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Anti-LDLR Modified TPZ@Ce6-PEG Complexes for Tumor Hypoxia-Targeting Chemo-/Radio-/Photodynamic/Photothermal Therapy

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Experimental data

Materials

1-ethyl-3-(3-(dimethylamino) propyl) carbodimide (EDC), Dimethyl sulfoxide (DMSO), sulfo-NHS (N-hydroxysulfosuccinimide) and Chlorine e6 (Ce6)were obtained from Adamas-beta. NH_2-mPEG-NH_2(2k) was obtained from ToYong Bio Co., Ltd. (Shanghai, China). Cell counting kit (CCK-8), Trypsin-EDTA (0.25%) and DMEM culture medium were from KeyGEN BioTECH (Jiangsu, China). Deionized water was used in all experiments and was prepared using a Millipore ultrapure water system (Billerica, MA).

Antibodies

The monoclonal rabbit anti-LDLR (ab51037), monoclonal mouse anti-HIF-1α (ab113642) and monoclonal rabbit anti-gamma H2A.X (ab81299) were obtained from Abcam. The polyclonal rabbit anti-LDLR antibody (10785-1-AP) was obtained from Proteintech. The monoclonal mouse anti human LDLR antibody (IgG_1 Clone # 472413) was obtained from R&D Systems.

Clinical samples

Tumor samples and adjacent normal samples of HNSCC patients were obtained from the Biobank of Nanjing Stomatological Hospital approved by the Ethics Committee of Nanjing Stomatological Hospital. Written informed consent was obtained from all patients for research use. Formalin-fixed, paraffin-embedded (FFPE) HNSCC samples were also obtained from the Biobank of Nanjing Stomatological Hospital. All slides were reviewed by a board-certified pathologist for the identification of lymph node metastasis status of HNSCC patients.

Quantitative real-time PCR of HIF-1 signaling pathway genes

Total RNA was extracted from frozen specimens of HNSCC patients using TRIZOL Reagent (Invitrogen, Cat.No.15596-026) following their RNA isolation protocol. RNA was reverse transcribed into cDNA using a PrimeScript RT Master Mix kit (Takara, Code No. RR036A). Quantitative real-time PCR was performed on an ABI7500 PCR system (Applied Biosystems, Warrington, UK) using 1 μl of cDNA for each sample. SYBR green (Applied Biosystems) was used to detect the products, and 20 pmol of primer was used for the reaction. All reactions were carried out with 20 μl of reaction mix and conducted in triplicate. The expression of a total of 88 genes from HIF-1 signaling pathway was detected. The basic

information and primers of these genes could be seen in Supplementary Table 1. The following conditions were employed for PCR: 50 °C for 2 min, then 95 °C for 15 min, followed by 40 cycles at 95 °C for 1 min each. All reactions were analyzed using the ABI7500 Applied Biosystems PCR software (v.2.0.5). The cycle threshold values of the target genes were initially normalized to the cycle threshold values of ACTB, and melt curves were examined to detect the specificity of the reactions. The comparative Ct method was used to calculate the relative changes in gene expression on the 7500 Fast Real Time PCR System.

Immunohistochemistry (IHC)

Immunohistochemical analysis for LDLR proteins expression was performed in 75 HNSCC samples tissue. Four-μm-thick tissue sections were cut, deparaffinised, and subjected to antigen recovery treatment with 100 mM citrate buffer target retrieval solution, pH 6.0 at 95 ºC, in a water bath for 20 minutes. Endogenous peroxidase activity was blocked by incubating with phosphate-buffered saline (PBS) and 3% hydrogen peroxidase for 30 minutes. After washing with PBS, the sections were incubated with monoclonal rabbit anti-LDLR (1:400, ab51037, Abcam) and overnight, followed by the Envision Dual Link System HRP method (Dako, K4061) for LDLR antibodies. Immunostaining evaluation was performed by two independent pathologists blinded to clinical data. The percentage of positive tumor cells was evaluated and graded as negative (0–10%), weakly positive (10–50%) and strongly positive (> 50%), irrespective of cell type26,42. A third pathologist would intervene if agreements could not be reached between the two pathologists.

Western blot

Cells cultured under hypoxic or normoxic conditions were harvested and lysed for 20 min using modified RIPA buffer supplemented with a protease inhibitor cocktail (Sigma-Aldrich). Protein extracts were loaded onto a 6% sodium dodecyl sulfate–polyacrylamide gel, electrophoresed, and transferred to a polyvinylidene difluoride membrane. Protein bands were probed with Mouse anti-HIF-1α monoclonal antibody (1:100, Abcam) and other experimental primary antibodies at 4 °C overnight after blocking with 5% separated milk, followed by alkaline phosphatase-linked secondary antibody (Cell Signaling Technology, USA) incubation for 1 h at 37 °C. Beta-tubulin was used as a loading control.

Immunofluorescence

Xenografts tumor: 90 min before sacrificed, mice bearing HSC3 tumors (~100 mm^3) were i.v. injected with pimonidazole hydrochloride (0.6 mg per 10g body weight). After sacrificed, tumors were embedded in optimal cutting temperature (OCT) compound and then cut into 10 μm sections. Sections were incubated with antipimonidazole antibody (dilution 1:100, Hypoxyprobe Inc.) and then followed with Dylight 488-goat anti-rabbit IgG secondary antibody (dilution 1:100, Earthox). To detected LDLR, LDLR rabbit polyclonal antibody (dilution 1:100, Proteintech) and Dylight 594-goat anti-rabbit IgG secondary antibody (dilution 1:100, Earthox) were used. After stained with DAPI (KeyGEN), slices were captured and analyzed by CLSM.

Tumors derived from HNSCC patients were embedded in optimal cutting temperature (OCT) compound and then cut into 10 μm sections. LDLR rabbit polyclonal antibody (dilution 1:100, Proteintech) and Dylight 488-goat anti-rabbit IgG secondary antibody (dilution 1:100, Earthox) were used to detected LDLR. Mouse anti-HIF-1α monoclonal antibody (dilution 1:100, Abcam) and Dylight 594-donkey anti-mouse IgG (dilution 1:100, Earthox) were used to detected HIF-1α. After stained with DAPI (KeyGEN), slices were captured and analyzed by CLSM.

Gene set enrichment analysis (GSEA)

Microarray dataset of HNSCC was downloaded from Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/). Dataset GDS1062 was performed on the Affymetrix Human Genome U133A Array (Affymetrix, Santa Clara, CA, USA). Then 18 HNSCC tissues from GDS1062 dataset were divided into two groups (high vs. low) according to the expression level of LDLR and expression value was used as the cut-off point. In order to explore the potential relationship between LDLR and metastasis-associated signatures, “CROMER_METASTASIS_UP” and “RICKMAN_METASTASIS_UP”. GSEA (http://software.broadinstitute.org/gsea/index.jsp) was performed between the two groups.

Synthesis

The Ce6-conjugated NH_2-mPEG-NH_2 (Ce6-PEG) was prepared by conjugating the amine-functionalized NH_2-mPEG-NH_2 with activated Ce6. Briefly, 17 mg of Ce6 was mixed with 15 mg of EDC and 15 mg of sulfo-NHS in 4 mL of anhydrous DMSO for 1 hour at room temperature. Then 120 mg of NH_2-mPEG-NH_2 in 4 mL of DMSO was added (molar ratio of NH_2-mPEG-NH_2: Ce6: EDC: NHS = 4:2:5:5). After reacting for 24 hours at room

temperature, the above solution was dialyzed against water by a 1kDa cut off membrane and then lyophilized to obtain Ce6-PEG nanomicelles.

The Ce6-PEG coated tirapazamine (TPZ) was self-assembled. Briefly, The Ce6-PEG was redispersed into methanol and the TPZ was dissolved into chloroform, ultrasound to clear. And mixed to vacuum rotary steam to dry out to obtain the final production Ce6-PEG-TPZ (CPT). Then redispersed for conjugation with anti-LDLR antibody using EDC·HCl as the cross-linker. The Ce-6-PEG was reacted with nanoparticles: anti-LDLR antibody: EDC·HCl in the molar ratio of 1:10:4,000 in a borate saline buffer (50 mM, pH 8.0) under continuous stirring for 2 hours at room temperature. The final production Ce6-PEG-TPZ-Anti-LDLR (CPTA) were dispersed in PBS (0.01 M, pH 7.4) after centrifugation and washed with 0.01 M PBS (pH 7.4) twice.

Characterization

TEM images of the nanomicelles were obtained using a JEM-200CX transmission electron microscope at an acceleration voltage of 300 kV. UV-vis-NIR spectra were obtained with a PerkinElmer Lambda 750 UV-vis-NIR spectrophotometer. Fluorescence spectra of different samples were obtained on a FluoroMax 4 luminescence spectrometer (HORIBA Jobin Yvon). The photothermal conversion efficiency (η) was calculated using the following equation. (c: specific heat capacity of CPT solution, 4.2×10^3J/ (kg K); m: mass of CPTA solution, 2.5×10^{-3}kg; Δ t: temperature increase of CPT solution; w: laser power, 1 W/cm^2; t: laser irradiation time, 600 s)

$$\eta(\%) = \frac{cm\Delta t}{wt} \times 100\% = \frac{4.2 \times 10^3 \times 2.5 \times 10^{-3}\Delta t}{1 \times 600} \times 100\%$$

Stability study

The colloid stability of CPT/CPTA was further evaluated after being dispersed in water, phosphate buffered saline (PBS), Dulbecco's modified Eagle's medium (DMEM), and fetal bovine serum (FBS, BI) for 2 weeks of incubation at room temperature. To test the stability of CPTA after treatment with laser or X-ray irradiation (660nm, 1W/cm^2, 30min), CPTA solutions were irradiated by laser and X-ray (2Gy), the UV-vis-NIR profiles and images were obtained.

Singlet oxygen detection

The method for singlet oxygen detection was based on the protocol reported previously. In brief, 100 mg of SOSG (Molecular Probes, USA) was dissolved in 330 mL of methanol to

obtain the stock solution of SOSG (0.5 mM). Then, 10μL of SOSG was added to 2 mL of CPT or CPTA solution containing 500μg/mL Ce6. Next, the sample was irradiated by a 660 nm laser at a power density of 1 W/cm^2. The fluorescence intensity of SOSG was measured with an excitation wavelength of 525nm every 3 minutes.

Cell culture experiment

Human head and neck squamous cells, HSC3, CAL27, JHU011 and SCC9 were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, P. R. China). HSC3, CAL27, JHU011 cells were cultured and maintained in DMEM supplemented with 10% fetal bovine serum (FBS, Biological Industries), 80μg/mL streptomycin, and 80 U/mL penicillin, $NaHCO_3$, 4.5 mg/mL glucose, and 0.584g/L L-Glutamine. SCC9 cells were cultured and maintained in RMPI-1640 supplemented with 10% fetal bovine serum (FBS, Biological Industries), 80μg/mL streptomycin, and 80 U/mL penicillin, $NaHCO_3$, 4.5 mg/mL glucose, and 0.584g/L L-Glutamine. To assess the viability of HSC3 cells treated with CPT/CPTA at different pO_2 S (21%, and 1%), 2 × 10^{3}cells/well were seeded into 96-well plates cultured for 24 h; the cells were then transferred to the indicated oxygen conditions for an additional 24 h. Various concentrations of CPT/CPTA (0, 1.25, 2.5, 5, 10, and 20μg/ml, TPZ) were then added to each well, followed by incubation for an additional 24 h. Cell viability was evaluated with the CCK-8 assay. To determine the LDLR antibody conjunction, the incubation was performed at 4 °C in which only specific binding could occur.

To examine the cellular uptake of CPT/CPTA, HSC3 cells were plated in 6-well plates at 1 × 10^{4}cells per well. After adhesion, CPT/CPTA was added into the wells at a concentration of 5μg/mL (TPZ) and cultured for different time periods (2, 4, 8, 12 and 24 hours). After washing three times with PBS (pH = 7.4), cells were fixed by 75% ethanol and labeled with Hoechst 33342 before imaging with a Nikon Digital Eclipse A1 Plus microscope.

To assess the viability of HSC3 cells treated with CPT/CPTA under the laser or not, 2×10^3 cells/well were seeded into 96-well plates cultured for 24 hours. Various concentrations of CPT or CPTA (0, 1.25, 2.5, 5 μg/mL, TPZ) were then added to each well, they were irradiated by a 660 nm laser at a power density of 1 W/cm^2 after 4 hours for 5 minutes, followed by incubation for an additional 4 hours, cell viability was evaluated with the CCK-8 assay.

To assess the apoptosis of HSC3 cells treated with CPT/CPTA under the laser or not, 1 × 10^4cells/well were seeded into 6-well plates. After adhesion, CPT/CPTA were added into the wells at a concentration of 5μg/mL (TPZ) and cultured for 4 hours. Then the 660 nm laser irradiated at a power density of 1 W/cm^2 after 4 hours for 5 minutes, followed by incubation for an additional 4 hours. Apoptotic cells were quantified by dual staining with Annexin V and PI. After washing with PBS, the cells were suspended in cold binding buffer. Annexin V (5 μL) and 100 μL PBS were added into each well for 10 minutes at 4°C. Subsequently, 200 μL binding buffer containing 5 μL PI was added. Finally, the entire mixture was incubated at 4°C for another 15 minutes. All the incubation was performed in the dark. Samples were detected with a FACS Verse flow cytometer (BD Biosciences) and the data obtained were analyzed by FlowJo.

HSC3 cells were maintained in a 35 mm confocal dish at a density of 2×10^4 per well for 24 hours. When HSC3 cells had grown to 30% in plates, 2 mL of CPT/CPTA (5 μg/mL) was added and further cultured for 4 hours. After 4 hours incubation, cells were irradiated with or without a 660 nm laser (1W/cm^2, 5 minutes). The synergistic treatment group was irradiated with X-rays immediately after irradiation with a 660 nm laser. After 4h, the cells were fixed with 4% paraformaldehyde for 10 minutes.

The Calcein-AM/PI staining was used for direct observation of living/dead cells. The cells were incubated with Calcein-AM/PI solution (4 μM for each fluorescent probe in PBS) for 1 h, and then washed for 3 times in PBS before imaged under Nikon Digital Eclipse A1 Plus microscope with green/red fluorescent exciters.

***In vitro* photodynamic therapy**

The Reactive Oxygen Species Assay Kit (Beyotime) was utilized to examine the cellular production of $^1O_2$. The operation according to the instructions. HSC3 cells were plated in six-well plates at 1 × 10^4cells per well. After adhesion, CPT / CPTA were added into the wells at a concentration of 5μg/ml (TPZ) and cultured for 4 hours. Then the DCFH-DA add to wells, then the sample was irradiated by a 660 nm laser at a power density of 1 W/cm^2 after 30 minutes. After washing three times with PBS (pH = 7.4), cells were fixed by 75% ethanol and labeled with Hoechst 33342 before imaging with a Nikon Digital Eclipse A1 Plus microscope.

***In vitro* clonogenic assays of radiotherapy**

Cells were treated with CPT or CPTA for 24 hours, followed by irradiation with 2 Gy using a 137 Cs source. Following irradiation, cells were seeded by limiting dilution at 500 cells per well into 6-well culture plates. After 14 days of culture, cell medium was removed, and cells were rinsed with PBS twice and then fixed with 2 mL of a mixture of 6.0% glutaraldehyde and 0.5% crystal violet added to each well at room temperature for 30 minutes. The glutaraldehyde crystal violet mixture was carefully immersed in tap water until all excess dye was removed, and plates were air-dried at room temperature. The resulting stained cell colonies were counted using a stereomicroscope.

DNA double-strand breaks of radiotherapy

HSC3 cells were maintained in a 35 mm confocal dish at a density of 2×10^4 per well for 24 hours and divided into 12 groups: (a) PBS only; (b) Laser only; (c)RT only; (d)CPT only; (e) CPTA only; (f) CPT & Laser; (g) CPTA & Laser; (h) CPT & RT; (i) CPTA & RT; (j) CPT &Laser+ RT; (k) CPT &Laser+ RT. When HSC3 cells had grown to 30% in plates, 2 mL of CPT/CPTA (5 μg/mL) was added and further cultured for 24 hours. The well without any treatments was regarded as the negative control group. After 24 hours incubation, cells were irradiated with or without a 660 nm laser (1 W/cm^2, 10 minutes) and X-ray (2 Gy). The synergistic treatment group was irradiated with X-rays immediately after irradiation with a 660 nm laser. After 24 hours, the cells were fixed with 4% paraformaldehyde for 10 minutes and Triton X-100 was used to permeate the cells. Next, the cells were further incubated in 0.5% bovine serum albumin for another 1 hours to prevent nonspecific protein interactions. Next, the cells were then treated with γ-H2A.X antibody (1:100, Abcam) overnight at 4℃. The Dylight 594-goat anti-rabbit IgG (dilution 1:100, Earthox) was added for 1 h. Hoechst 33342 was used to stain the cell nuclei.

Animal models

All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of the Medical School of Nanjing University and Experiments were approved by the Animal Ethics Committee of Nanjing Stomatological Hospital. To set up the tumor xenograft model, Balb/c male nude mice (6 weeks old, average body weight: 22 g; Nanjing Biomedical Research Institute of Nanjing University, Nanjing, China) were subcutaneously injected in the bottom back with 5×10^6 HSC3 cells/mouse. After approximately 2 weeks post injection, the tumor nodules reached a volume of 100 mm^3. The

tumor size was monitored by a Vernier caliper, and the tumor volume (V) was calculated as $V = L \times W^2/2$, where L and W are the length and width of the tumor, respectively.

Hemolysis test

Hemolysis assay was carried out by using mice RBCs as model cells. RBCs were isolated and washed with PBS five times. Then different concentrations of CTP & CPTA were added into the cell suspensions of the obtained RBCs for 3 hours. The released hemoglobin in the supernatant was determined by measuring its absorbance at 540 nm. The extent of hemolysis was calculated relative to 100% hemolysis treated with ultrapure water.

Biodistribution

When the tumor volume reached around 100 mm^3, mice were injected CPT/CPTA (TPZ, 60nmol/kg). Then the fluorescence spectra of different mice were obtained on a FluoroMax 4 luminescence spectrometer (HORIBA Jobin Yvon). At 4 hours, the mice were euthanized, and the tumor and major organs were collected to test the fluorescence spectra. The mouse with an injection of PBS was used as a blank control.

***In vivo* antitumor evaluation**

The tumor-bearing mice were weighed and randomly divided into different groups when the tumor volume reached around 100mm^3. The mice were then divided into 12 groups to receive different disposes: (a) PBS (phosphate buffered saline) only; (b) Laser only; (c)RT only; (d)CPT only; (e) CPTA only; (f) CPT & Laser; (g) CPTA & Laser; (h) CPT & RT; (i) CPTA & RT; (j) CPT & Laser+ RT; (k) CPT & Laser+ RT. Each group included four mice. From day 0, the corresponding mice were intravenously injected CPT/CPTA (TPZ, 60nmol/kg). After 4 hours, the corresponding mice received was irradiated by a 660 nm laser at a power density of 1 W/cm^2 for 10 minutes. After 4 hours, the mice received the 2 Gy of X-ray radiation according to group requirements on a Siemens Primus clinical linear accelerator (6 MeV) using a 1cm × 1cm radiation field to cover the entire tumor without the need of critical anatomies at a source-to-skin distance of 100 cm. All treatments were given only once. During the next 15 days, the tumor volume and the whole body weight of each mouse were measured with a vernier caliper every other day.

Histology analysis

At day 15, the mice were euthanized, and the tumor and major organs were collected, and fixed in the 10% neutral-buffered formalin and embedded in paraffin. For the hematoxylin

and eosin staining, the formalin-fixed tumors were embedded in paraffin blocks and visualized by an optical microscope (DM5500B, Leica). And the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining was constructed following the protocol and visualized by Nikon Digital Eclipse A1 Plus microscope.

Statistical analysis

The correlations of LDLR expression and lymph node metastasis were evaluated with Fisher's exact test. The Kaplan-Meier survival curve was generated based on LDLR expression and statistical significance was determined by log-rank tests. All other statistical analyzes were performed using Statistical Product and Service Solutions software. A p-value of < 0.05 was considered statistically significant. GraphPad Prism 5.0 software was used to create the figures.

Supporting Information

Table S1. Names of HIF-1 signaling pathway genes and their corresponding primer ID.

Gene_symbol	Primer_id	Gene_symbol	Primer_id
ADORA2B	9000000	KCTD11	31454
LDLR	9000031	LRP2BP	96905
MIF	9000040	MRPL14	100063
NME1	9000044	PPM1J	109404
ANKRD9	9000092	TMEM30B	49172
TMEM189	9000095	TRMT5	49057
BNIP3	6247505	B4GALT2	6032754
SLIRP	9000087	MNAT1	6002838
HILPDA	9000084	PGK1	6020364
PGAM1	9000049	PTGFRN	6045092
TUBB2A	9000069	PVR	6009940
VAPB	9000079	GEMIN2	6016229
CXCL8	52117	TEAD4	6098208
NDRG1	6212020	TFAP2C	6014618
PGF	6219000	TPD52L2	6019209
PAWR	6247898	ANGPTL4	6078448
PYGL	6210024	ANLN	6002193
SLC2A1	6218206	CNIH4	6055569
TNS4	6223823	HAUS2	6049632

VEGFA	6028587	NUDT15	6047688
BCAR1	6202814	PLEKHG3	6039185
ALDOA	64498	RUVBL2	6089738
CA9	40382	SLCO1B3	6027400
KRT17	86118	SNX24	6007243
LDHA	46794	TANC2	6081550
MTX1	124792	TIMM23	9000256
AK3	19178	VEZT	6003341
DPM2	98160	CA12	6205575
MRPS17	55695	COL4A5	6094310
PDZD11	65415	GMFB	6253804
PLAU	76834	GSS	6210287
PPARD	87674	P4HA1	6220253
PSMA7	85282	PFKFB4	6216348
PSMD2	117520	PSMB7	6226907
RAN	119328	HOMER1	6234660
RNPS1	27759	PPP4R1	6235890
S100A10	121228	IGF2BP2	6205459
S100A3	122110	RNF24	22932
SLC16A1	109499	CORO1C	6214786
SLC6A8	104090	GPN3	6214910

SPTB	52955	XPO5	6221036
TPBG	114093	RPL27	91408
TPI1	21102	HPRT1	96179
MRGBP	87188	ACTB	19435
CDCA4	85137	OAZ1	21566
EIF2S1	6044854	B2M	34448
HES2	8687	GAPDH	17872

Table S2. Differentially expressed genes in HNSCC patients with lymph node metastasis compared to those without metastasis.

Dysregulated	Gene
Upregulated	PSMA7
	RNPS1
	SLC6A8
	TIMM23
	TMEM189
	TRMT5
	LDLR
	NME1
Downregualted	ANGPTL4
	PFKFB4

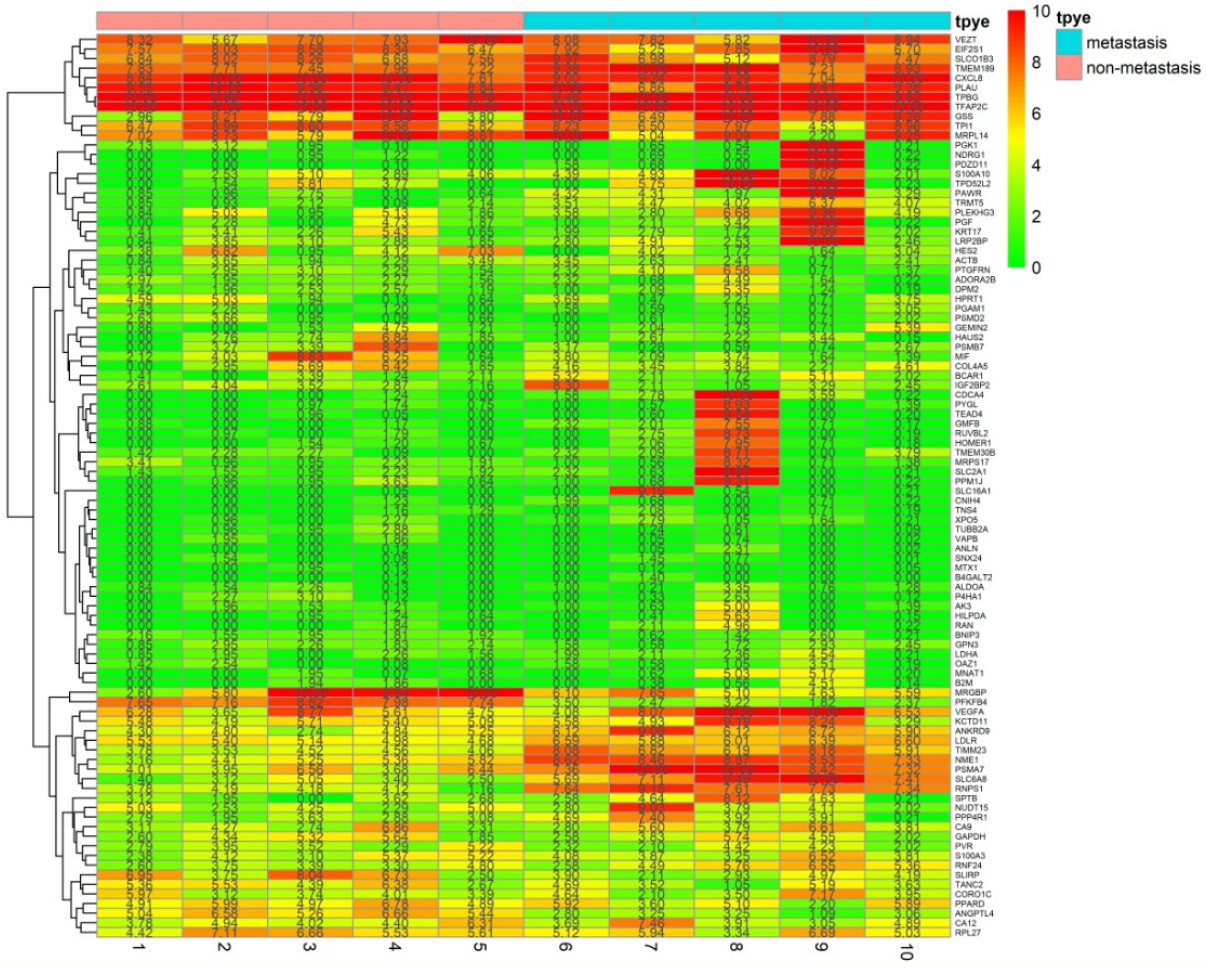

Figure S1. Original picture of Figure 1A.

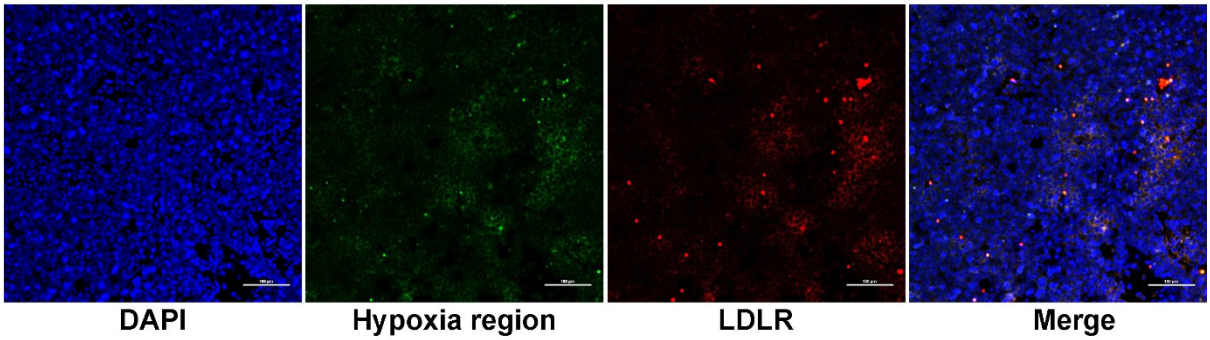

Figure S2. The immunofluorescence images of xenograft tumor. Scale bar: 100 μm.

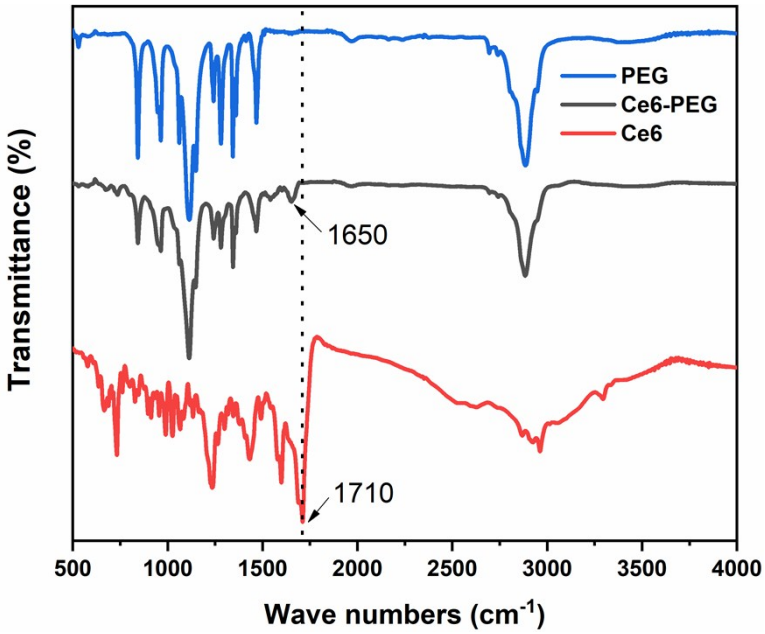

Figure S3. The fourier transform infrared (FTIR) image of PEG, Ce6, and CE6-PEG.

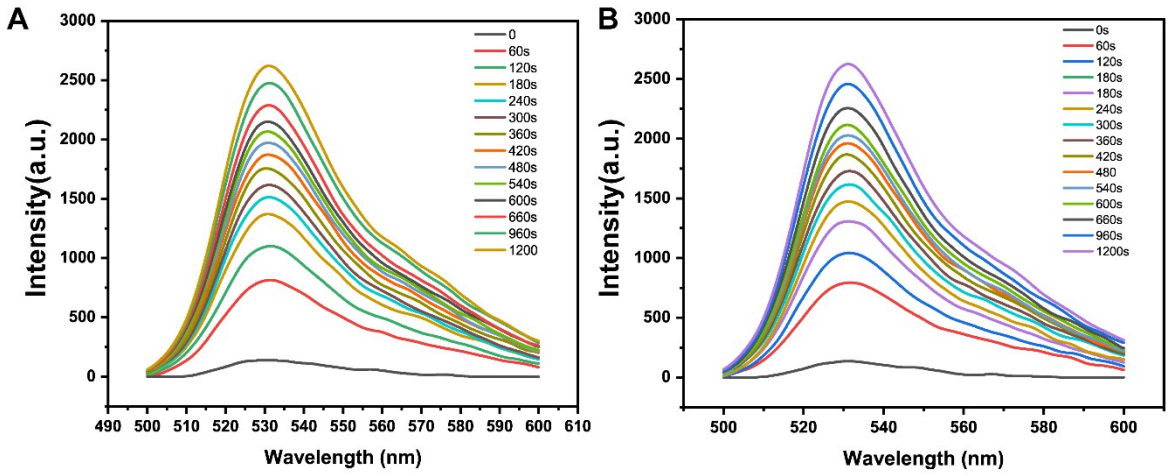

Figure S4. The photodynamic profiles of (A) CPT and (B) CPTA in PBS (500 μg/mL). The intensity of SOSG after received different laser irradiation (1 W/cm^2) time.

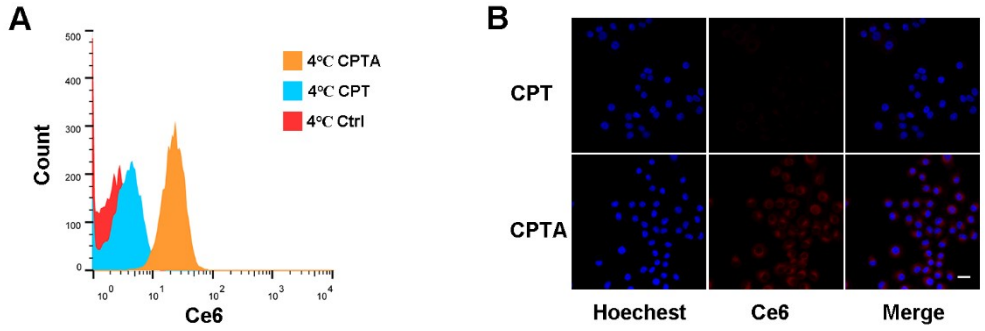

Figure S5. The binding tests of CPT and Anti-LDLR. (A) CLSM images of HSC3 cells treated with CPT & CPTA after 1 hour in 4℃. Scale bar: 50μm. (B) Flow cytometry showing the uptake of CPT & CPTA in HSC3 cells after 1 hour in 4℃. Scale bar: 50 μm.

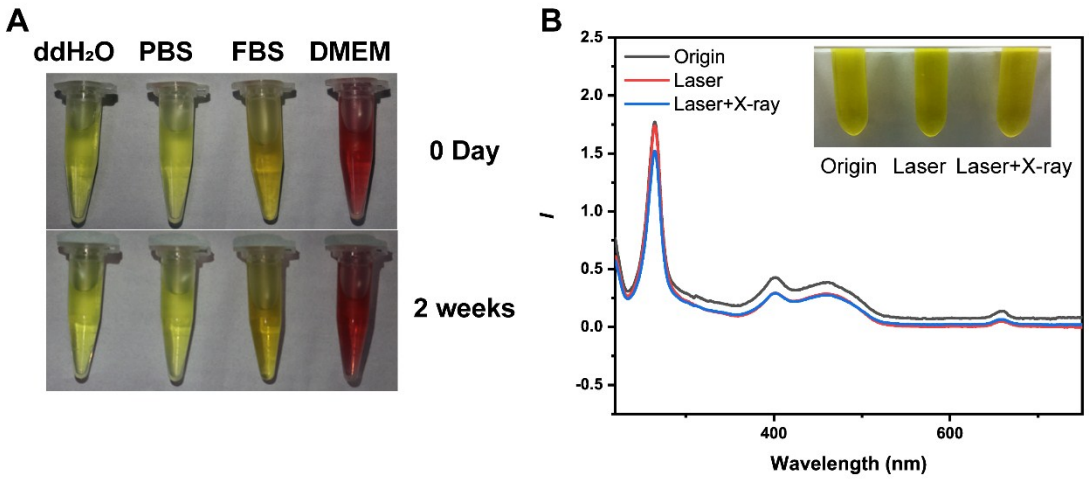

Figure S6. (A)The appearance of CPTA in different media at room temperature after two weeks.(B) The appearance and UV-vis-NIR profiles of CPTA solutions after laser and X-ray irradiation.

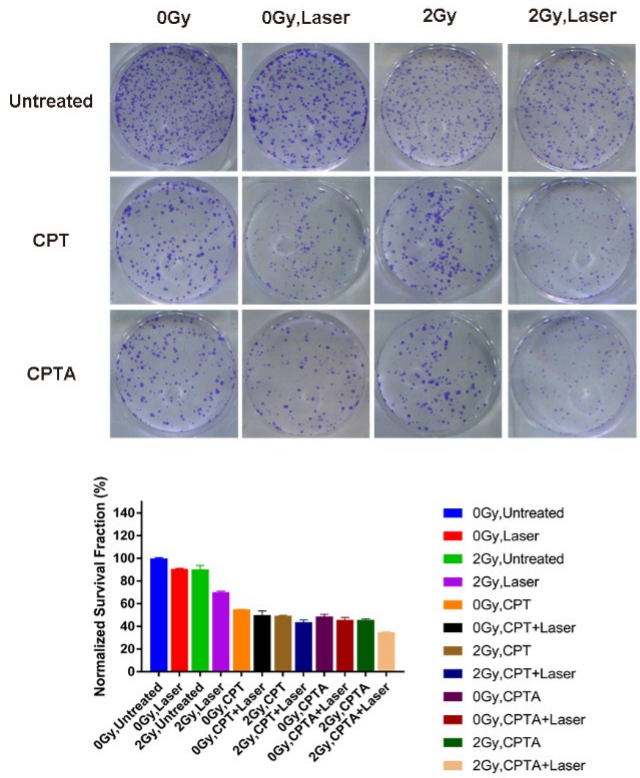

Figure S7. The colony formation assays of HSC3 cells after different treatment.

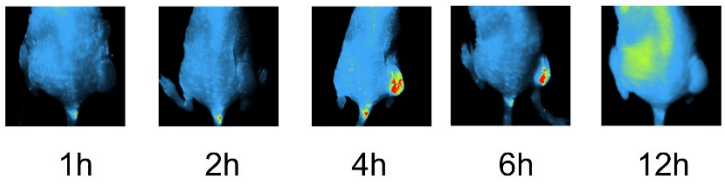

Figure S8. The in vivo fluorescence imaging at different time points.

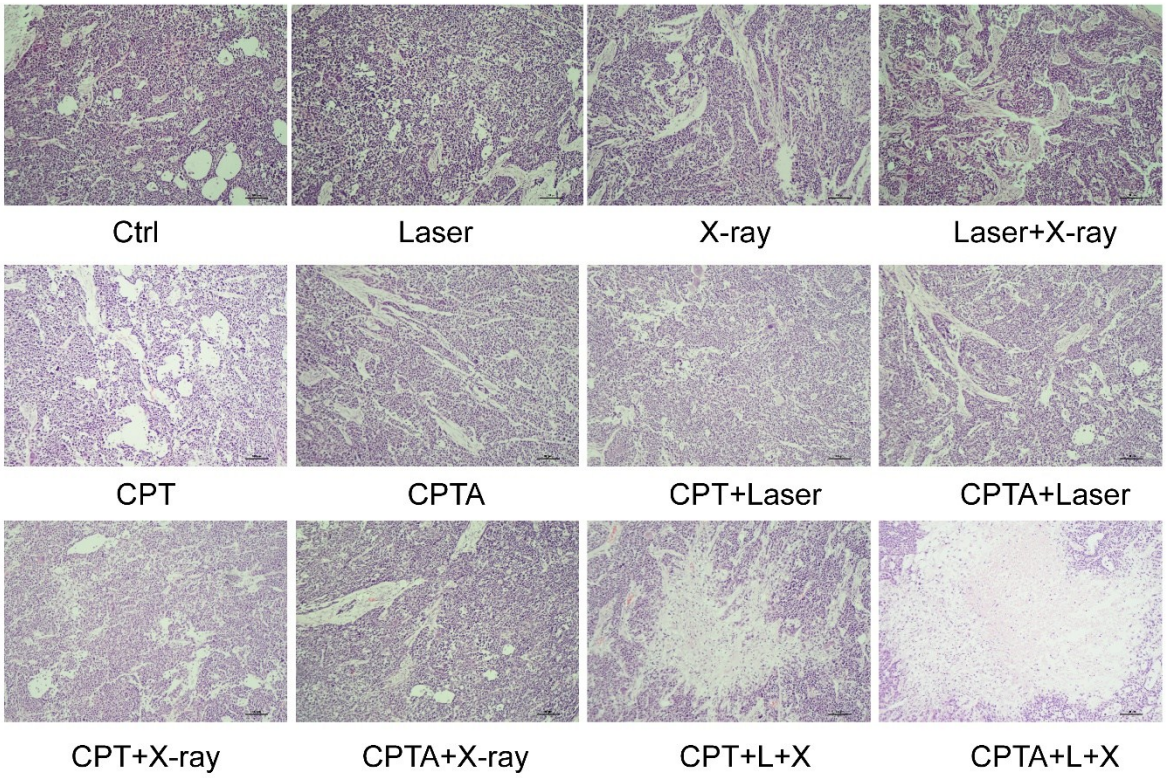

Figure S9. The H&E staining histological images of tumor after different treatment. Scale bar: 100μm

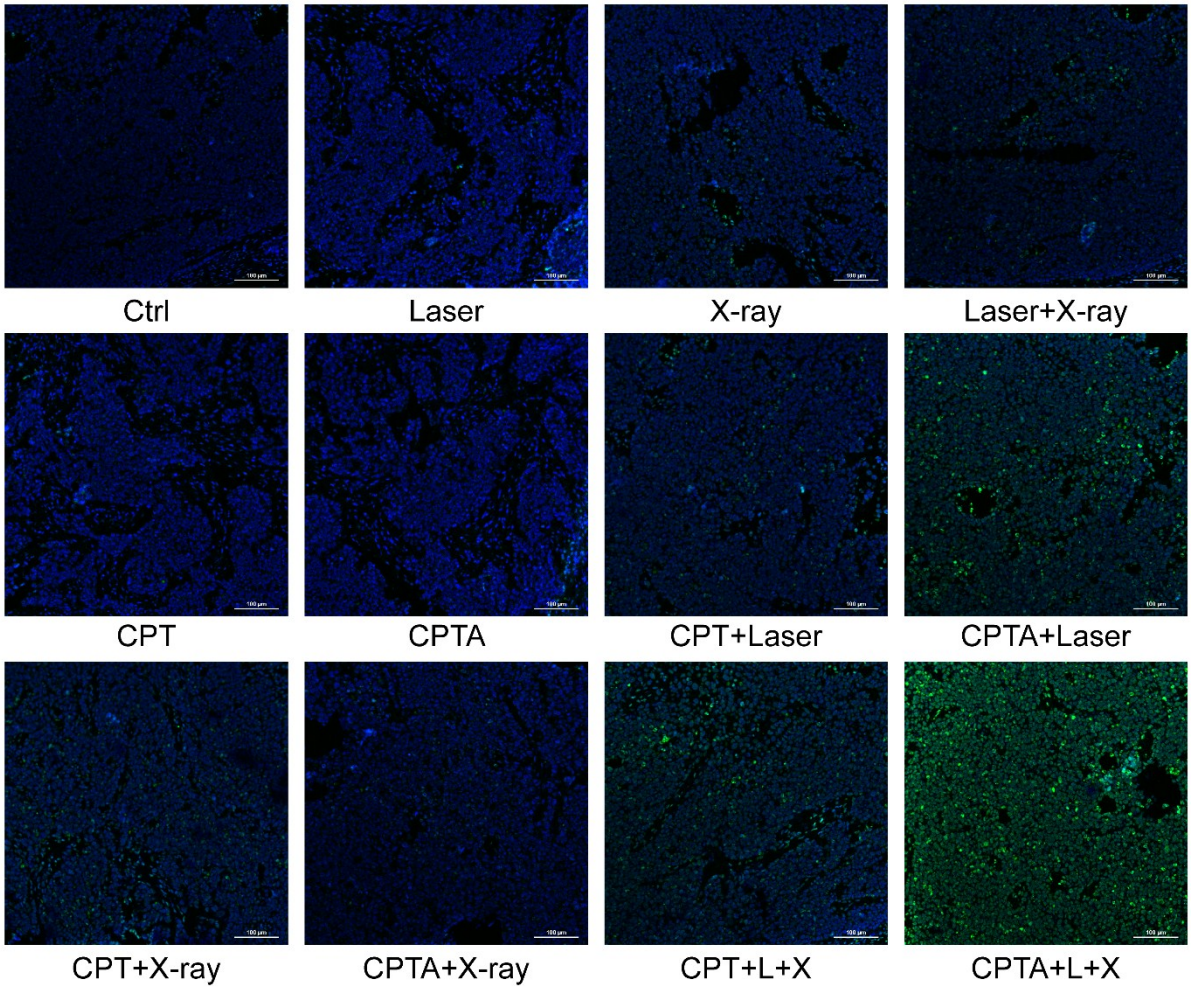

Figure S10. TUNEL staining of tumor after different treatment. Scale bar: 100μm.

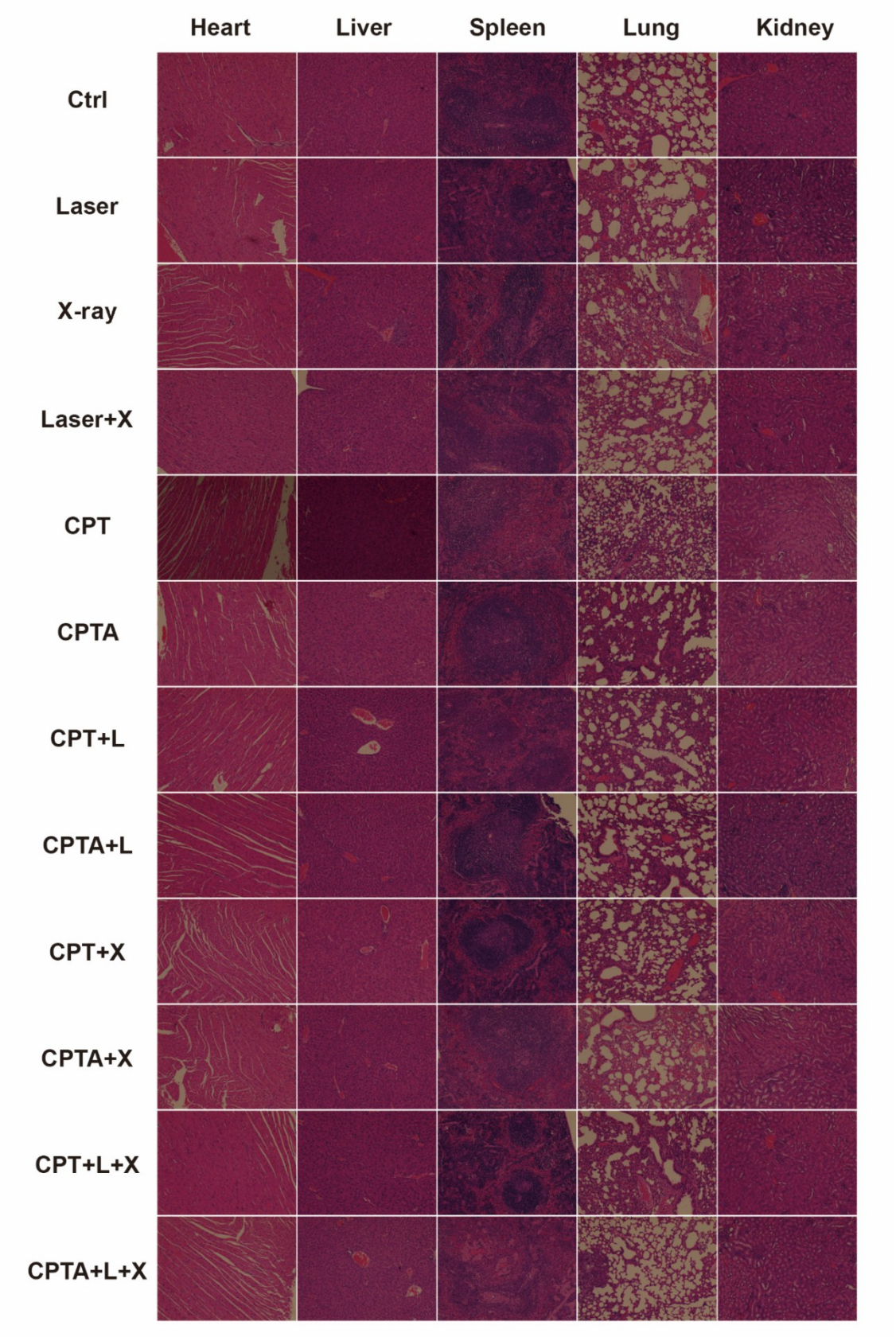

Figure S11. H&E stained histological images of major organs collected after 15 days in mice of different groups.