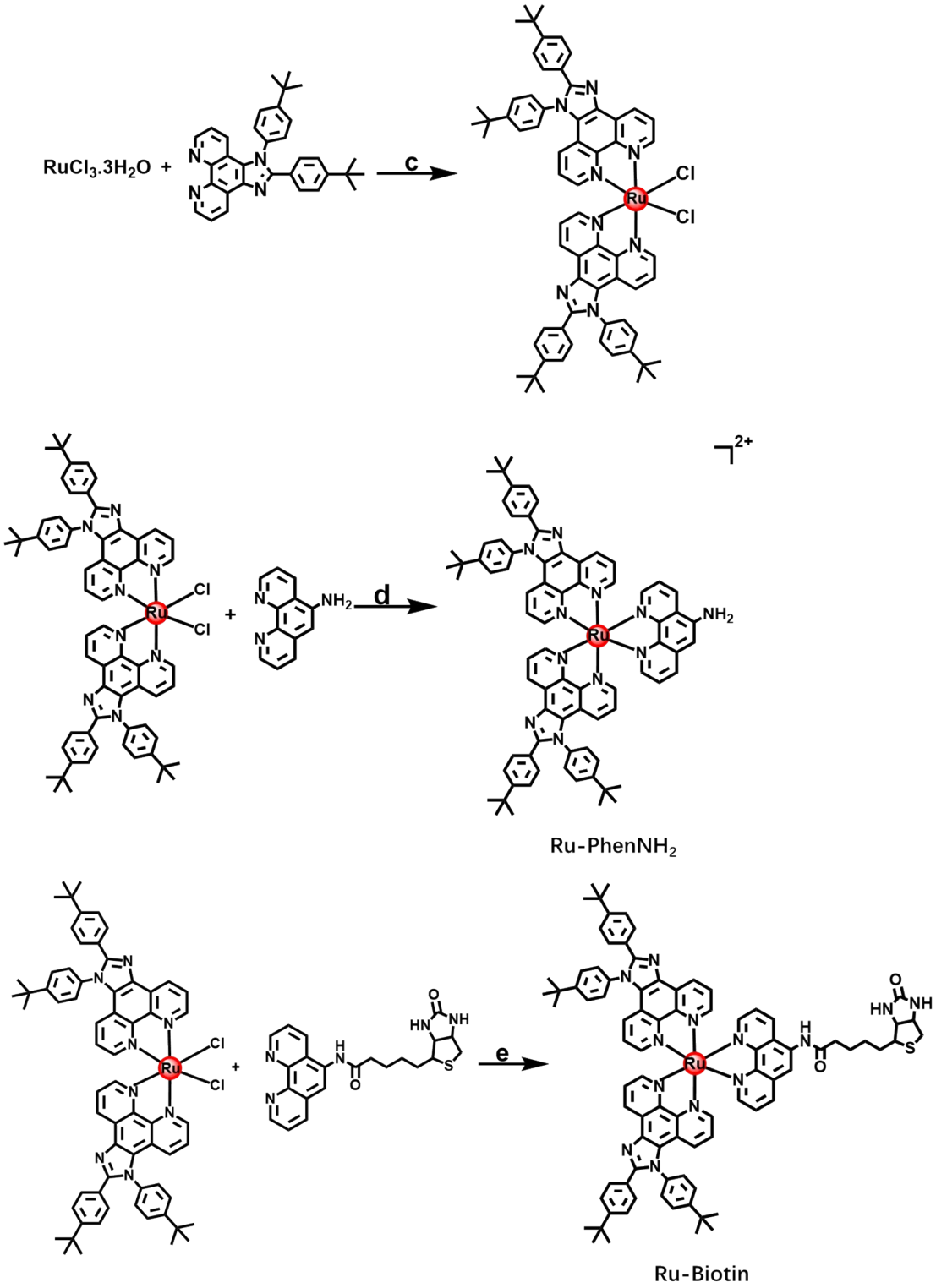
All of the reagents were purchased from commercial sources and were used as received unless stated otherwise. Electrospray ionization mass spectra (ESI-MS) was recorded on an LCQ system (Finnigan MAT, USA). Microanalyses (C, H, and N) were carried out using an Elemental Vario EL CHNS analyzer (Germany). The ^{1}H NMR were recorded on Varian Mercury Plus 600 Nuclear Magnetic Resonance Spectrometer. The ESR spectra were measured using a Bruker e-scan ESR spectrometer. The electronic absorption spectra and emission spectra were recorded using a Perkin–Elmer Lambda 850 UV/Vis spectrometer and Perkin–Elmer LS 55 luminescence spectrometer respectively. All the compounds were dissolved in DMSO just before the experiments and the final DMSO concentration was less than 1% (v/v). Ruthenium chloride hydrate, biotin, 1,10-phenanthroline-5,6-dione, 4-tert-butylcldehyde, 4-tert-butylaniline, cisplatin, and diazepam were purchased from Sigma-Aldrich. DPBF (1,3-diphenyliso-benzofuran), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Alfa Aesar. The 2',7'-dichlorofluorescin diacetate (DCFH-DA) kit and the Annexin V-FITC apoptosis assay kit were obtained from the Beyotime Institute of Biotechnology (China). The Caspase 3/7 Activity Assay Kit was obtained from Promega. All other reagents and solvents were of high purity.

Synthesis and characterization

Ligands Synthesis



Ru(II) Complexes Synthesis



Scheme S1 Synthetic routies of ligands and Ru(II) complexes. Reaction conditions: (a) NH_4Ac, HAc, 120 °C reflux, 24 h, Ar. (b) $SOCl_2$, DMF, DMAP, at room temperature, 18 hours. (c) DMF, 140 °C reflux, 24 h, Ar. (d) EtOH/H_2O (2:1, v/v), 120 °C reflux, 8 h, Ar. (e) EtOH/H_2O (2:1, v/v), 120 °C reflux, 8 h, Ar.

L1: 1,10-Phenanthroline-5,6-dione (1.00 g, 4.76 mmol, 1 eq), 4-tert-Butylaniline (711 mg, 4.76 mmol, 1 eq), 4-tert-Butylbenzaldehyde (773 mg, 4.76 mmol, 1 eq) and ammonium acetate (3.66 g, 47.4 mmol, 10 eq) were added to acetic acid (20 ml) in a round bottomed flask. The reaction was refluxed at 125 ^{o}C with stirring for 3 h. The reaction mixture was poured into ice water (400 ml), neutralized with sodium hydroxide. The crude product was purified by column chromatography on alumina with CH_2Cl_2-methanol (80:1 to 50:1, v/v) as eluent. Yield: 1.01 g, 44 %. Calculated for ESI-MS: 484.26 and obtained (CH_3OH) m/z: 485.58 $[M+H]^+$. Elemental analysis: calcd (%) for $C_{33}H_{32}N_4$: C 81.78, H 6.66, N 11.56, found: C 81.54, H 6.83, N 11.63. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.30 (s, 2H), 9.12 (s, 1H), 7.85 (d, *J* = 5.2 Hz, 1H), 7.67 (d, *J* = 7.9 Hz, 2H), 7.56 (d, *J* = 8.0 Hz, 2H), 7.50 – 7.42 (m, 3H), 7.34 (d, *J* = 7.6 Hz, 3H), 1.47 (s, 9H), 1.31 (s, 9H).

L2 and L3: The ligands **L2** and **L3** was synthesized according to the literature method.[1,2]

Ru[(L1)$_2$]Cl$_2$: $RuCl_3$. $3H_2O$ (230 mg, 870 μmol, 1 eq), **L2** (840 mg, 1.74 mmol, 2 eq), and LiCl (240 mg, 5.66 mmol, 6.5 eq) were heated at reflux and stirred in dimethylformamide (4 mL) for 8 h. After the mixture was cooled to room temperature, acetone (8 mL) was added and the solution cooled at 0 °C overnight. Filtration yielded a microcrystalline product. The solid was washed three times with water (10 mL) followed by three portions of diethyl ether (10 mL), the solid was then dried and carried forward without further purification.

Ru-PhenNH$_2$: A mixture of Ru[(**L1**)$_2$]Cl_2 (260 mg, 220 μmol, 1 eq) and the **L2** ligand (42 mg, 220 μmol, 1 eq) was dissolved in EtOH/H_2O (30 mL, 2:1, v/v) and heated at 90 ^{o}C for 8 h under argon. After cooling to rt the mixture was evaporated under reduced pressure, and the crude product was purified by column chromatography on alumina with CH_2Cl_2-toluene (1:10 to 10:1, v/v) as a gradient eluent system to yield **Ru-PhenNH$_2$** as a red solid. (210 mg, 157 μmol, 53%) Calculated for ESI-MS: 1265.51 and obtained (CH_3OH) m/z: 633.23 $[M-2Cl]^{2+}$, 1264.96 $[M-2Cl+H]^+$, Elemental analysis: calcd (%) for $C_{78}H_{73}N_{11}Cl_2Ru$: C 74.03, H 5.81, N 12.17, found: C 74.22, H 5.67, N 12.23. ^{1}H NMR (400 MHz, DMSO) δ 9.80 (d, *J* = 5.7 Hz, 1H), 9.53 (d, *J* = 5.6 Hz, 1H), 9.39 (d, *J* = 8.3 Hz, 1H), 8.27 – 8.23 (m, 4H), 8.17 (d, *J* = 8.5 Hz, 3H), 8.13 – 8.08 (m, 2H), 7.94 (dd, *J* = 9.4, 4.0 Hz, 4H), 7.82 – 7.78 (m, 4H), 7.66 (s, 1H), 7.55 (d, *J* = 6.9 Hz, 1H), 7.50 (d, *J* = 6.7 Hz, 2H), 7.44 (t, *J* = 6.7 Hz, 1H), 7.15 (s, 1H), 7.02 (d, *J* = 6.9 Hz, 2H), 6.95 (d, *J* = 9.2 Hz, 1H), 6.86 (s, 1H), 6.82 (d, *J* = 7.1 Hz, 1H), 6.75 (d, *J* = 7.4 Hz, 1H), 6.66 (dd, *J* = 17.5, 9.9 Hz, 2H), 6.27 (d, *J* = 9.4 Hz, 2H), 5.66 (d, *J* = 7.5 Hz, 1H), 1.38 (d, *J* = 28.5 Hz, 36H).

Ru-Biotin: This complex was synthesized in a similar manner to that described for the **Ru-PhenNH$_2$**, except that **L3** (92 mg, 220 μmol, 1 eq) was used instead of **L2** to yield **Ru-biotin** (220 mg, 141 μmol, 64%). Calculated for ESI-MS: 1489.56 and obtained (CH_3OH) m/z: 746.28 $[M-2Cl]^{2+}$, 1490.98 $[M-2Cl+H]^+$, Elemental analysis: calcd (%) for $C_{88}H_{87}N_{13}O_2RuS$: C 70.94, H 5.75, N 12.22, found: C 70.87, H 5.86, N 12.43. ^{1}H NMR (400 MHz, DMSO) δ 9.15 (dd, *J* = 28.1, 15.7 Hz, 4H), 8.77 – 8.65 (m, 2H), 8.09

(d, *J* = 3.9 Hz, 4H), 7.96 (s, 4H), 7.87 (s, 4H), 7.78 (d, *J* = 8.3 Hz, 2H), 7.73 (s, 5H), 7.58 (d, *J* = 7.9 Hz, 4H), 7.50 (s, 1H), 7.45 – 7.40 (m, 4H), 7.34 (dd, *J* = 16.4, 8.4 Hz, 2H), 6.43 (d, *J* = 28.2 Hz, 2H), 4.32 (s, 1H), 4.17 (s, 1H), 3.16 – 3.13 (m, 1H), 2.83 (d, *J* = 12.2 Hz, 1H), 2.61 (s, 1H), 1.90 (s, 2H), 1.74 (d, *J* = 12.8 Hz, 2H), 1.40 (d, *J* = 5.4 Hz, 18H), 1.37 (s, 1H), 1.27 (d, *J* = 2.0 Hz, 18H), 1.23 (s, 1H), 1.08 (d, *J* = 8.6 Hz, 2H).

Two-photon absorption cross section measurements

The two-photon absorption cross section measured as previously reported by Makarov et al.[3] The complexes and Rhodamine B were dissolved in methanol at a concentration of 400 M. The TPA cross-sections were obtained by two-photon excitation fluorescence using an OpeletteTM 355II. (pulse width < 100 fs, 80 MHz repetition rate, tuning range 700-1050 nm, spectra Physics Inc., USA).[4] The two-photo absorption spectra of the **Ru-PhenNH$_2$** or **Ru-Biotin** were determined over a broad spectral region (750-900 nm) relative to Rhodamine B as the standard. The quadratic dependence of two-photon-induced luminescence intensity on the excitation power was verified at an excitation wavelength of 820 nm. The TPA cross-section of **Ru-PhenNH$_2$** or **Ru-Biotin** can be calculated by the following equation.

$$\sigma_S = \sigma_R \frac{\phi_R C_R I_S n_S}{\phi_S C_S I_R n_R} \quad \text{..... (1)}$$

Where I is the integrated fluorescence intensity, C is the concentration of the tested complex, n is the refractive index, and Φ is the quantum yield. Subscript "S" stands for sample and "R" stands for reference, i.e. Rhodamine B. In the experiment, we ensured that the excitation flux and the excitation wavelengths were the same for both the sample and the reference.

Electron spin resonance (ESR) assay

The ESR spectra were recorded on a Bruker Model A300 spectrometer at room temperature (20 mW microwave power, 100 G-scan range, and 1 G field modulation). An area light source was used for irradiation for 5 min (450 ± 10 nm, 20 mW cm^{-2}). **Ru-PhenNH$_2$** or **Ru-Biotin** were dissolved in aerated methanol containing 10 mM 2,2,6,6–tetramethylpiperidine (TEMP) as a $^1O_2$ spin trap and sucked into capillary tubes by siphon effect in the dark. The control group was methanol solution containing TEMP alone.

Singlet Oxygen Production

The $^1O_2$ quantum yields (Φ_Δ) of **Ru-PhenNH$_2$** and **Ru-Biotin** were detected according to the literature procedure. The ruthenium complexes and DPBF (50 μM) solutions were degassed with nitrogen for 10 min, before photoirradiation at 450 nm (20 mW·cm^{-2}). The absorbance of DPBF at 418 nm was recorded every 2 s. The absorbance at 450

nm of ruthenium complexes was kept at 0.1. $[Ru(bpy)_3]^{2+}$ was utilized as the standard (Φ_Δ= 0.81 in methanol)[5]. The $^1O_2$ quantum yields of the ruthenium complexes was calculated according to Eq. (2)

$$\Phi_\Delta{}^{\mathbf{Ru}} = \Phi_\Delta{}^{MB} \times (s^{\mathbf{Ru}} \times F^{MB}) / (s^{MB} \times F^{\mathbf{Ru}}) \quad (2)$$

Where *s* is the slope of a linear fit of the change of absorbance at 418 nm against the irradiation time (s) and F is the absorption correction factor, which is given by $F = 1 - 10^{-OD}$ (OD is the optical density at the irradiation wavelength).

Octanol/water partition coefficients (Log *P*) measurement

Water and octanol were mixed for 24 h to full saturation and the two layers were separated. The complexes were dissolved in the octanol phase which was previously saturated with water to give a 5 mL solution. The same volume of water phase previously saturated with octanol was added into the solution. The mixture was shaken vigorously at room temperature for 24 hours. The concentration of **Ru-PhenNH$_2$** or **Ru-Biotin** was measured by UV-vis spectroscopy. The evaluation was carried out in triplicate. The partition-coefficient of each complex be determined by the equation.

$$\text{Log } P_{o/w} = \log \left(\frac{[solute]_{octanol}}{[solute]_{water}} \right)$$

Cell culturing and imaging

HeLa (human cervical carcinoma), HLF-a (Human Lymphatic Fibroblasts), A549 (human lung carcinoma), and A549R (cisplatin-resistant human lung carcinoma) cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The cell lines were cultured at 37 °C with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL) supplemented with 10% inactivated fetal bovine serum (FBS, Gibco BRL) and 50 U/mL streptomycin and 50 ng/mL penicillin (Gibco BRL). For the specific cell uptake experiment, after the mixed A549R cells or HLF-a cells were cultured at 37 °C under 5% CO_2 for 24 h, the cells were further incubated with **Ru-PhenNH$_2$** or **Ru-Biotin** (5 μM) for 3 h before fluorescence imaging was performed. For the receptor inhibition experiment, A549R cells were treated with 100 μM of D-biotin in DMEM medium for and incubated for 2 h prior before treatment with **Ru-Biotin** and further incubation for 3 h, followed by fluorescence imaging.

Cell uptake studies

A549R/HLF-a cells were plated at a density of 10^6 cells/mL in 10 mL of DMEM for 24 h, then the **Ru-PhenNH$_2$** or **Ru-Biotin** (5 μM) was added to the culture medium and incubated for 3 h. The culture was removed and the cells were washed three times with cold PBS and trypsinization. The cell numbers were subsequently accurately counted. The cells were collected and digested in a solution of 20% HNO_3 (1 mL) and 10% H_2O_2

(1 mL) for 48 h. Each sample was then diluted with Milli-Q water to obtain 2% HNO_3 sample solutions. The ruthenium content was determined using inductively coupled plasma mass spectrometry (ICP-MS).

Stability in FBS and culture media

The stability of the Ru(II) complexes in plasma was assessed by using a procedure analogous to a recently reported method. A Ru complex solution and of a diazepam solution were mixed and added to the plasma solution. The resulting mixture was incubated for 24 h at 37℃ with a shake at the speed of 280 rpm. The mixed solution was added with acetonitrile solution, and the mixture was centrifuged for 15 min at 2000 g. The acetonitrile was removed, and the residue was suspended in acetonitrile solution. The filtrate was analyzed by HPLC-UV spectroscopy with an HPLC system (Thermo, USA) connected to a UV/Vis spectrophotometer. The moving phase is linear gradient of A (acetonitrile; HPLC grade) in B (distilled water containing 0.02 % trifluoroacetic acid (TFA) and 0.03 % HCOOH).

Caspase-3/7 activation detection

Caspase-3 activity was measured using Caspase-Glo 3/7 assay kit (Promega, USA), A549R cells were seeded in white 96-well plates at a density of 1×10^4 cells 24 h (100 μL), and the negative control was incubated with pure culture medium. Cisplatin (50 μM) as the positive control and complexes **Ru-PhenNH$_2$** or **Ru-Biotin** (2.5 μM and 5 μM) as the experimental group were incubated for 3 h at 37 °C. In the dark group, the plates were put in the dark. In the light group, the plates were exposed to LED area light irradiation (450 nm, 20 mW cm^{-2}, light dose = 12 J cm^{-2}); The incubation proceeded for additional 12 h in the dark for all plates. Upon completion, 100 μL of Caspase-Glo 3/7 reagent was added to each well, and incubated for 1 h at room temperature in the dark to give a final volume of 200 μL. Each column represents the average of triplicates from three independent experiments. The luminescence intensity was measured by a multifunction microplate reader (infinite M200 PRO, TECAN).

Detection of two-photon induced intracellular singlet oxygen

A549R cells were treated with **Ru-PhenNH$_2$** or **Ru-Biotin** (5 μM) for 3 h in the dark, the culture medium was removed and PBS containing DCFH-DA (5.0 μM) was then added into the plate for 30 min. The culture medium was refreshed with fresh PBS and subjected to two-photon irradiation (820 nm, 0.27 mW cm^{-2}, 80 MHz, 100 fs) using a laser source equipped in an LSM 810 Carl Zeiss Laser Scanning Confocal Microscope. The whole procedure was processed in the dark. Fluorescence imaging was performed before and after the irradiation using an excitation wavelength of 488 nm and emission wavelength between 510 nm and 550 nm.

Annexin V-FITC/propidium iodide double staining assay

A549R cells were treated with 5 μM of the **Ru-PhenNH$_2$** or **Ru-Biotin** (0.5% DMSO, v%) and incubated for 3 h at 37 °C. The culture medium was removed and the cells were washed with new medium three times before the addition of fresh medium. Cells were further incubated with annexin V-FITC and propidium iodide following the protocols of the manufacturer (Life Technologies) and imaged before, and 30 minutes after, the TPPDT treatment (820 nm, 0.27 mW cm^{-2}, 80 MHz, 100 fs, irradiation for 2 min). The fluorescence was recorded using a laser source equipped in an LSM 810 Carl Zeiss Laser Scanning Confocal Microscope. Annexin V-FITC channel, (λ_{ex} = 488 nm, λ_{em} = 525 nm). Propidium iodide (λ_{ex} = 536 nm, λ_{em} = 617 nm).

Live/dead viability/cytotoxicity assay

A549R cells were seeded in white 96-well plates at a density of 1 × 10^4 cells, 24 h (100 μL). In the dark group, the cells were pre-incubated with 50 μM **Ru-PhenNH$_2$** or **Ru-Biotin** for 3 h. In the light group, the cells were pre-incubated with 5 μM for 3 h before being irradiated at 450 nm by LED area light (450 nm, 20 mW cm^{-2}, light dose = 12 J cm^{-2}). Both groups were then incubated for 45 h and stained by Calcein-AM/PI under inverted fluorescence microscope (Zeiss), Calcein-AM: λ_{em} = 515 nm, λ_{ex} = 490 nm; PI: λ_{em} = 617 nm, λ_{ex} = 536 nm. Scale bar = 100 μm.

Cytotoxicity assay on 2D cancer cell monolayer

Cells were seeded into 96-well microtiter plates at 1×10^4 cells per well. After incubation for 24 h, a series of different concentrations of the complexes and cisplatin were added to the cultures. The plates were incubated in the dark for 3 h. The dark control were kept in the dark. The tested compounds were removed by washing the cells with fresh medium. The light group was exposed to Laser Scanning Confocal Microscope (820 nm, 0.27 mW cm^{-2}, 80 MHz, 100 fs). All the plates were then incubated for an additional 45 h in the dark. 20 μL of MTT solution (5 mg/mL in 1 × PBS) was added to each well and then placed at 37 °C for 4 h. The cultures were removed and 150 μL of DMSO was added to each well. The optical density of each well was measured on a microplate spectrophotometer at a wavelength of 595 nm.

Generation and Imaging of MCTSs

A suspension of 1% agarose in DMEM was sterilized by high pressure and high temperature for 20 min. The gel was added into 96-well microassay culture plates (50 μL/well), then exposed under UV irradiation for 3 h. Cells at 2 × 10^4 cells/mL were transferred to the prepared 96-well microassay culture plates with a volume of 200 μL/well and formed MCTSs aggregates approximately 400 μm in diameter after 3 days.

The cell solution in the inlet was replaced with fresh cell culture media every two days to maintain the growing of MCTSs. After formation of the MCTSs, each MCTS in a 96-well plate was imaged with a phase contrast microscope (Zeiss Axio Observer D1, Germany) or confocal microscopy (LSM 810, Carl Zeiss, Göttingen, Germany) to record their color, integrity, diameter, and volume. For the imaging of multicellular tumor spheroid. To investigate the depth of the complexes under one- and two-photon lasers, the 3D MCTSs with diameters of 450-550 μm were incubated with **Ru-PhenNH$_2$** or **Ru-Biotin** (5 μM) for 6 h. The MCTSs were imaged by a confocal microscope (Zeiss LSM 810 NLO, 10× objective). The excitation wavelengths were 450 nm and 820 nm. An emission filter of 550 ± 20 nm was used.

References

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2. M. Slim and H. F. Sleiman, *Bioconjugate. Chem.*, 2004, **15**, 949-953.
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Supporting Tables and Figures:

Table S1 Photophysical properties of complexes.

Complexes	ϕ_{em}[a]	T(ns)[b]	$\phi_{\Delta}(^1O_2)$[c]	Em /nm[d]
Ru-PhenNH$_2$	0.048	314	0.84	610
Ru-Biotin	0.077	372	0.87	610
[Ru(bpy)$_3$]$^{2+}$	0.040	420	0.59	608

[a] refers to the fluorescence quantum yield. [b] refers to the lifetime of fluorescence. [c] refers to the quantum yield of $^1O_2$ (ROS). [d] refers to the wavelength of emission spectra in PBS. These photophysical experiments were tested in PBS.

Table 2 (photo)cytotoxicity IC_{50} (μM) values of the complexes towards different cell lines[a].

Complexes	A549R			HLF-a		
	Dark	820 nm	PI[b]	Dark	820 nm	PI[a]
Cisplatin	73.4±4.3	72.6±3.8	0.98	16.6±1.2	17.1±0.6	0.97
Ru-PhenNH$_2$	>100[c]	13.1±0.2	>7.63	>100	14.2±0.5	>7.04
Ru-Biotin	72.9±5.8	3.3±0.1	22.1	>100	36.4±2.1	>2.74

[a] Irradiated at 820 nm light (0.27 mW cm^{-2}, 80 MHz, 100 fs, 2 min) by confocal microscopy and further incubation for 45 h in a fresh medium. [b] PI (refers to photocytotoxicity index) is the ratio of dark-to-light toxicity. Data represent mean values from three replicates. [c] The concentration cannot be higher due to its poor solubility.

Table S3 (Photo)cytotoxicity IC_{50} (μM) values of the complexes towards different cell lines[a].

Complexes	HeLa			A549		
	Dark	820 nm	PI[b]	Dark	820 nm	PI
cisplatin	15.7 ± 0.8	15.6 ± 1.2	0.99	15.3 ± 0.9	15.1 ± 0.5	0.98
Ru-PhenNH$_2$	> 100[c]	10.4 ± 0.5	> 9.6	> 100	11.2 ± 0.2	> 8.9
Ru-Biotin	86.87 ± 3.9	3.8 ± 0.2	22.86	79.5 ± 2.1	3.94 ± 0.1	20.17

[a] Irradiated at 820 nm by confocal (820 nm, 0.27 mW cm^{-2}, 80 MHz, 100 fs, 2 min). and further incubation for 45 h in a fresh medium. [b] PI (refers to photocytotoxicity index) is the ratio of dark-to-light toxicity. Data represent mean values from three replicates. [c] The concentration cannot be higher due to its poor solubility.

Table S4. (Photo)cytotoxicity IC_{50} (μM) values of the complexes towards different cell lines[a].

Complexes	A549R			HLF-a		
	Dark	450 nm	PI[b]	Dark	450 nm	PI
cisplatin	73.4 ± 4.3	74.8 ± 4.1	0.98	16.6 ± 1.2	16.3 ± 0.8	0.98
Ru-PhenNH$_2$	> 100[c]	12.4 ± 0.3	>8.06	> 100	13.3 ± 0.7	> 7.5
Ru-Biotin	72.9 ± 5.8	2.63 ± 0.1	27.7	> 100	32.27 ± 2.3	> 3.1

[a] Irradiated at 450 nm by an LED area light (450 nm, 20 mW cm^{-2}, light dose = 12 J cm^{-2}) and further incubation for 45 h in a fresh medium. [b] PI (refers to photocytotoxicity index) is the ratio of dark-to-light toxicity. Data represent mean values from three replicates. [c] The concentration cannot be higher due to its poor solubility.

Table S5 (Photo)cytotoxicity IC_{50} (μM) values of the complexes towards different cell lines[a].

Complexes	HeLa			A549		
	Dark	450 nm	PI[b]	Dark	450 nm	PI
cisplatin	15.7 ± 0.8	16.5 ± 1.1	0.95	15.3 ± 0.9	15.9 ± 0.5	0.96
Ru-PhenNH$_2$	> 100[c]	11.3 ± 0.5	> 8.84	> 100	10.8 ± 0.2	>9.3

Ru-Biotin	86.87 ± 3.9	2.4 ± 0.3	36.2	79.5 ± 2.1	2.2 ± 0.1	36.1

[a] Irradiated at 450 nm by an LED area light (450 nm, 20 mW cm^{-2}, light dose = 12 J cm^{-2}) and further incubation for 45 h in a fresh medium. [b] PI (refers to photocytotoxicity index) is the ratio of dark-to-light toxicity. Data represent mean values from three replicates. [c] The concentration cannot be higher due to its poor solubility.

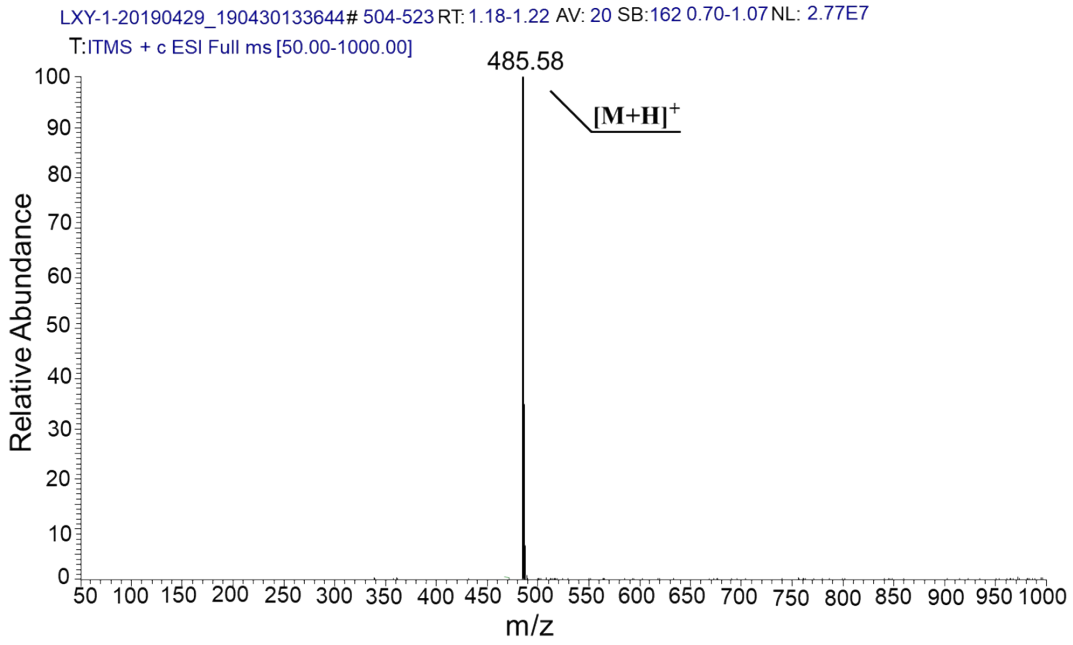

Figure S1 ESI-MS spectrum of **L1**.

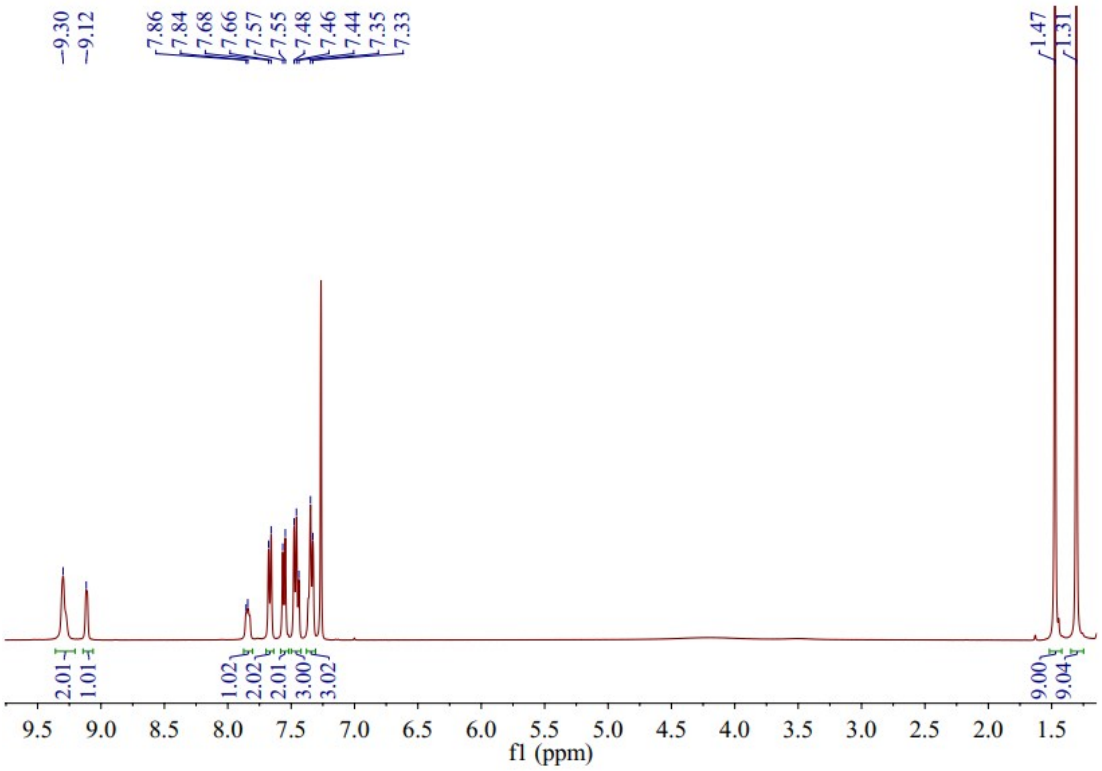

Figure S2 ^{1}H NMR spectrum of **L1**.

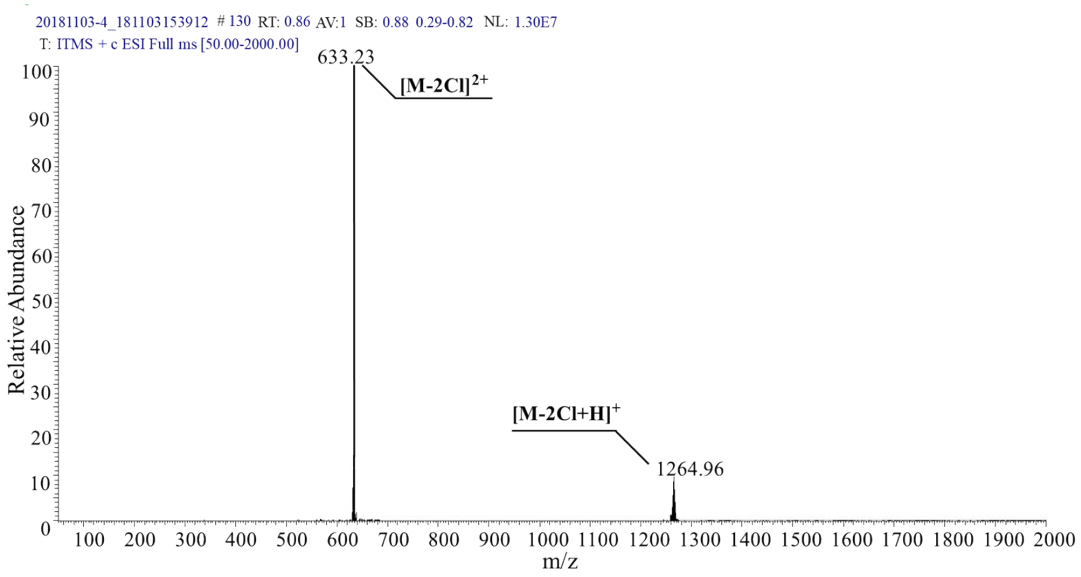

Figure S3 ESI-MS spectrum of **Ru-PhenNH$_2$**.

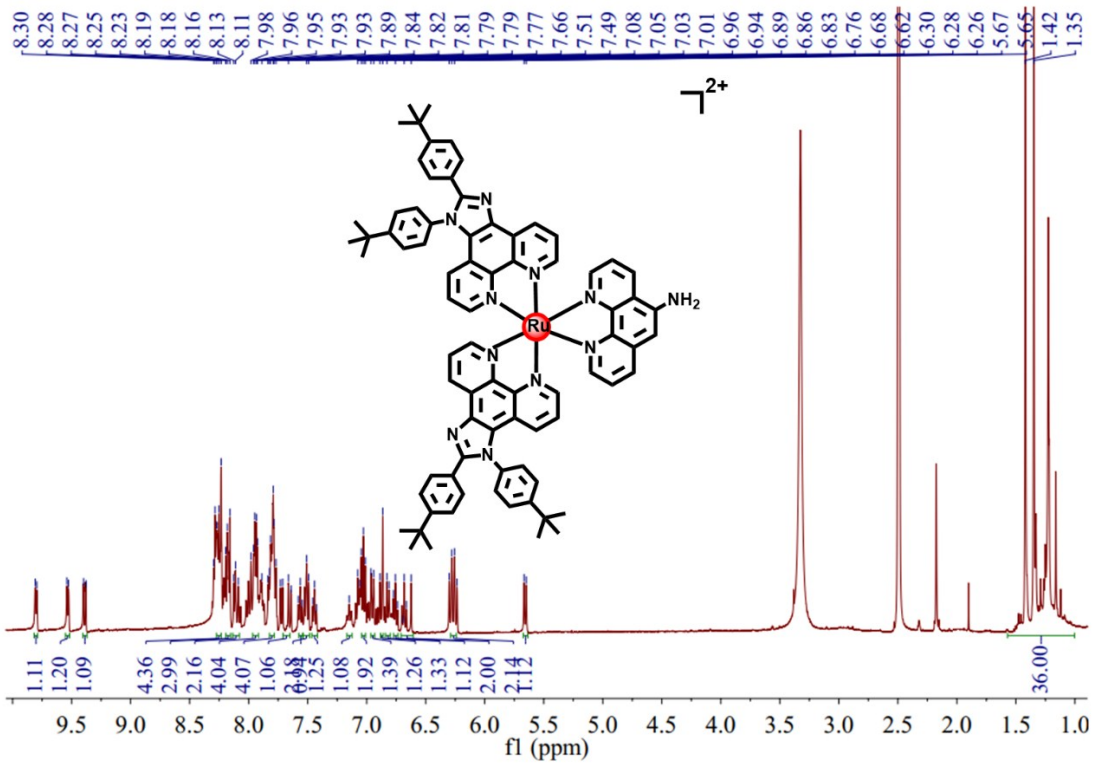

Figure S4 ^{1}H NMR spectrum of **Ru-PhenNH$_2$**.

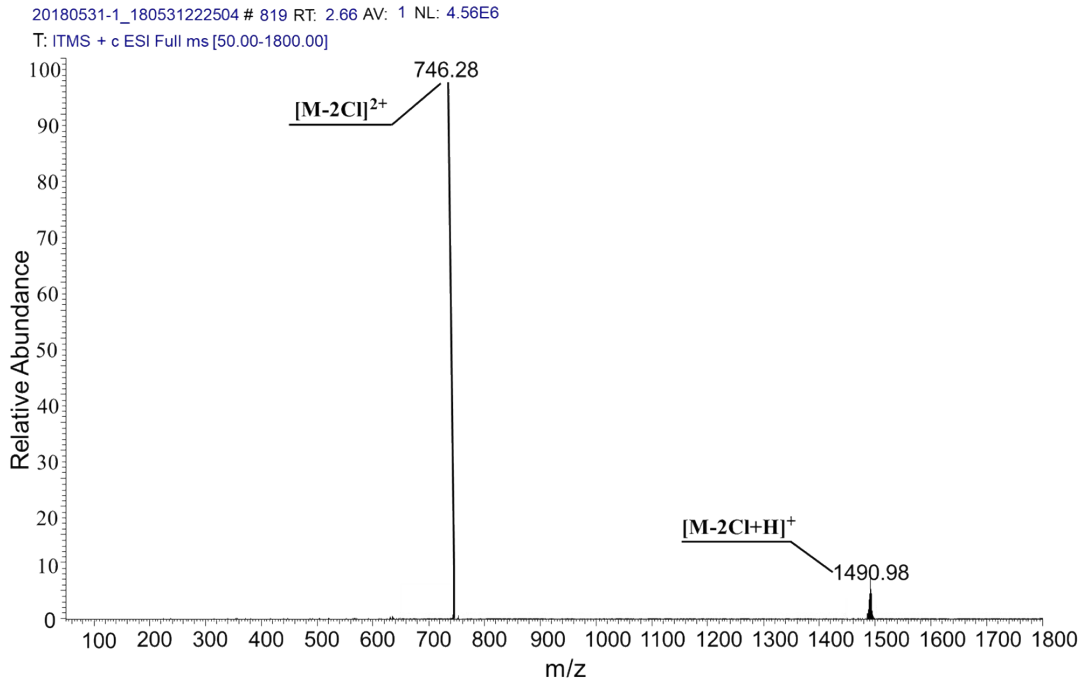

Figure S5 ESI-MS spectrum of **Ru-Biotin**.

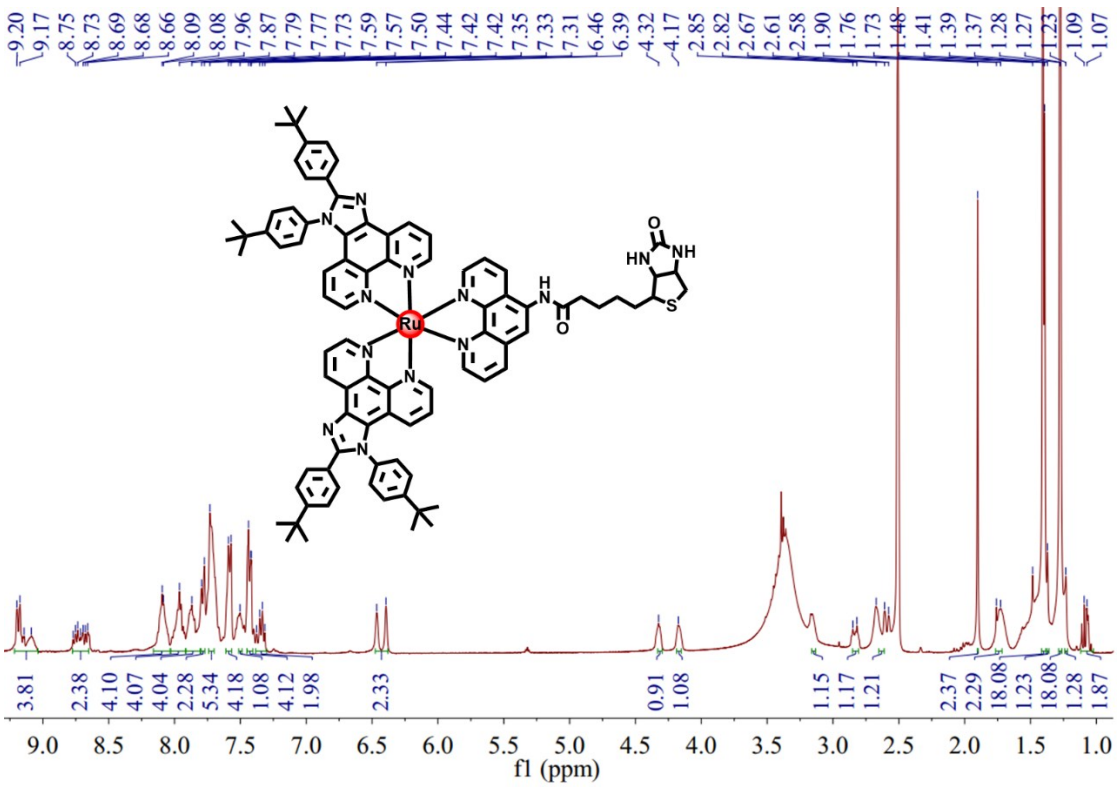

Figure S6 ^{1}H NMR spectrum of **Ru-Biotin**.

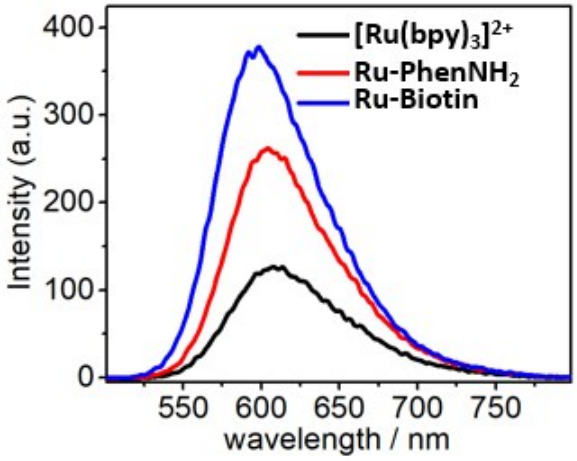

Figure S7 The emission spectra of 10 μM **Ru-PhenNH$_2$** and **Ru-Biotin** in PBS (λ_{ex} = 450 nm).

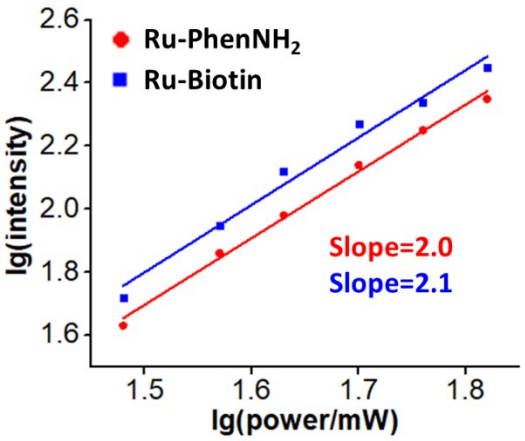

Figure S8 The logarithmic plots of the power dependence of relative two-photon induced luminescence intensity of **Ru-PhenNH$_2$** and **Ru-Biotin** as a function of pump power at an excitation wavelength of 820 nm, respectively.

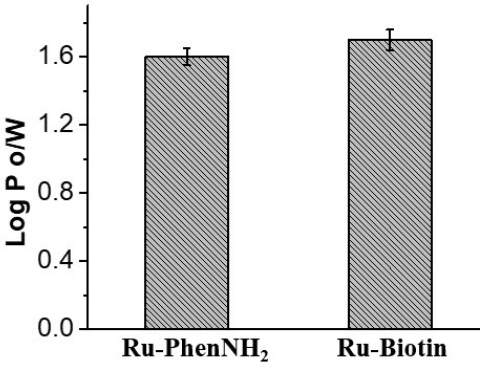

Figure S9 Octanol/water partition coefficients of **Ru-PhenNH$_2$** and **Ru-Biotin**, the error bars denote standard deviation calculated from three replicate trials.

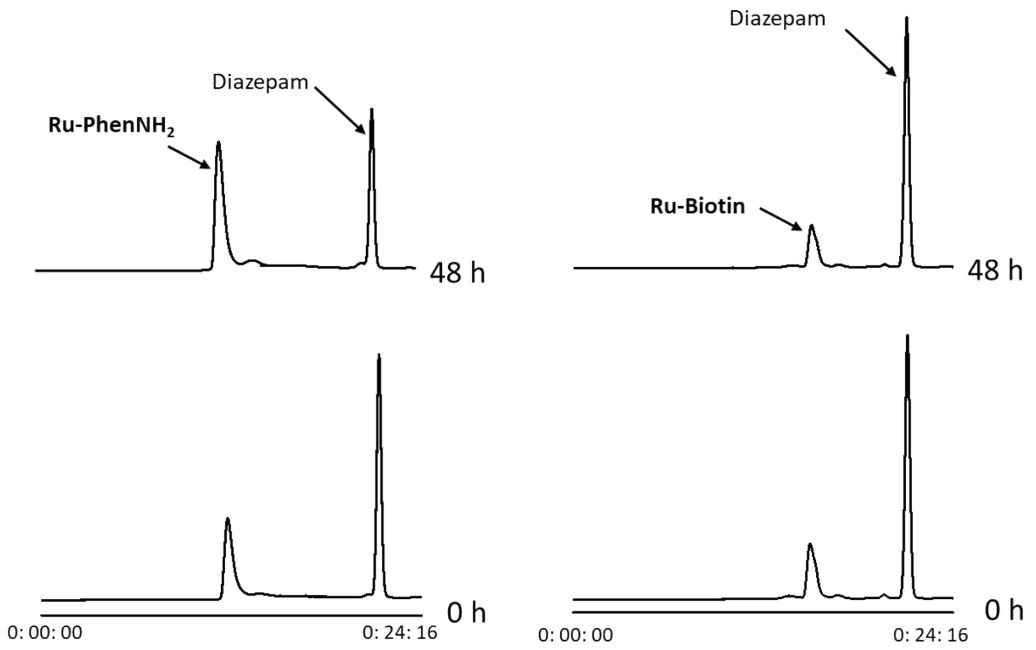

Figure S10 HPLC analysis of **Ru-PhenNH$_2$** and **Ru-Biotin** incubated in FBS for 0 h or 48 h. The contents were tracked by HPLC-UV (Diazepam was used as internal standard)

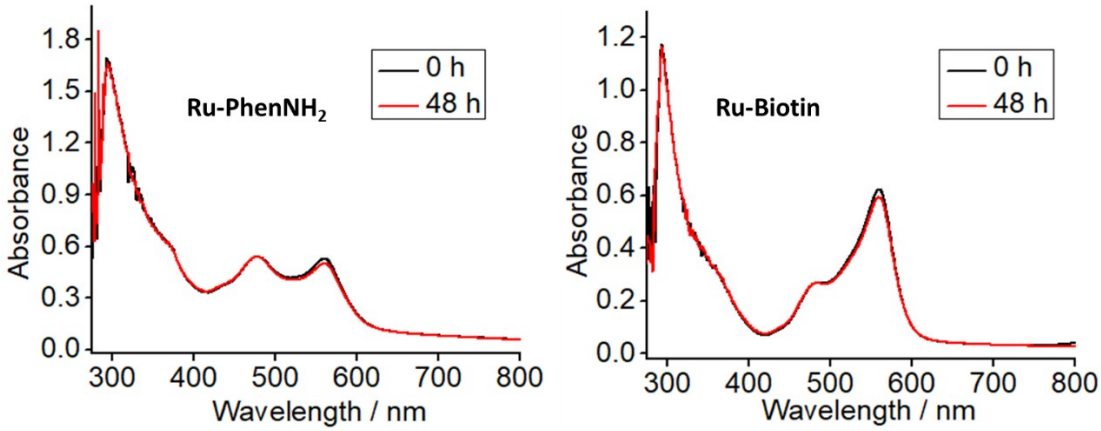

Figure S11 Absorption spectra of **Ru-PhenNH$_2$** and **Ru-Biotin** (10 μM) incubated in DMEM (10% FBS) for 0 h or 48 h.

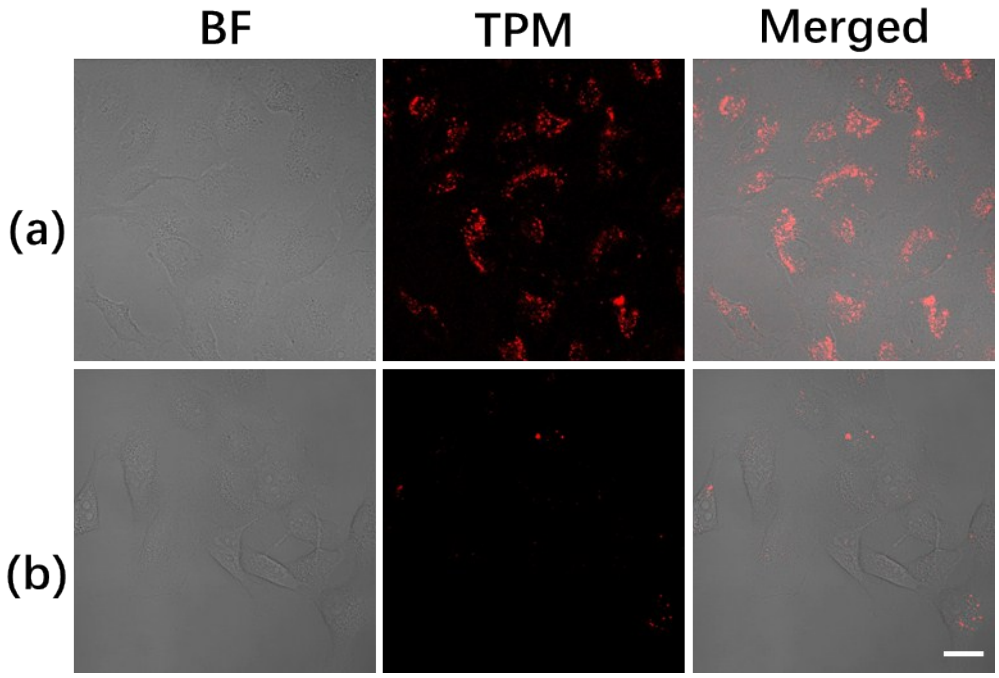

Figure S12 (a) Fluorescence images of A549R cells incubated with **Ru-Biotin** (5 μM) for 3 h. (b) Fluorescence images of A549R cells pretreated with 100 μM of biotin for 2 h, and incubated with **Ru-Biotin** (5 μM) for another 3 h. Scale bar = 20 μm.

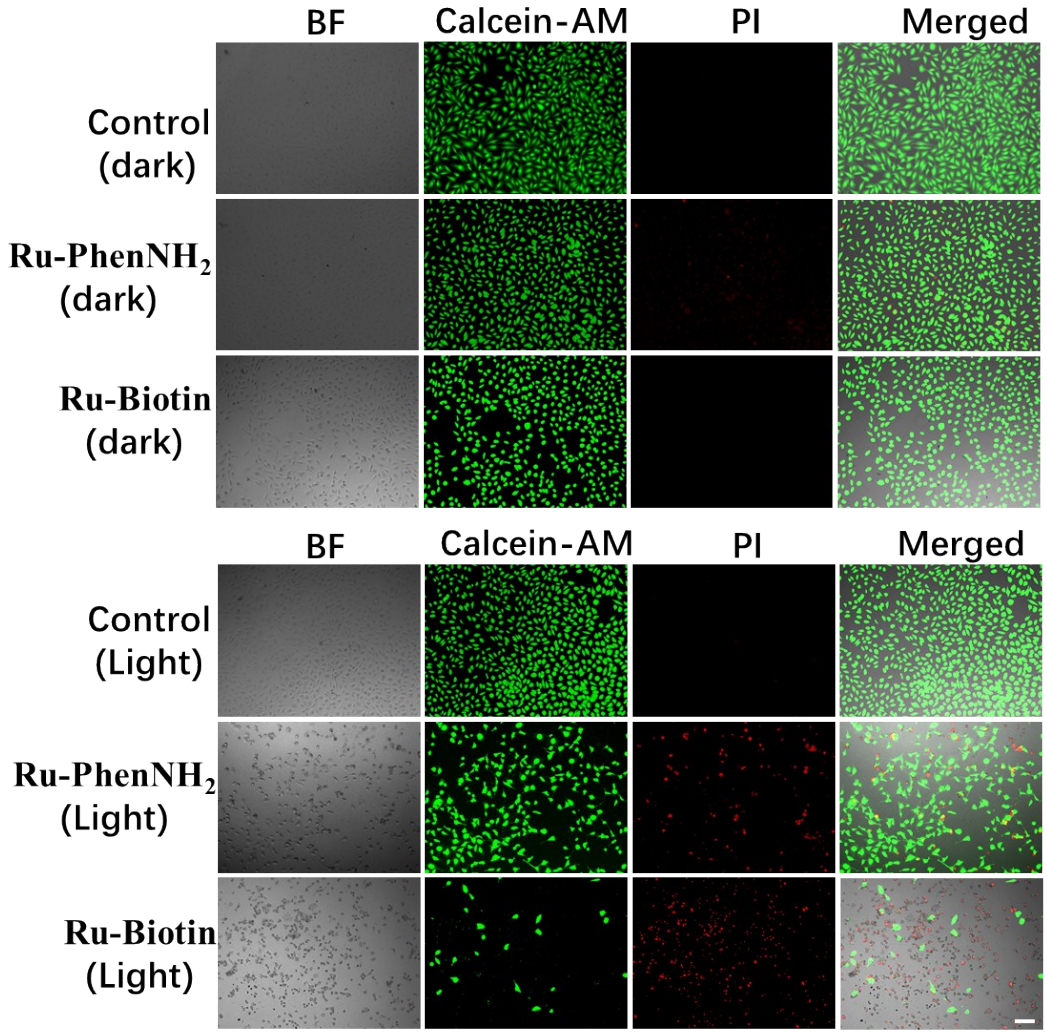

Figure S13 Inverted fluorescence microscope (Zeiss) images of A549R cells pre-incubated with 5 μM **Ru-PhenNH$_2$** and **Ru-Biotin** for 3 h under the dark condition or irradiated at 450 nm by LED area light (20 mW cm^{-2}, light dose = 12 J cm^{-2}), then incubate for 45 h and stained by Calcein-AM/PI under inverted fluorescence microscope (Zeiss), Calcein-AM: λ_{em} = 515 nm, λ_{ex} = 490 nm; PI: λ_{em} = 617nm, λ_{ex} = 536 nm. Scale bar = 100 μm.

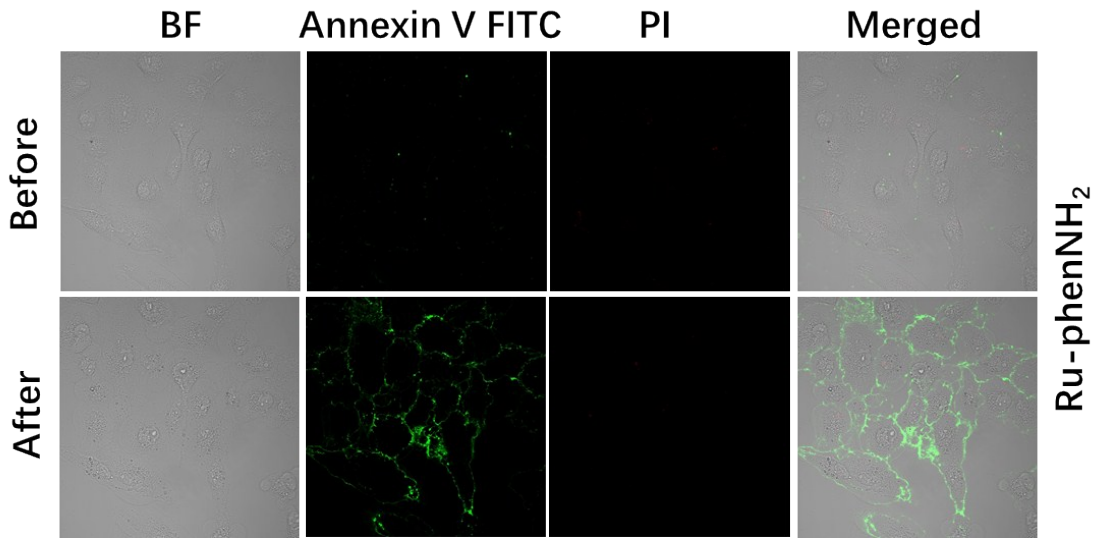

Figure S14 A549R cells were incubated with **Ru-PhenNH$_2$** (5 μM) for 3 h. AV/PI co-staining on A549R cells before or after two-photon irradiation at 820 nm (0.27 mW cm^{-2}, 80 MHz, 100 fs) for 2 min; Annexin V-FITC: λ_{em} = 500 - 530 nm, λ_{ex} = 488 nm; PI: λ_{em} = 600 - 630 nm, λ_{ex} = 536 nm. Scale bar: 30 μm.

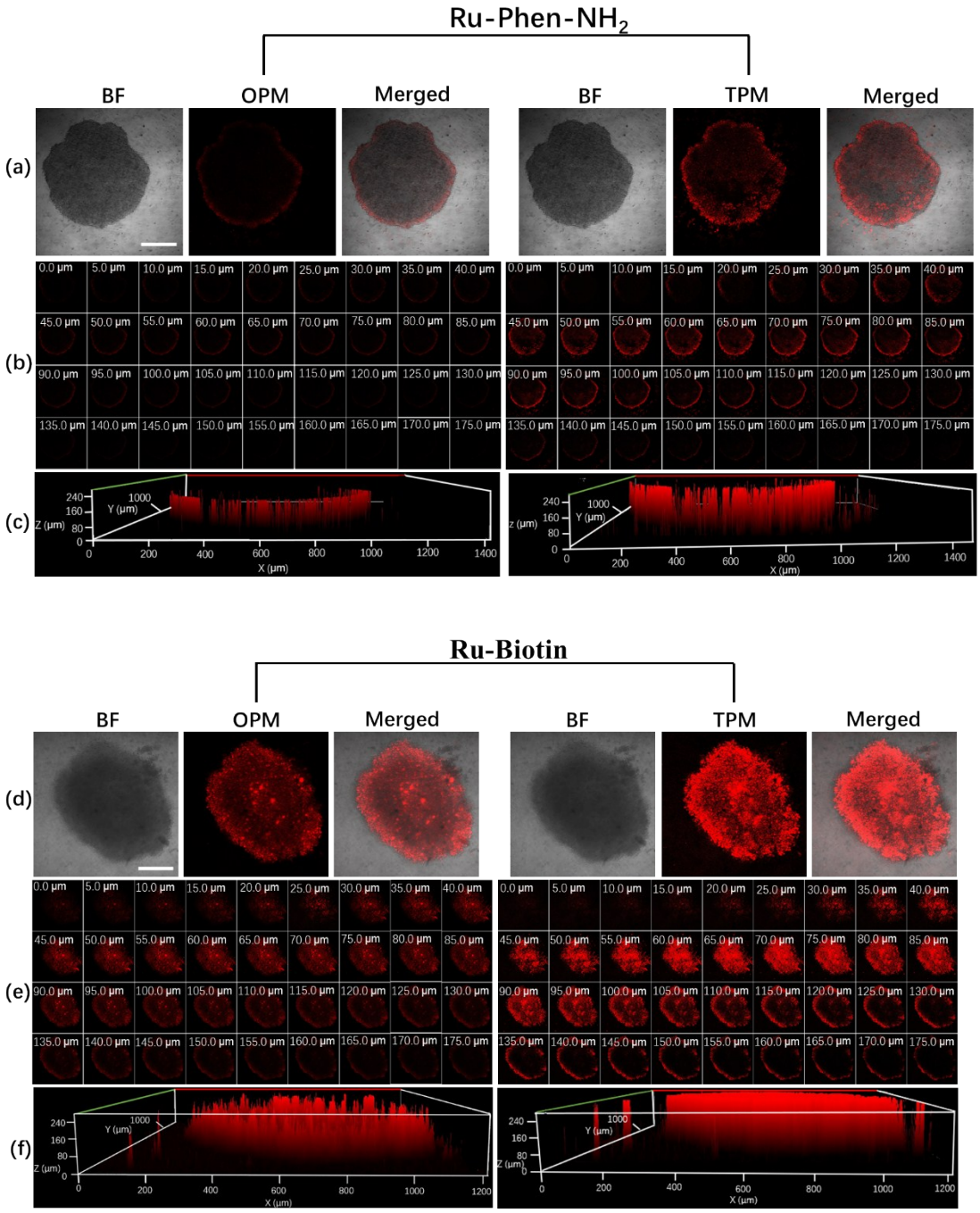

Figure S15 (a) and (d) One- and two-photon excited Z-stack images of 3D tumor MCTSs after incubation with **Ru-PhenNH$_2$** and **Ru-Biotin** (5 μM, 6 h). (b) and (e) The substrate of OPM and TPM Z-axis scanning images captured every 5 μm from the top to bottom of an intact 600 μm spheroid. (c) and (f) The one- and two-photon 3D Z-stack images of an intact spheroid. The excitation wavelengths of OPM and TPM were set with 450 nm and 820 nm, respectively. Scale bars: 200 μm.

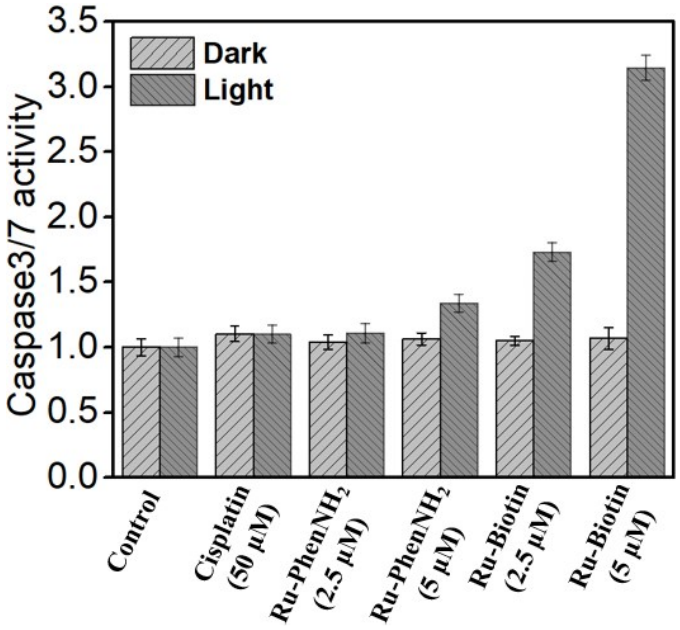

Figure S16 Caspase 3/7 actively measurements for A549R were pre-incubated with cisplatin (50 μM) and **Ru-PhenNH$_2$** and **Ru-Biotin** (2.5 μM, 5 μM) for 3 h with/without 450 nm. Vertical axis indicates the amount of caspase-3/7 activity for each treatment, measured amount of caspase-3/7 activity for control (untreated) complexes was considered equivalent to 1 so that those of treated complexes are reported compared to 1. Data are reported as mean values ± S.D. of three independent experiments.