Supplementary Information For

Two-photon photodynamic ablation of tumour cells using an RGD peptide-conjugated ruthenium(II) photosensitiser

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Experimental Section

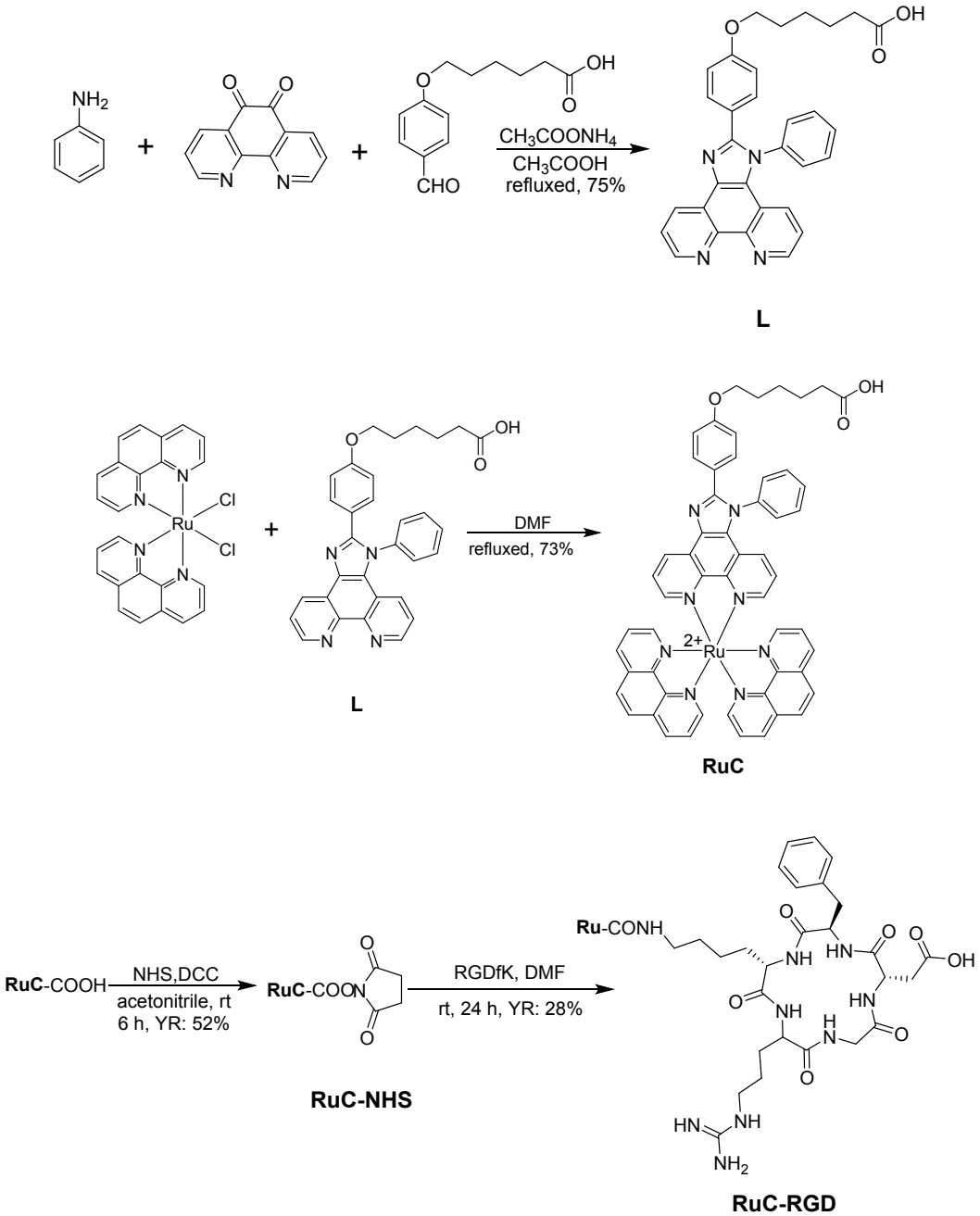
Materials

All reagents were purchased from commercial sources and used without further purification unless otherwise specified. Ruthenium chloride hydrate, 4-formylbenzoic acid, aniline, anhydrous *N,N*-dimethylformamide (DMF), N-hydroxysuccinimide (NHS), *N,N'*-dicyclohexylcarbodimide (DCC), 1,3-diphenyliso-benzofuran (DPBF), phosphate buffer saline (PBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), H_2TPP, 2,7-dichlorodihydro-fluorescein diacetate (DCFH-DA), and 1,10-phenanthroline (phen) were all purchased from Sigma-Aldrich (St Louis, MO, USA). Dulbecco's modified eagle medium (DMEM) with high glucose, fetal bovine serum (FBS), penicillin, streptomycin, MitoTracker Green (MTG), LysoTracker Green (LTG), and Hoechst 33342 were purchased from Thermo Fisher Scientific (Waltham, MA, USA). RGD peptides, Cyclo (RGDfK), were purchased from ChinaPeptides (Co., Ltd. Shanghai, China) The ligand 1,10-phenanthroline-5,6-dione[1], 6-(4-formylphenoxy)hexanoic acid[2] and *cis*-$Ru(phen)_2Cl_2$[3] were prepared according to literature methods.

General instrumentation

An LCQ system (Finnigan MAT, U.S.A.) was used for electrospray ionisation mass spectrometry (ESI-MS) analyses. A nuclear magnetic resonance (NMR) spectrometer (Bruker Avance III, 400 mHz and 500 mHz) was used to obtain NMR spectra; tetramethylsilane (TMS) was used as standard. ESR spectra were measured with 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. The inductively coupled plasma mass spectrometry (ICP-MS) experiments were carried out on an Agilent 7700x instrument (Thermo Elemental Co., Ltd.). Visible one-photon irradiation (λ_{irr} = 450 nm, 40 mW/cm^2, 100% power) in PDT was provided by a commercially available LED visible area light source (Height LED Instruments, China). Two-photon absorption cross section measurements and two-photon *in vivo* PDT were performed in an open light pathway by the excitation of a modelocked Ti: Sapphire laser (pulse width 35 fs, Coherent Co., Ltd., USA).

Synthesis and characterization



Scheme S1. Synthetic routes of RGD peptide conjugated Ru(II) complex (**RuC-RGD).**

Synthesis of ligand L

6-(4-(1-phenyl-1*H*-imidazo[4,5-*f*][1,10]phenanthrolin-2-yl)phenoxy)hexanoic acid (**L**)

A mixture of glacial acetic acid (10 mL), aniline (0.093 g, 1 mmol), 6-(4-formylphenoxy)hexanoic acid (0.236 g, 1 mmol), ammonium acetate (1.542 g, 20 mmol), and 1,10-phenanthroline-5,6-dione (0.21 g, 1 mmol) was refluxed under argon for 24 h. The reaction mixture was then cooled to room temperature and poured into water (20 mL). The solution was neutralised with a 25% NH_3 solution. Dichloromethane (DCM) was added to the solution and the organic materials were extracted in the organic layer that was rotary evaporated to produce a yellow crude product. The crude product was then purified using column chromatography (DCM/ethanol) to obtain the ligands (yield = 75%). The ligand was characterised by ^{1}H NMR and ESI-MS measurements. ^{1}H NMR (DMSO-d_6): δ (ppm) 9.14-8.90 (m, 3H), 7.87 (dd, J = 7.9, 4.3 Hz, 1H), 7.75 (s, 4H), 7.56-7.44 (m, 3H), 7.34-7.13 (m, 2H), 6.91 (d, J = 8.3 Hz, 2H), 3.96 (t, J = 6.2 Hz, 2H), 2.23 (t, J = 7.2 Hz, 2H), 1.76-1.65 (m, 2H), 1.62-1.50 (m, 2H), 1.47-1.36 (m, 2H); ESI-MS: m/z = 503.15 $[M+H]^+$.

Synthesis of RuC

A mixture of ligand **L** (0.100 mg, 0.2 mmol) and the ruthenium complex precursor $Ru(phen)_2Cl_2$ (0.106 mg, 0.2 mmol) in *N,N*-dimethylformamide (DMF, 10 mL) was heated to 150 °C for 8 h under nitrogen to obtain a deep-red solution. The reaction mixture was then cooled and diluted with 15 mL water. Saturated aqueous ammonium hexafluorophosphate solution was added to the diluted solution under vigorous stirring and then filtered. The dark red solid was collected, washed with a small amount of water and ether, dried under vacuum, and purified on alumina by column chromatography with acetonitrile ethanol as eluent. The solvent was removed under reduced pressure, which yielded red microcrystalline (Yield = 73%). ^{1}H NMR (DMSO-d_6): δ (ppm) 9.19 (d, J = 8.2 Hz, 1H), 8.78 (dd, J = 16.9,

9.1 Hz, 4H), 8.40 (d, *J* = 11.2 Hz, 4H), 8.15-8.01 (m, 5H), 7.96 (d, *J* = 4.9 Hz, 1H), 7.89-7.71 (m, 10H), 7.57-7.47 (m, 3H), 7.39 (d, *J* = 8.3 Hz, 1H), 6.96 (d, *J* = 8.2 Hz, 2H), 3.96 (t, *J* = 6.0 Hz, 2H), 1.86 (t, *J* = 7.0 Hz, 2H), 1.72-1.62 (m, 2H), 1.50-1.40 (m, 2H), 1.39-1.28 (m, 2H); ESI-MS: m/z = 481.95 $[M-2PF_6]^{2+}$.

Synthesis of RuC-RGD

The carboxyl-ended ruthenium complex (0.23 mmol) was dissolved into anhydrous acetonitrile (30 mL) with vigorous stirring. NHS (0.43 mmol) and DCC (0.38 mmol) were then added into the solution and the reaction was allowed to stir at room temperature for 6 h. The product was purified on a silica chromatographic column using 10% methanol-dichloromethane as the mobile phase. The material was lyophilised to yield Ru-NHS as a dark orange solid (Yields = 52%). Ru-NHS (0.016 mmol) was dissolved into degassed anhydrous DMF (0.5 mL) followed by addition of 0.04 g RGDfK (in 4.5 ml degassed PBS pH 7.4). The solution was stirred at room temperature for 24 h. The final product was purified using HPLC and lyophilized in a vacuum to yield an orange red **RuC-RGD** power at a yield of 28%.

RuC-RGD: ^{1}H NMR (DMSO-d_6): δ (ppm) 9.17 (d, *J* = 8.2 Hz, 1H), 8.83-8.74 (m, 4H), 8.40 (d, *J* = 13.9 Hz, 4H), 8.31-7.95 (m, 12H), 7.89-7.69 (m, 13H), 7.61-7.45 (m, 4H), 7.38 (d, *J* = 8.6 Hz, 1H), 7.24-7.12 (m, 6H), 6.95 (d, *J* = 8.8 Hz, 2H), 4.67 (dd, *J* = 14.5, 8.4 Hz, 1H), 4.46 (dd, *J* = 14.1, 7.2 Hz, 1H), 4.22 (d, *J* = 6.2 Hz, 1H), 4.10 (d, *J* = 7.5 Hz, 1H), 4.04 (d, *J* = 7.2 Hz, 1H), 3.96 (d, *J* = 6.3 Hz, 2H), 3.25 (d, *J* = 12.2 Hz, 1H), 3.12-3.07 (m, 2H), 3.02-2.93 (m, 3H), 2.75 (dd, *J* = 16.1, 8.2 Hz, 2H), 2.35 (dd, *J* = 16.0, 5.5 Hz, 1H), 2.08 (t, *J* = 7.2 Hz, 2H), 1.70 (dd, *J* = 14.1, 7.2 Hz, 2H), 1.52 (m, 6H), 1.38 (dd, *J* = 14.4, 7.2 Hz, 4H), 1.10-1.03 (m, 2H), 0.89-0.75 (m, 2H). ^{13}C NMR (DMSO-d_6) δ (ppm) 172.53, 172.38, 172.28, 171.39, 170.79, 170.35, 169.76, 160.39, 158.90, 158.65, 158.40, 158.14, 157.31, 154.16, 153.21, 153.18, 153.10, 153.07, 151.52, 150.70, 147.71, 147.66, 147.58, 147.51, 146.11, 146.09, 138.10, 137.35, 136.61, 131.56, 131.32, 131.17, 130.97, 130.93, 129.60, 129.27, 129.22, 128.51, 128.21, 128.10, 127.18, 126.83, 126.72, 126.63,

126.02, 125.82, 121.80, 121.59, 118.72, 116.33, 114.91, 68.02, 55.13, 54.59, 51.99, 49.23, 43.67, 40.77, 38.63, 37.84, 35.80, 31.78, 28.99, 28.80, 25.63, 25.50, 23.33, 0.57. ESI-MS: m/z = 775.2 $[M-2PF_6]^{2+}$.

Cell lines and cell culture

Human glioblastoma (U87MG) and human breast cancer (MCF-7) cell lines were obtained from the Cell Bank (Cell Institute, Sinica Academia Shanghai, Shanghai, China). Cells were cultured (in 25 cm^2 culture flasks) in DMEM supplemented with 10 % fetal bovine serum, penicillin (100 units/mL), and streptomycin (50 units/mL) in a CO_2 incubator (95% relative humidity, 5% CO_2) at 37 °C. The cells were used when they attained the logarithmic growth phase.

Cellular uptake and distribution

Inductively coupled plasma mass spectrometry (ICP-MS) was employed to quantify the amount of **RuC** or **RuC-RGD** in different cell lines and subcellular compartments, as described in our previous report[4]. Cells were cultured in 25 cm^2 culture plates (Corning) and incubated for 24 h with the **RuC** or **RuC-RGD** at a concentration of 20 μM. The **RuC** + **RGD** and **RuC-RGD** + **RGD** groups were pre-incubated with excess RGD peptide (10 μg/mL) before the **RuC** or **RuC-RGD** were added. After incubation, the whole cell, the mitochondria, the cytoplasm (without mitochondria), and nuclear fractions were obtained separately using mitochondrial and nuclear isolation kits. The samples were then digested with 60% HNO_3 for 24 h and analysed by ICP-MS. Aliquots were removed and used to determine the protein concentration using the BCA protein assay reagent (Pierce, Rockford, IL). The data are reported as the mean ± standard deviation (n = 3) and the results are reported as nanograms of ruthenium per milligram of cellular protein.

Cellular localisation analysis

U87MG cells were seeded on 35 mm glass-bottomed culture dishes when a confluency of approximately 60% was achieved. The cells were then incubated for 24 h with **RuC**

or **RuC-RGD** at a concentration of 20 μM. A mitochondria-specific green fluorescent probe MitoTracker Green (MTG, 5 μg/mL), or a lysosome a-specific green fluorescent probe LysoTraker Green (LTG, 5 μg/mL) and nuclei stain probe Hoechst 33342 (10 μg/mL) were then added to the dishes, which were incubated for 30 min in the dark. The cells were then washed with 1 mL of PBS before the samples were observed under a confocal scanning microscope (LSM 880, Carl Zeiss, Oberkochen, Germany). MitoTracker Green (λex = 488 nm, λem = 510-540 nm), LysoTraker Green (λex = 488 nm, λem = 510-540 nm) and Hoechst 33342 (λex = 405 nm, λem = 410-450 nm).

Determination of two-photon absorption cross-sections

Measurements of two-photon cross-sections were performed by a multiphoton excited phosphorescence method using rhodamine B as the reference using Ti: Sapphire laser (Coherent Legend Elite, pulse width = 35 fs, repetition rate =1k Hz, tuning range 720-920 nm, Coherent Inc. U.S.A.). Two-photon luminescence was measured in fluorometric quartz cuvettes. The quadratic dependence of the two-photon-induced luminescence intensity on the excitation power was verified at excitation wavelengths of 820 nm for **RuC** and **RuC-RGD**, respectively. The two-photon absorption cross-section of the probes was calculated at each wavelength according to Equation (1): [5]

$$\delta_2 = \delta_1 \frac{\phi_1 C_1 I_2 n_2}{\phi_2 C_2 I_1 n_1} \qquad (1)$$

where, I is the integrated luminescence intensity, C is the concentration, n is the refractive index, and φ is the quantum yield. The subscript '1' refers to the reference samples, and '2' the experimental samples.

Formation and imaging of MCTSs

Formation and imaging of multicellular tumour spheroids (MCTSs) were performed as described in our previous report[6]. A suspension of 1% agarose in DMEM was sterilised by high pressure and high temperature for 20 min. The gel

was added into 96-well micro 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 micro culture plates with a volume of 200 μL/well. MCTSs aggregates that were approximately 400 μm in diameter were formed after 3 days. The cell culture media in the wells was refreshed every two days. To investigate the depth of the complexes under one-photon (OP) and two photon (TP) lasers, the 3D MCTSs were treated with **RuC-RGD** (20 μM) for 24 h. The MCTSs were imaged by a confocal microscope (LSM 880, Carl Zeiss, Oberkochen, Germany). The excitation wavelength of OP was 450 nm and that of TP was 820 nm. The OP and TP excited luminescent images of sections along the z-axis were captured and stacked in the z-stack imaging mode.

(Photo) cytotoxicity in monolayer cells and 3D MCTSs

(Photo) cytotoxicity of **RuC** and **RuC-RGD** in monolayer cells was measured using the MTT assay[6].Exponentially grown MCF-7 and U87MG cells were seeded into 96-well culture plates at a density of 1×10^4 cells/well and incubated at 37 °C for 24 h. The cells were then treated with of the indicated concentrations of the test compounds and incubated for 24 h. Then the test culture media were replaced with fresh medium. The OP light group was exposed to LED area light irradiation (450 nm, 20 mW/cm^2, 10 min), whereas the TP light group was exposed to a Laser Scanning Confocal Microscope (820 nm, 25 mW, 120 s) while the dark control was placed in the dark. All groups were incubated for an additional 24 h. MTT solution (5 mg/mL) was added to each well and incubated for 4 h, and the resulting formazan was dissolved in DMSO. The optical density was measured on a microplate spectrophotometer at a wavelength of 595 nm. The half-maximal inhibitory concentration (IC_{50}) values were determined by a series of the inhibition rate versus the dose used to treat the cells[4].

To evaluate the (photo)toxicity to the 3D MCSTs, the 3D U87MG MCTSs were incubated with increasing concentrations of **RuC** or **RuC-RGD** in the dark for 24

h. Then the test culture media were replaced with fresh medium. After that, MCTSs were treated with 820 nm TP irradiation (25 mW, 120 s), then the samples were incubated in the dark for an additional for 24 h. The cytotoxicity of **RuC** or **RuC-RGD** to 3D MCST was measured by determining the cell ATP concentration with the CellTiter-Glo® 3D Cell Viability kit (Promega).

For detecting the kinetics of 3D MCTSs regrowth, the MCTSs were treated with **RuC** (20 μM) or **RuC-RGD** (20 μM) on day 2 respectively. After being incubated for 24 h, the culture media was replaced with fresh culture media, and the laser group was exposed to 820 nm TP irradiation (25 mW, 120 s) on day 3. The samples were then incubated in the dark for an additional 3 days. The images of 3D MCTSs were recorded every 24 h over 6 days using an inverted fluorescence microscope (Zeiss, Model Axio Observer D1, Germany).

3D MCTSs growth inhibition and viability assay

The U87MG 3D MCTSs viability assay was performed using the Live/Dead viability/cytotoxicity kit for mammalian cells (Life Technologies) as described in a previous report[6]. The ubiquitous intracellular esterase in live cells could convert nonfluorescent cell-permeant calcein acetoxymethyl (AM) to the intensely green fluorescent calcein (the determination of cell viability is dependent on these properties, whereby MCTSs show stronger green fluorescence when more cells survive). After MCTSs were treated with **RuC-RGD** (20 μM, 24h) and an 820 nm laser irradiation (25 mW, 120 s), the MCTS were incubated with calcein AM (2 μM) solutions for 60 min and imaged using an inverted fluorescence microscope (Zeiss, Model Axio Observer D1, Germany).

Western blot assays

Western blot assays were performed as described in our previous report[4]. Briefly, U87MG MCTSs were treated with **RuC-RGD** (5, 10, and 20 μM) in the dark for 24 h. The tested culture media was then replaced with fresh medium and the MCTSs were treated with 820 nm TP irradiation (25 mW, 120 s), following which

the samples were incubated in the dark for an additional 24 h. The MCTSs were collected and washed twice with ice-cold PBS and lysed in RIPA buffer to extract the total cellular proteins. The cytosol protein extracts were then prepared for the analysis of cytochrome c level in the cytosol (C-Cyto-C). The protein extracts were resolved using 10% SDS-PAGE gels loaded with equal amounts of proteins per lane. Next, the proteins were transferred onto PVDF transfer membranes (Millipore, Bedford, MA), which were then blocked with 5% non-fat milk in TBST buffer, and shaken on a shaker for 1 h at room temperature. The membranes were then probed with primary antibodies at a 1:1000 dilution in 5% non-fat milk overnight at 4 °C and then with secondary antibodies (goat anti-rat, Santa Cruz Biotechnology, Santa Cruz, CA) conjugated with horseradish peroxidase at a 1:2000 dilution for 1 h at room temperature. The protein bands were visualised using ECL detection reagents according to the instructions for the SuperSignal West Pico kit.

Reactive oxygen species (ROS) detection

To detect the intracellular generation of ROS by **RuC-RGD** under irradiation of TP laser, 3D U87MG MCTSs were treated with **RuC-RGD** (20 μM, 24h) in the dark. The culture medium was then changed with PBS containing DCFH-DA (5.0 μM) and incubated for 30 min, following which the culture medium was replaced with PBS and subjected to TP irradiation (25 mW, 120 s). Fluorescence imaging was obtained before and after the irradiation. The excitation wavelength was 488 nm and the emission wavelength was between 510 and 550 nm.

Quantification of singlet oxygen ($^1O_2$) generation

The $^1O_2$ quantum yields (Φ_Δ) of **RuC** and **RuC-RGD** were examined by monitoring the photooxidation of DPBF sensitised according to a previous report[7]. The solutions containing **RuC** (10 μM) or **RuC-RGD** (10 μM) and DPBF (50 μM) were aerated with nitrogen for 10 min. The solutions were then irradiated at 450 nm (20 mW/cm^{-2}) for different times. The absorbance at 450 nm of ruthenium

compounds was kept at 0.1. The absorbance of DPBF at 418 nm was recorded every 2 s. $[Ru(bpy)_3]^{2+}$ was used as the standard and the $^1O_2$ formation quantum yield in methanol was 0.81[7]. The $^1O_2$ quantum yields of the ruthenium complexes was determined according to equation (2):

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

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

Electron spin resonance (ESR) assay

To examine the generation of ROS by **RuC** and **RuC-RGD** under irradiation, 2,2,6,6-tetramethylpiperidine (TEMP) was used as spin trap reagents. **RuC** (10 μM) or **RuC-RGD** (10 μM) were dissolved in aerated methanol containing 10 mM TEMP. The ESR spectroscopy (Bruker model A300 spectrometer, 298 K) of these samples was recorded under the photoirradiation of a 450 nm area light source for 5 min. A methanol solution containing TEMP alone was used as the control group.

***In vivo* therapy**

Balb/c_(nu/nu) female nude mice aged 4-5 weeks were purchased and bred in the experimental animal center, School of Life Sciences, Sun Yat-sen University. All experimental protocols received prior approval by the SYSU Animal Care and Use Committee. U87MG xenograft tumours were established by inoculating 2×10^6 cells via subcutaneous injection (s.c.). When the volumes of tumours reached approximately 200 mm^3, the tumours were cut into approximately 1 mm^3 fragments under sterile conditions, then the fragments were transplanted to other mice. When the volumes of tumours reached approximately 60 mm^3, nude mice with well-grown tumours were randomly divided into five groups (six mice per group) before the experiments.

Administration of the photodynamic therapy was as follows: (group 1, control): mice were intravenously injected with the physiological saline solution (0.2 mL/20

g body weight); (group 2, laser): mice were intravenously injected with the physiological saline solution (0.2 mL/20 g body weight) and were irradiated by an 820 nm laser (50 mW, 1 kHz, pulse width 35 fs, 120 s/mm along z axis) 2 h after the injection; (group 3, **RuC** + laser): mice were intravenously injected with **RuC** (0.2 mL/20 g body weight; a dose of 6 μmol/kg body weight) and irradiated by an 820 nm laser (50 mW, 1 kHz, pulse width 35 fs, 120 s/mm along z axis) 2 h after the injection; (group 4, **RuC-RGD**): mice were intravenously injected with **RuC-RGD** (0.2 mL/20 g body weight; a dose of 6 μmol/kg body weight); (group 5, **RuC-RGD + laser**): mice were intravenously injected with **RuC-RGD** (0.2 mL/20 g body weight; a dose of 6 μmol/kg body weight) and irradiated by an 820 nm laser (50 mW, 1 kHz, pulse width 35 fs, 120 s/mm along z axis) 2 h after the injection. The same laser treatment was administered to groups 2, 3, and 5. The first time of administration of PDT was set as day 0, and PDT was repeated four times on days 0, 6, 12, and 18. The mice were anesthetised prior to receiving two-photon PDT. Mice with tumours were photographed with a digital colour camera on days 0, 10, and 20. The tumour sizes and body weight of the mice were measured every four days. For tissue distribution analysis, organs including heart, liver, spleen, lung, kidney, intestines, brain, and tumours from three different mice in the groups 3 and 5 were collected after the animals were sacrificed. Dissected organs and tumours were weighed and digested with concentrated nitric acid, and the concentration of ruthenium was determined by ICP-MS. Tumour volume (V) was calculated as $V = (\text{length} \times \text{width}^2) / 2$. Tumour inhibition rate (TIR) was calculated as TIR = (tumour volume of control group - tumour volume of treated group) / tumour volume of control group) × 100%[8].

Histological examination

All the animals were sacrificed at the end of the *in vivo* PDT test and the tumours from different treatment groups were collected immediately for histological examination by haematoxylin and eosin (H&E) stain[8]. The samples were

immersed in 4% paraformaldehyde at 4 °C and 6 μm sections were prepared from paraffin-embedded samples, which were then stained with H&E. The primary organs including, ovary, intestine, brain, lung, heart, spleen, kidney, and liver were also resected. The tissue structure and cellular morphology of the sections were observed and photographed using an Olympus microscope.

Statistical analysis

Data are presented as mean ± standard deviation. Data from each experiment was statistically analysed by the Student-Newman-Keuls analysis of variance. All statistical analyses were performed using the SPSS version 19.0 software. Differences were considered significant at $P < 0.05$.

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Supporting Figures and Tables

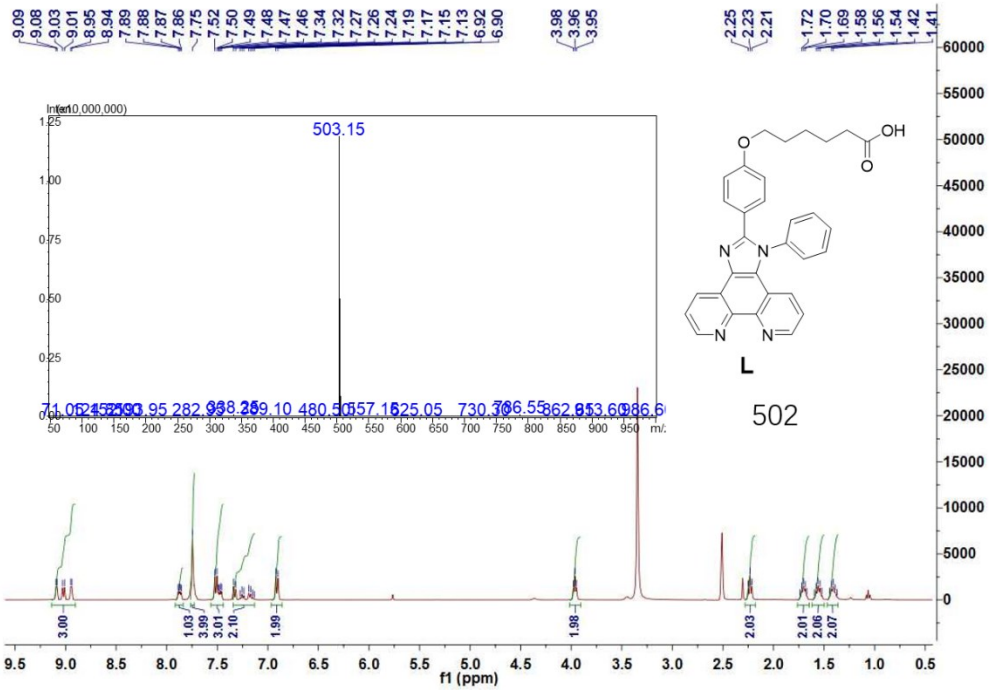

Fig. S1. ESI-MS spectrum and ^{1}H NMR spectrum of **L**

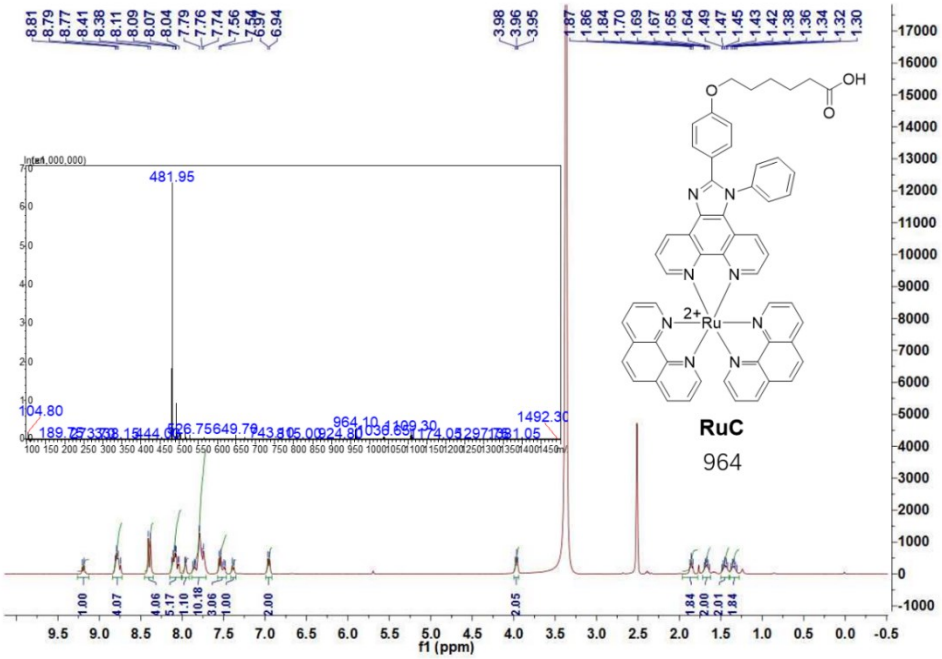

Fig. S2. ESI-MS spectrum and ^{1}H NMR spectrum of **RuC**.

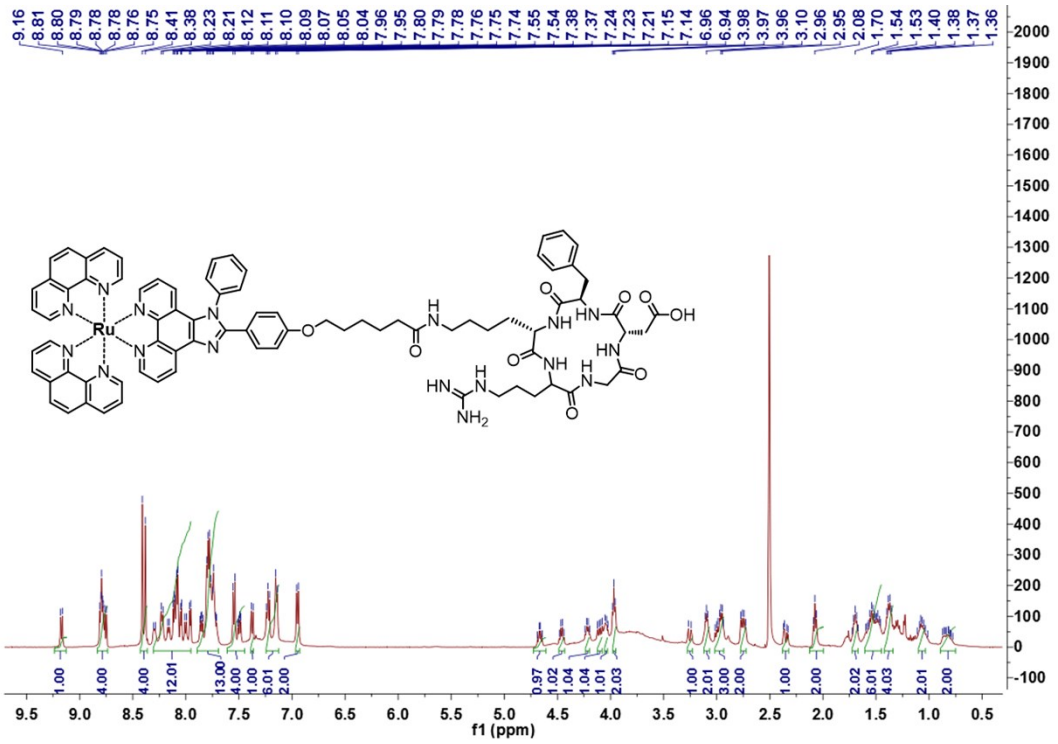

Fig. S3. ^{1}H NMR spectrum of **RuC-RGD**.

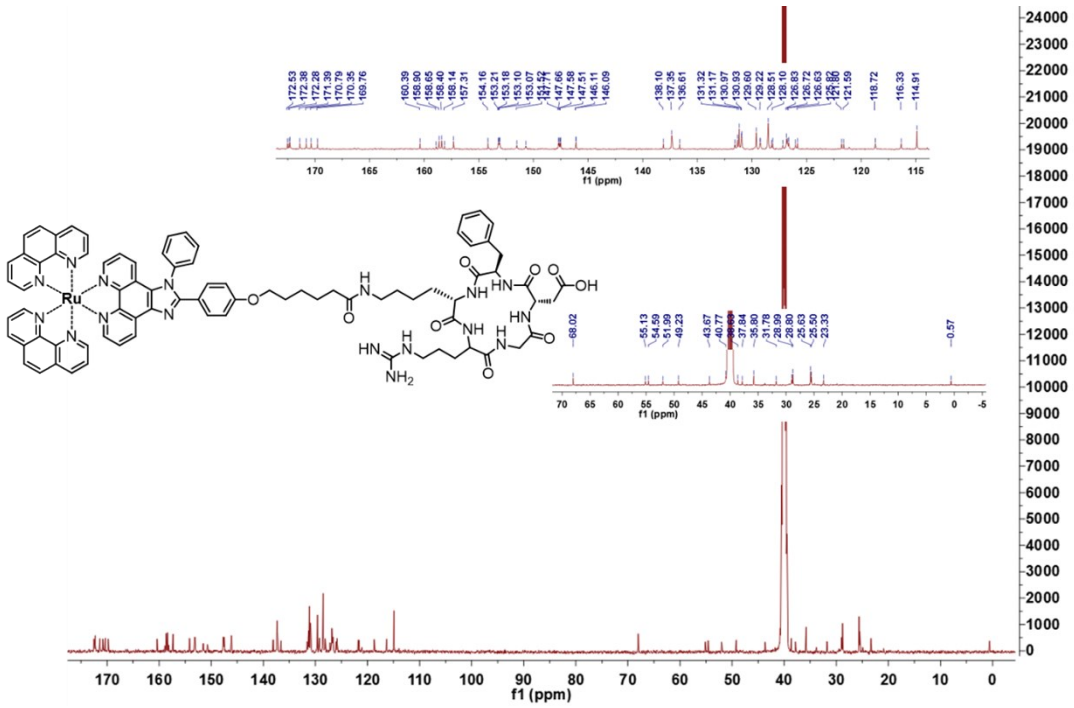

Fig. S4. ^{13}C NMR spectrum of **RuC-RGD**.

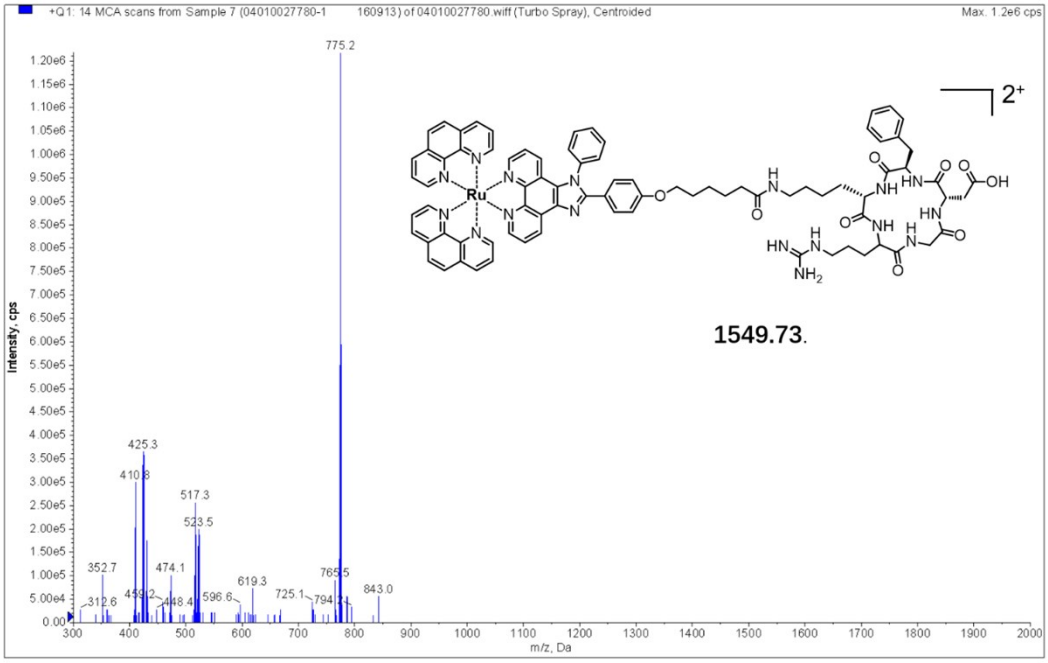

Fig. S5. ESI-MS spectrum of **RuC-RGD**.

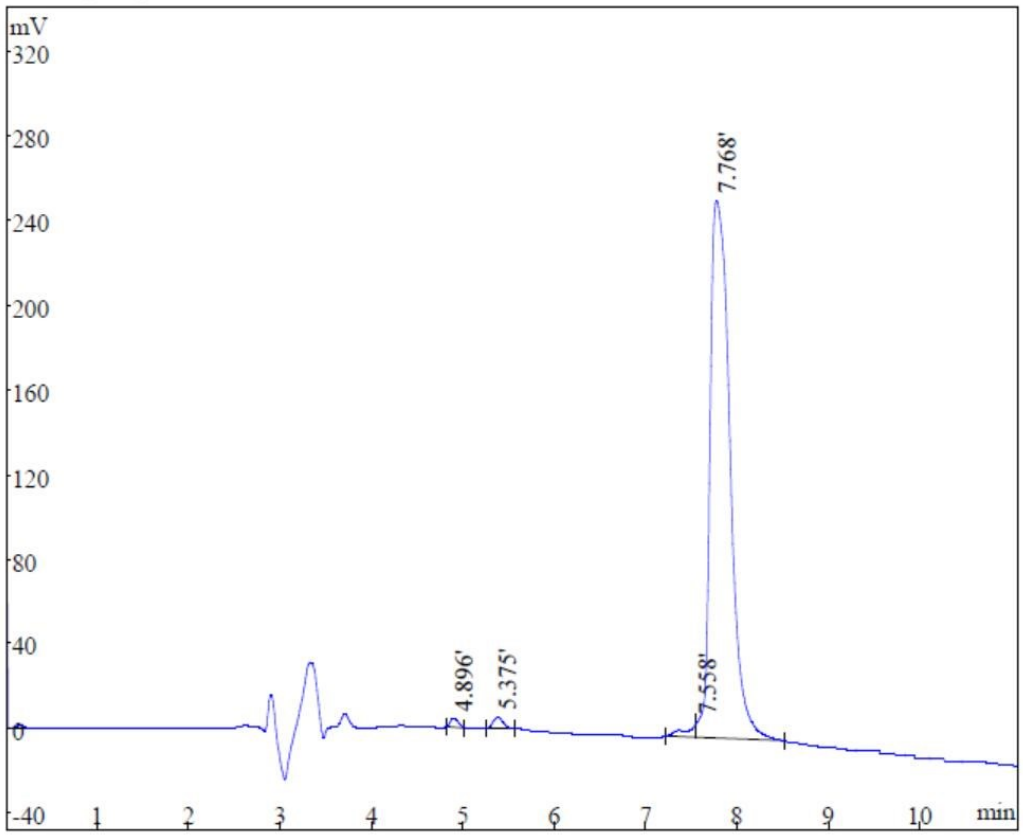

Fig. S6. HPLC traces of **RuC-RGD**.

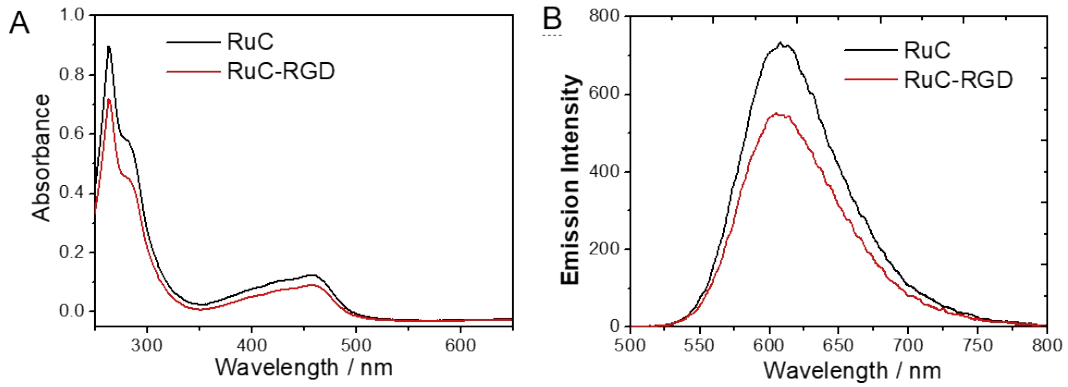

Fig. S7. A: The absorption spectra of 10 μM **RuC** and **RuC-RGD** in PBS. B: The emission spectra of 10 μM **RuC** and **RuC-RGD** in PBS.

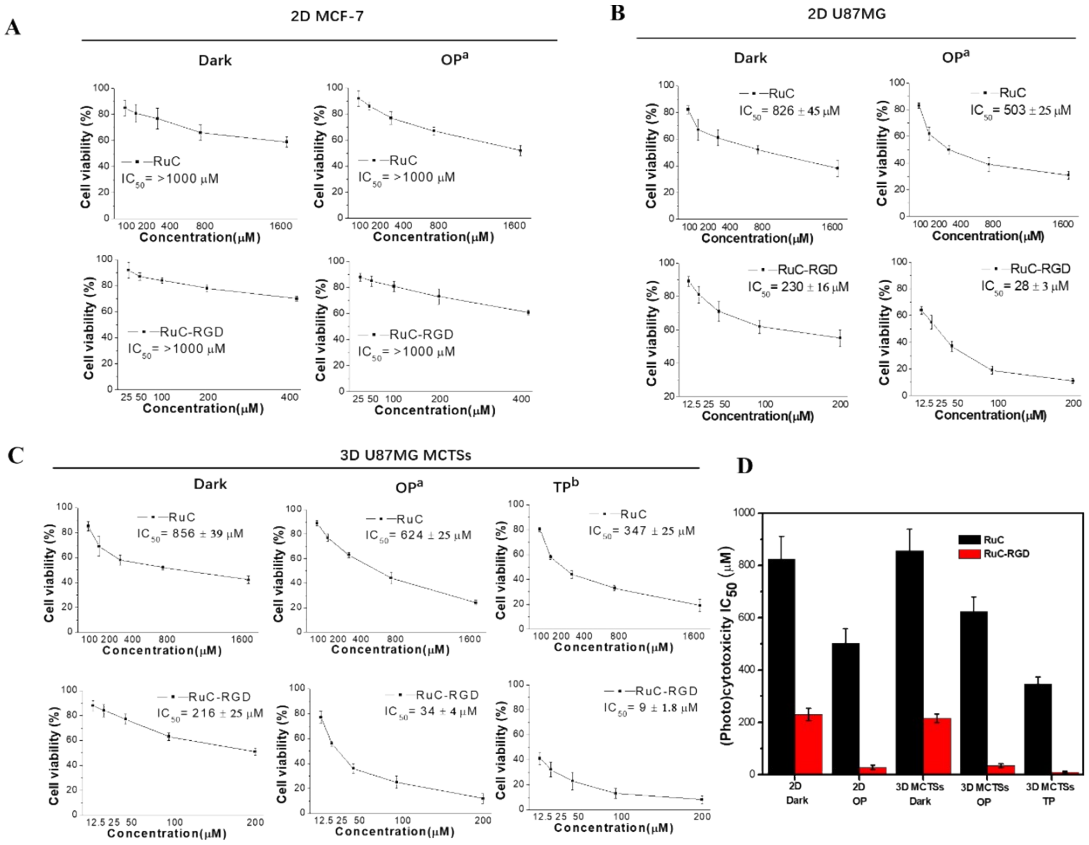

Fig. S8. A) MCF-7 2D monolayer cells viability after being treated with the indicated concentrations of **RuC** or **RuC-RGD** under dark or OP light for 24 h. B) U87MG 2D monolayer cells viability after being treated with the indicated concentrations of **RuC** or **RuC-RGD** under dark or OP light for 24 h. C) U87MG 3D MCTSs viability after being treated with the indicated concentrations of **RuC** or **RuC-RGD** under dark or irradiation for 24 h. D) (Photo) cytotoxicity (IC_{50} [μM]) to U87MG monolayer cells and MCTSs. [a]Irradiated by an LED area light (450 nm, 20 mW/cm^2, 10 min). [b]Irradiated at 820 nm TP irradiation (25 mW, 120 s). Data represent mean values from three replicates.

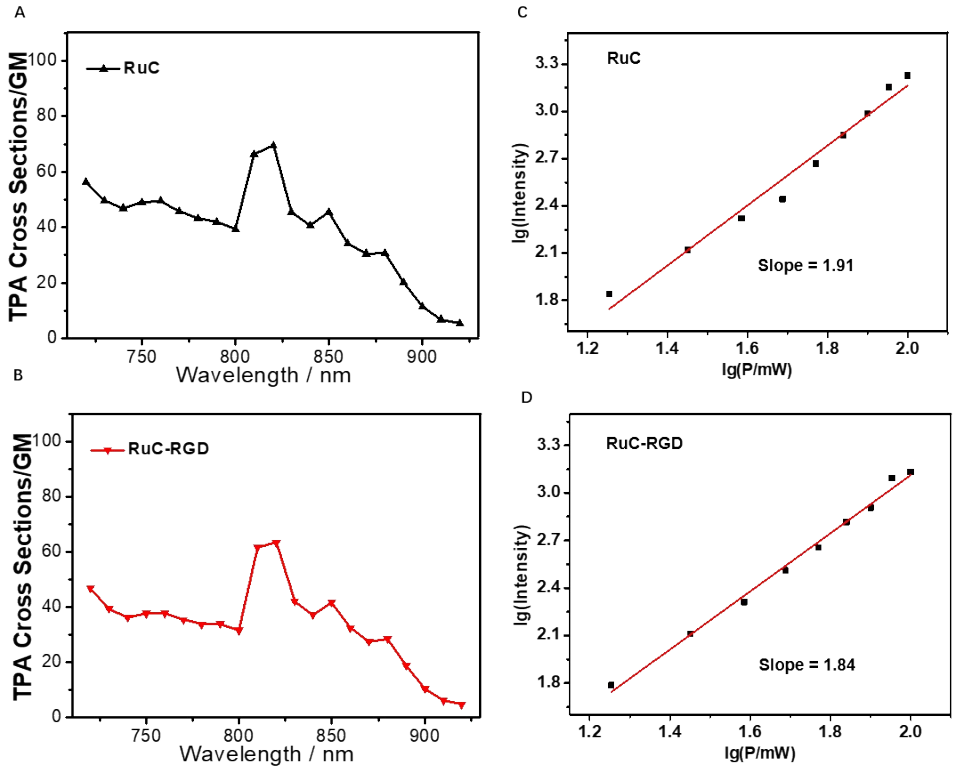

Fig. S9. A-B: Two-photon absorption cross sections of **RuC** and **RuC-RGD** at different excitation wavelengths from 700 to 950 nm in methanol. C-D: The logarithmic plots of the power dependence of relative two-photon induced luminescence intensity of sections **RuC** and **RuC-RGD** as a function of pump power at an excitation wavelength of 820 nm. The solid lines are the best-fit straight lines with gradient, slope = 1.84 and 1.91, indicating that **RuC** and **RuC-RGD** is two-photon excitation active.

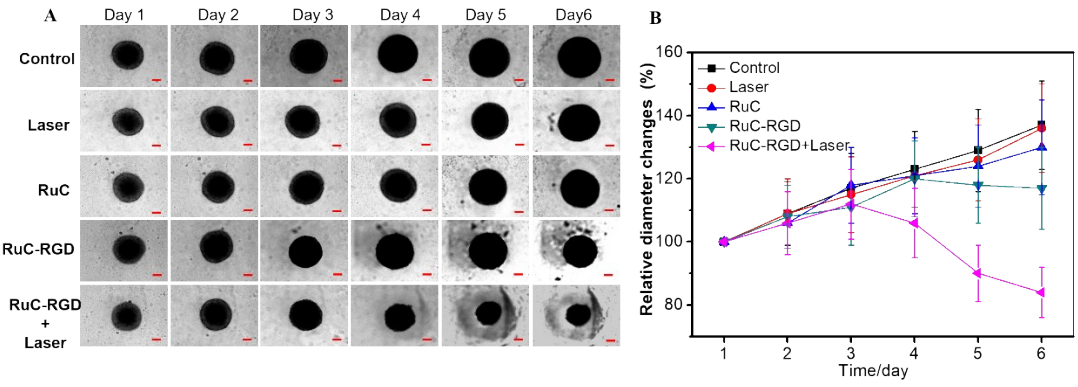

Fig. S10. A) Diameter change of 3D U87MG MCTSs after different treatment. The MCTSs were subjected to two-photon laser irradiation (25 mW, 120 s) at 820 nm on Day 3. B) Variation over time of the relative changes in the diameter of U87MG MCTSs in different treatment groups. Scale bar: 100 μm.

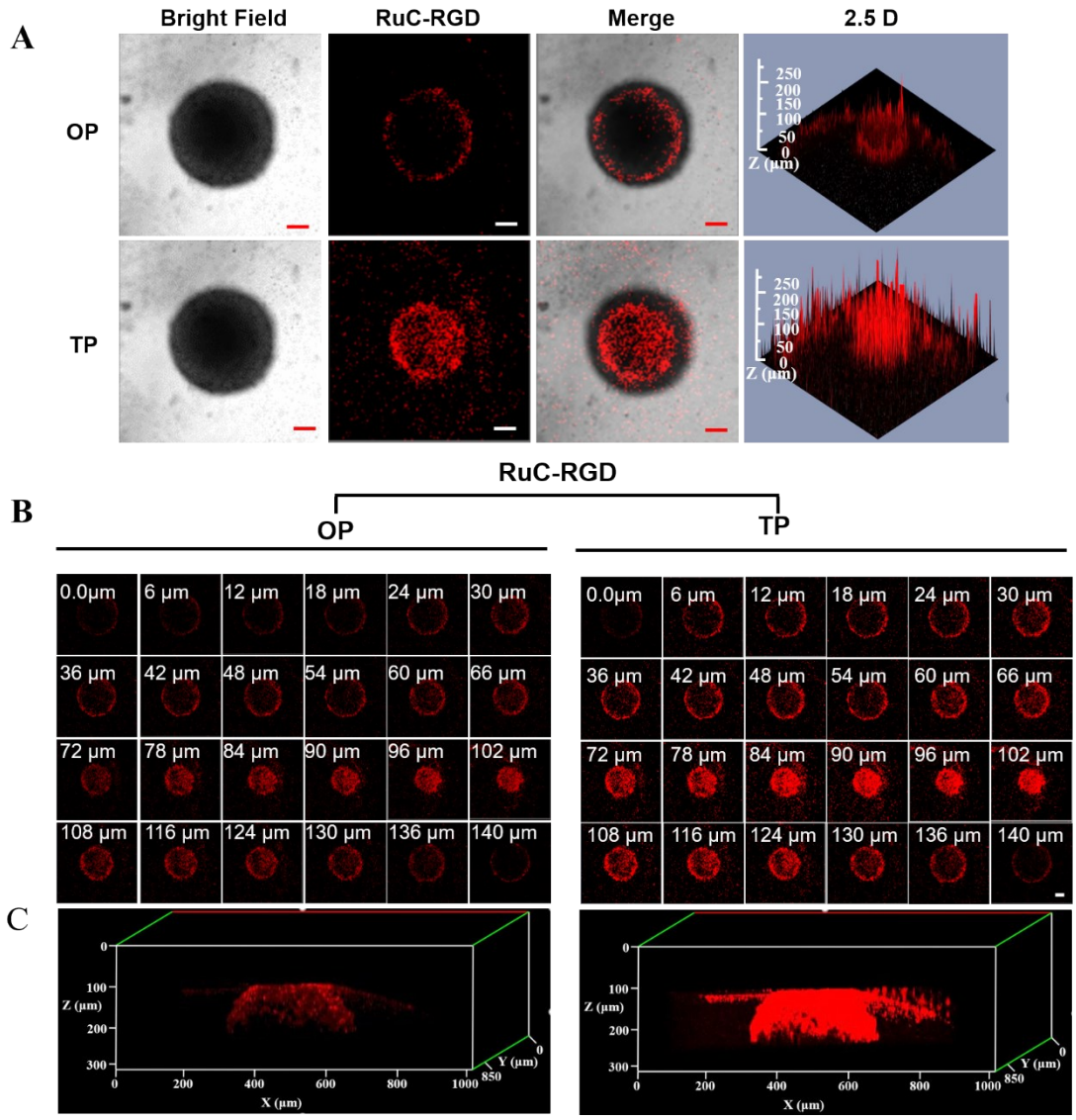

Fig. S11. A) One- and two-photon excited images of U87MG MCTSs after incubation with RuC-RGD (20 μM) for 24 h. B) Z-stack images of OP and TP Z-axis scanning images captured every 6 μm from the top. C) The OP and TP 3D Z-stack images of an intact spheroid. 3D U87MG MCTSs were incubated with **RuC-RGD** (20 μM) for 24 h. The excitation wavelengths of OP and TP were 450 and 820 nm, respectively. Scale bars: 100 μm.

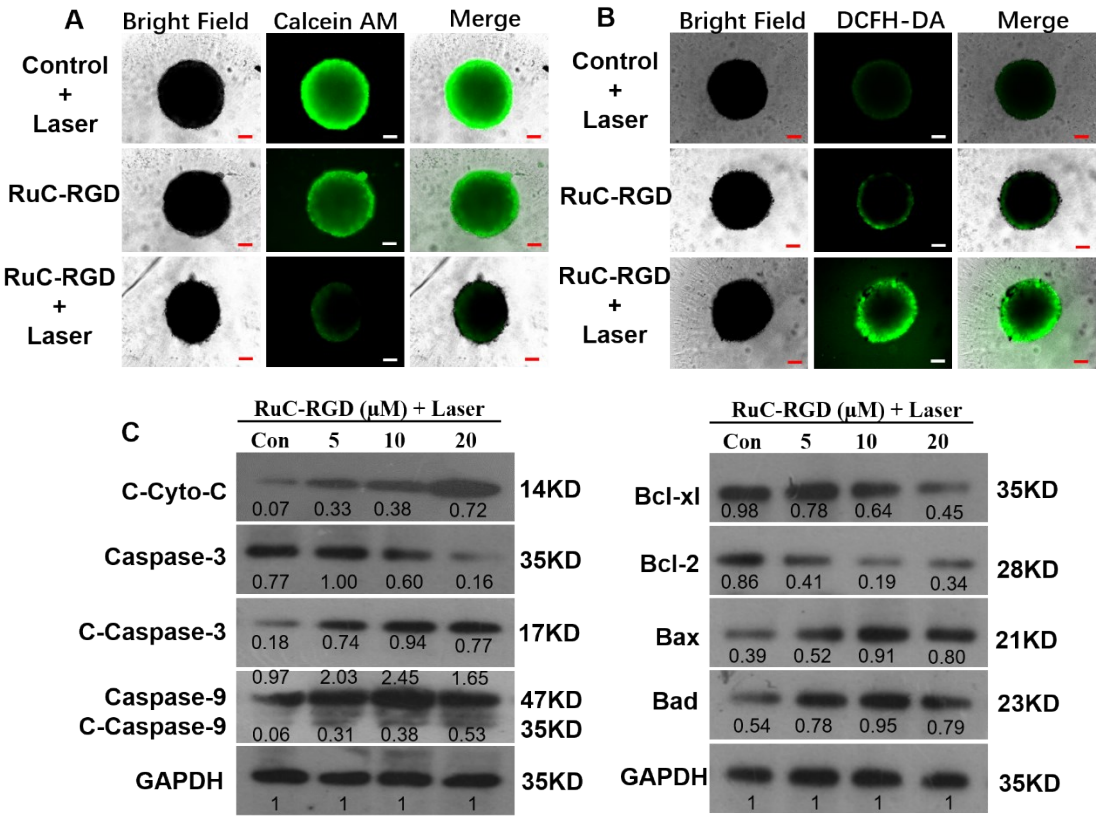

Fig. S12. (A) Images of calcein acetoxymethyl (λex = 488 nm, λem = 515 nm) staining on 3D U87MG MCTSs. The MCTSs were treated with RuC-RGD (20 μM, 24 h) and without or with 820 nm laser irradiation (25 mW, 120 s). (B) Images of DCFH-DA (λex = 488 nm, λem = 515-540 nm) staining on 3D U87MG MCTSs. The MCTSs were treated with RuC-RGD (20 μM, 24 h) and without or with 820 nm laser irradiation (25 mW, 120 s). (C) Western blot analysis of U87MG MCTSs treated with RuC-RGD and 820 nm laser irradiation (25 mW, 120 s). Scale bars: 100 μm.

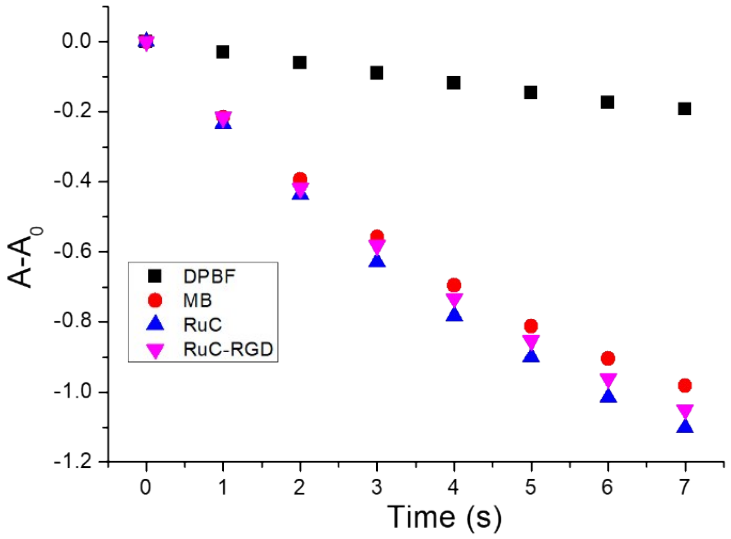

Fig. S13. Photooxidation of DPBF by complexes under light irradiation. Changes in absorption spectra of DPBF at 418 nm upon irradiation at 450 nm, and MB was used as a standard.

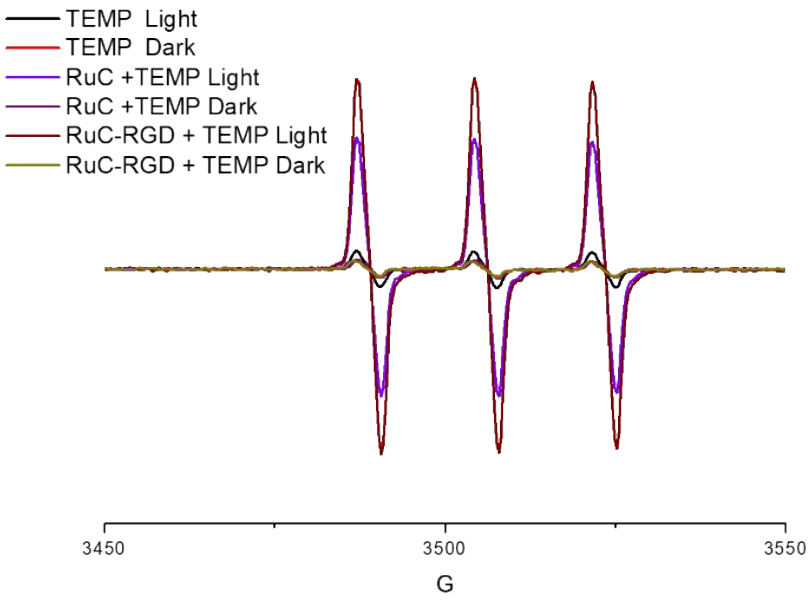

Fig. S14. The EPR signals of **RuC** and **RuC-RGD** trapped by TEMP.

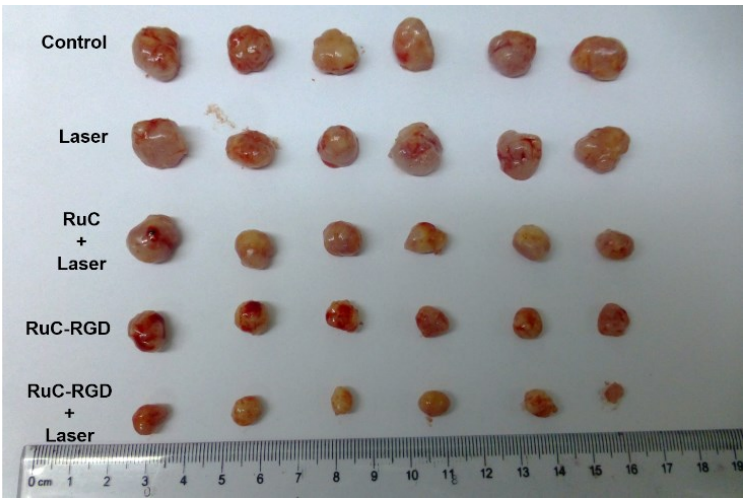

Fig. S15. Tumors were dissected from the mice after different treatment at day 20.

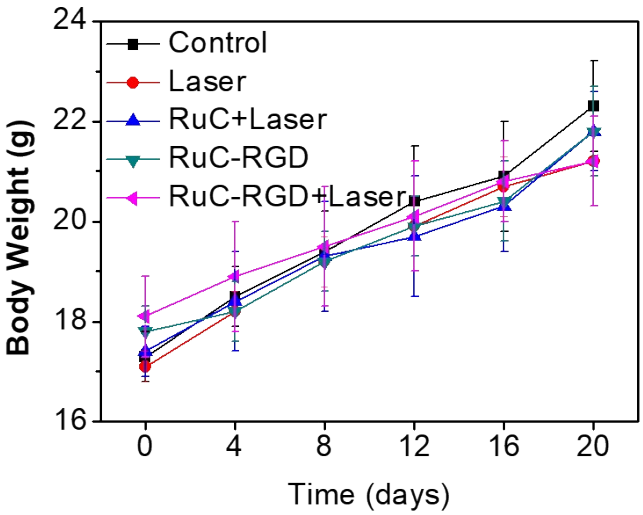

Fig. S16. Body weights growth curves of the mice in different groups.

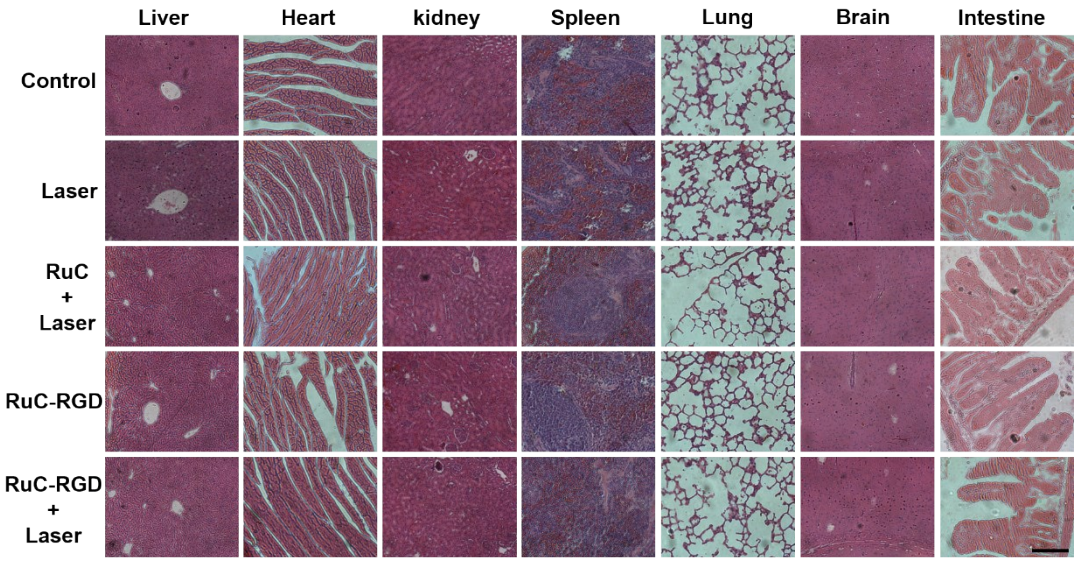

Fig. S17. H&E staining of the major organs (liver, heart, kidney, spleen, lung, brain and intestine) of U87MG tumor-bearing mice after different treatments. Scale bar: 100 μm.

Table S1. Photophysical data for **RuC** and **RuC-RGD** in PBS at 298 K

Complexes	λ_{ab} [a]	ε [b]	λ_{em} [c]	φ [d]	δ/GM [e]	Φ_{Δ} [f]
RuC	457	2.45	613	0.1184	69.6 GM	0.54
RuC-RGD	457	1.77	605	0.1240	63.5 GM	0.52

[a] λ_{ab} maximum values of the one-photon absorption in nm. [b] Extinction coefficient in 1×10^4 $M^{-1}\cdot cm^{-1}$. [c] λ_{em} maximum values of the one-photon emission spectra in nm. [d] Luminescent quantum yield. [e] Two-photon absorption cross section at 820 nm, measured in methanol. [f] Singlet oxygen quantum yield

Table S2. (Photo) cytotoxicity in different monolayer cells and 3D MCTSs (IC_{50}[μM])

Compound	2D MCF-7		2D U87MG			3D U87MG				
	Dark	OP[a]	Dark	OP[a]	PI[c]	Dark	OP[a]	PI[c]	TP[b]	PI[c]
RuC	>1000	>1000	826 ± 45	503 ± 25	1.6	856 ± 39	624 ± 25	1.3	347 ± 25	2.5
RuC-RGD	>1000	>1000	230 ± 16	28 ± 3.0	8.2	216 ± 25	34 ± 4.0	6.4	9 ± 1.8	24

[a] Irradiated by an LED area light (450 nm, 20 mW/cm^2, 10 min). [b] Irradiated at 820 nm TP irradiation (25 mW, 120 s). [c] PI (refers to photocytotoxicity index) is the ratio of dark-to-light toxicity. Data represent mean values from three replicates.