Supporting Information

Hyperthermia Induced Stellate Cell Deactivation to Enhance Dual Chemo and pH-Responsive Photothermal Therapy of Pancreatic Cancers

Qinglian Hu[1], Minjie Xu[1], Jiayu Feng[1], Hui Xie[3], Jingyu Li[1], Ying He[1], Guping Tang[4], Bing Guo[2]**

1. Materials and Methods

1.1 Materials

5-bromo-2,3,3-trimethyl-3*H*-indole (compound **1**), tributyl(thiophen-2-yl)stannane, (4-methoxyphenyl)boronic acid, croconic acid tetrakis(triphenylphosphine)palladium(0) ($Pd(PPh_3)_4$), tetrabutyl ammonium bromide (Bu_4NBr), and potassium carbonate (K_2CO_3) were purchased from Bidepharm, China. 1,2-dihexadecanoyl-sn-glycero-3-phosphocholine (DPPC)1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-PEG2000) were purchased from Xi'an ruixi Biological Technology Co., Ltd. Gemcitabine (GEM) was purchased from Shanghai Aladdin Biochemical Technology Co., Ltd. Cholesterol, thiazolyl blue tetrazolium bromide (MTT) and Dimethyl sulfoxide (DMSO) were purchased from Sangon Biotech (Shanghai) Co., Ltd. MilliQ water (18.2 MΩ cm-1) was used for all experiments. All other chemicals were used as received.

1.2 Characterization

NMR spectra were obtained using a Bruker 500NMR spectrometer. UV-vis (Shimadzu Model UV-1700 spectrometer) was used to test the absorption properties of NP suspension. The size and zeta-potential of synthesized NPs samples was measured on NanoBrook Omni Particle Size Analyzer (Brookhaven Instruments, USA). The morphology of the NPs samples was characterized by transmission electron microscope (TEM) on a Tecnai G2 F30 S-Twin system (Philips-FEI, Netherlands), operating at an acceleration of 100 kV. The

photoluminescence spectra and fluorescent decay kinetics was measured with a FLS 980 fluorescence spectrometer (Edinburgh Instruments, Livingston, UK) equipped with an Xe lamp and μs flash Xe lamp as the excitation source.

1.3 Synthesis of 2,3,3-trimethyl-5-(thiophen-2-yl)-3*H*-indole, compound 2

A mixture of 5-bromo-2,3,3-trimethyl-3*H*-indole (compound **1**, 760 mg, 4.0 mmol), tributyl(thiophen-2-yl)stannane (1866 mg, 5.0 mmol), and $Pd(PPh_3)_4$ (92 mg, 0.08 mmol) was added in toluene (25mL). The reaction was stirred at 90 ℃under argon atmosphere for 24h. When the reaction was finished, the solvent was removed *via* a rotary evaporator. The crude product was purified by silica column with hexane/ethyl acetate (v: v = 2/1) as the eluent to afford 2,3,3-trimethyl-5-(thiophen-2-yl)-3*H*-indole, **(compound 2**) as yellow solids (821mg, yield 85%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.49-7.41 (m, 3H), 7.21-7.15 (m, 2H), 6.99-6.97 (m, 1H), 2.20 (s, 3H), 1.24 (s, 6H).

1.4 Synthesis of CTh

A mixture of compound **2** (265 mg, 1 mmol) and croconic acid (71 mg, 0.5 mmol) was dissolved in dried butanol/toluene (10/10 mL) was stirred at 110 ℃ o under argon atmosphere for 3 h. When the reaction was finished, the mixture was cooled down to room temperature and further stored in -20 fridge for 2 days to make crystallization of the product. The crude product was further collected by filtration and washed with hexane, to yield compound **CTh** as brown red crystals (91 mg, yield 31%). ^{1}H NMR (400 MHz, CDCl3) δ 15.78-14.45 (m, 2H), 7.54-7.43 (m, 4H), 7.25-7.06 (m, 8H), 7.03-7.00 (m, 2H), 6.01-5.91 (m, 2H), 1.50-1.49 (m, 12H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 186.25, 185.41, 176.50, 175.95, 174.21, 143.94, 143.75, 142.05, 141.75, 140.65, 140.55, 140.38, 132.13, 131.89, 129.02, 128.29, 128.21, 126.63, 126.58, 125.29, 125.16, 124.93, 123.42, 123.34, 123.12, 120.27, 120.20, 113.81, 113.68, 113.06, 95.11, 94.47, 91.76, 50.46, 50.29, 49.86, 26.29, 25.82, 25.61. MALDI: calculated for $C_{35}H_{28}N_2O_3S_2$: 588.15, found 588.62.

1.5 Synthesis of C-G NPs

CTh, DPPC and GEM were dissolved in DMSO at the concentration of 5 mg mL^{-1}. DSPE-PEG_{2000} and cholesterol was dissolved in tetrahydrofuran (THF) at the concentration of 10 mg mL^{-1}. A mixture of **CTh** (2 mg), DSPE-PEG_{2000}(4 mg), DPPC (4 mg), cholesterol (4 mg), GEM (1 mg) and THF (1 mL) was added into MilliQ water (10 mL) with ultrasound (40 W) drop by drop. The solvent was stirred at room temperature for 48 h and dialysis 48 h. Finally, the volume of the solvent was concentrated into 1 mL (1 mg mL^{-1}) via ultrafiltration. The prepared **C-G** NPs were used for subsequent experiments or stored at 4 ℃.

1.6 Characterization of C-G NPs

Dynamic light scattering (DLS) measurements was performed on Malvern Zetasizer Nano ZS. Transmission electron microscopy (TEM) images was performed on Hitachi TEM system.

1.7 *In Vitro* photothermal and photothermal stability Assay

To evaluate the photo-thermal conversion efficiency of **CTh** NPs or **C-G** NPs, we added the different concentrations of **CTh** NPs or **C-G** NPs solution into 96-well plate at 100 μL/well, then irradiated the well vertically with 808 nm laser above the plate (1 W cm^{-2}). The temperature changes of every well were recorded by an infrared camera (FLIR Systems AB) at every 30 s. To further test the photothermal stability of **C-G** NPs, the **C-G** NPs aqueous solution at concentration of 10 μg mL^{-1} was irradiated with 808 nm laser (1 W cm^{-2}) for 5 min and naturally cooled for 10 min. The temperature was recorded using an infrared camera.

1.8 Measurements of the photothermal conversion efficiency (η)

The photothermal conversion efficiency (η) of **C-G** NPs was calculated using those following equations: T_{Max} and *Tsurr* denote the maximum equilibrium temperature and ambient temperature, respectively. Q_{Dis} is the heat wastage form the light loss of the solvent, and the Q_{Dis} was determined using MilliQ water. I is the incident laser power (I=1.0 W cm^{-2}), A808 is the absorbance at 808 nm, and τ_s is the system time constant of the sample. m_D and C_D index the solution mass (0.1 g) and heat capacity (4.2 J g^{-1}) of MilliQ water used as the solvent, respectively.

$$\eta = \frac{hS(T_{Max} - T_{Surr}) - Q_{Dis}}{I(1 - 10^{-A808})}$$

$$hS = \frac{m_D C_D}{\tau_s}$$

$$\tau_s = -\frac{\tau}{\ln \frac{T_{RT} - T_{Surr}}{T_{Max} - T_{Surr}}}$$

1.9 *In Vitro* GEM release from C-G NPs with NIR light

To investigate *in vitro* GEM release from **C-G** NPs under 808 nm laser, we pipetted 1 ml 150 μg/mL **C-G** NPs into a 24-well plate and irradiated for 10 min. At 2, 4, 6, 8 and 10 min, 100 μL of the solution was taken out and passed through a 0.22 μm Millipore filter. Absorbance at 268 nm was detected to measure the concentration of GEM using a spectrophotometer (Shimadzu, Japan). The concentration of GEM was calculated by comparing the absorbance of the samples to the standard curve.

1.10 Cell Culture

Both PANC-1 cells (human pancreatic cancer cell line) and human pancreatic stellate cells (PSCs) were obtained from Hunan Fenghui Biotechnology Company. The PANC-1 cells and PSC cells both cultured in dulbecco's modified eagle's medium (DMEM) with 10% fetal bovine serum, 100 units mL^{-1} penicillin and 100 units mL^{-1} streptomycin. Cells were cultured at 37 ℃ and 5% CO_2 atmosphere.

1.11 Cell Viability Assay

In order to detect the inhibitory effect of PTT-GEM combinational treatment, we seeded the PANC-1 cell into 96-well at 10,000 cells per well and cultured 12 h. Then we replaced the complete medium with different concentrations of **CTh** NPs, GEM NPs or **C-G** NPs. The laser-irradiation groups were exposed to 808 nm NIR at 1 W cm^{-2} for 5 min and cultured 24 h. We removed the supernatant and added 0.5 mg mL^{-1} MTT solution into 96-well at 100 μL per well. After 4 hours, we replace the MTT solution with 150 μL DMSO and oscillated in 37 ℃ for 15 min. Finally, we determined the absorption at a wavelength of 490 nm on a microplate reader (ThemoMultiscan MK3, USA).

1.12 Calcein-AM/PI staining and imaging

PANC-1 cells (50,000 cells per well) were seed in 24-well plate and cultured at 37 ℃ and 5% CO_2 atmosphere for 24 h. Then we replaced the complete medium with free medium containing various concentrations of **CTh** NPs or **C-G** NPs (20 μg mL^{-1} and 20 μg mL^{-1}). Afterwards, every well was exposed to 808 nm laser (1 W cm^{-2}) for 5 min. After another 12 h of incubation, the cells were stained with Calcein-AM/PI (Yeasen, China) according to the operation manual and imaged using an inverted optical microscope (Nikon, Japan).

1.13 Trans-well Co-cultivation Assay

To evaluate the effect of PANC-1 cells to PSCs, we seeded the PANC-1 cells into Trans-well upper chamber at 50,000 cells per well and PSCs into plate at 100,000 cells per well and cultured 12 h. Then, we replaced the complete medium of upper chamber with **CTh** NPs, GEM NPs or **C-G** NPs and serum-free medium of plate and cultured 12 h. The upper chambers were exposed to 808 nm NIR for 5 min and cultured 6 h. Finally, we collected the supernatant of upper chamber, PANC-1 cells and PSCs for following experiments.

1.14 Establishment of PANC-1 subcutaneous tumor model

All animal experiments were performed strictly according to the requirements and guidelines of the Institutional Ethical Committee of Animal Experimentation of Zhejiang University of Technology. Male nude mice were obtained from Shanghai SLAC Laboratory Animal (Shanghai, China). PANC-1 cells (1,000,000) and PSCs cells (1,000,000) in 0.1 mL DMEM medium were subcutaneous injected into the left abdominal area of mice of weight 18 g. Mice were cultured in suitable condition at 27 ℃ and 12 h light-dark cycle, and fed enough water and chow.

1.15 *In vivo* Tumor Penetration Capability Assay

To detect whether the tumor penetration capability of GEM enhanced after NIR irradiation. We replaced GEM with Cy7 dye and synthesize **CTh**-Cy7 NPs. While the volume of subcutaneous tumor was reached 80 mm^3, **CTh**-Cy7 NPs were injected peritumorally and the tumors were irradiated 5 min using 808 nm pulse laser (1 W cm^{-2}) after 6 h. Then we sacrificed

the mice, took out the tumor and fixed with embedding medium for frozen tissue. The fixed tumor was sliced into 20 μm slides and observed using an inverted optical microscope (Nikon, Japan).

1.16 *In vivo* Photo-thermal Conversion Efficiency Assay

In order to evaluate the photo-thermal conversion efficiency of **CTh** NPs or **C-G** NPs *in vivo*. While the tumor volume was reached 80 mm^3, a total of 15 nude mice were randomly divided into three groups: Control, "**CTh** NPs + Laser" and "**C-G** NPs + Laser" (n = 5). Mice of Control group were injected 100 μL PBS intratumorally. Mice of other two group were injected with **CTh** NPs or **C-G** NPs as the dose of 2 mg kg^{-1} of **CTh**. For PTT treatment, we irradiated the tumor vertically with 808 nm NIR laser (1 W cm^{-2}) for 10 min. The temperature changes were recorded by infrared camera (FLIR Systems AB) at every 30 s.

1.17 Enzyme-linked immunosorbent assay (ELISA)

PANC-1 was irradiated with 808 nm laser for 5 min and subsequently treated with serum-starved medium for 24 h. The supernatants were collected and the secretion level of TGF-β1 was determined through an ELISA kit (Multi science, China) according to the manufacturer's protocols. The concentration of TGF-β1 of sample was calculated by comparing the OD of the samples to the standard curve.

1.18 Western Blot Assay

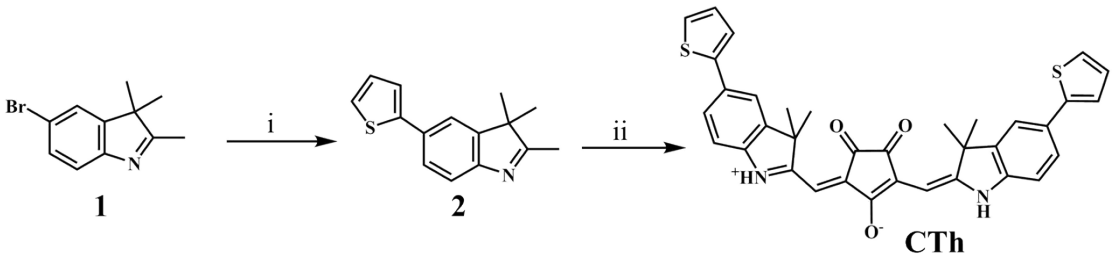
Firstly, the tumor issues or cellular proteins were prepared using RIPA lysis and quantified by BCA kit. Secondly, the proteins were separated by SDS-PAGE electrophoresis and transferred to a PVDF membrane. After being blocking 1 h, the proteins were incubated overnight with antibodies against rabbit TGF-β1 (Huabio, China), rabbit α-SMA (Huabio, China), rabbit Collagen I (Huabio, China) and mouse β-actin (Huabio, China). Subsequently, the PVDF membrane were incubate with secondary antibodies. Finally, signals were detected by the ECL according to the manufacturer's instructions.

1.19 RT-qPCR assay

The mRNA of tumor tissues and cell was preparing with chloroform and isopropanol and reverse-transcribed to cDNA through reverse transcription kit (TOYOBO, China). The expression of genes was determined through RT-qPCR and analyzed by $2^{-\Delta Ct}$ comparing with standardized house-keeping genes GAPDH.

1.20 Statistical Analysis

The data were shown as means ± SD. Statistical assessment was made using two-tailed paired Student's test for two groups and one-way analysis of variance (ANOVA) for multiple groups.



Scheme S1. The synthetic route for **CTh**. (i) tributyl(thiophen-2-yl)stannane, $Pd(PPh_3)_4$, toluene, 90 °C, argon atmosphere, 24 h; (ii) croconic acid, butanol/toluene, 110 °C, argon atmosphere, 3 h.

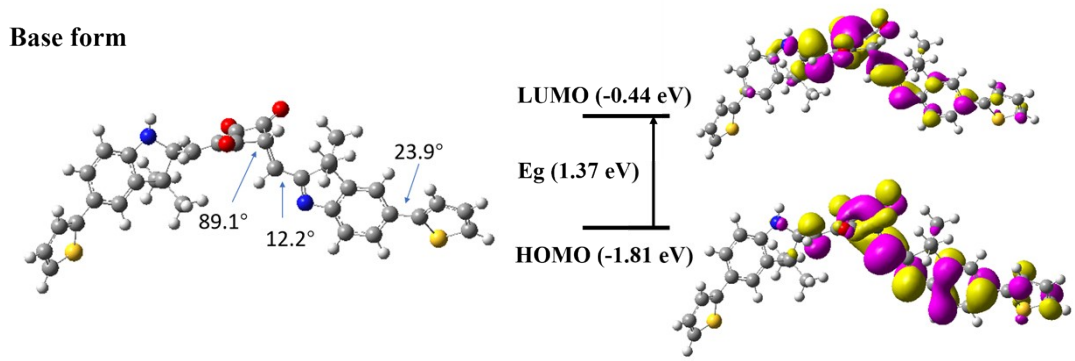


Figure S1. Molecular simulation, HOMOs and LUMOs of **CTh** with base form in DFT calculations.

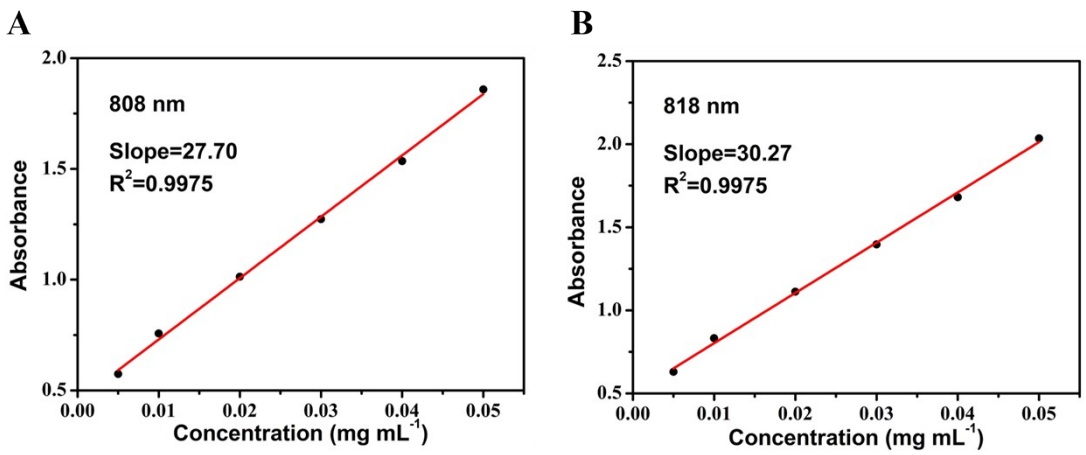


Figure S2. Linear relationship between absorption and concentration of **CTh** at 808 nm (A) and 818 nm (B)

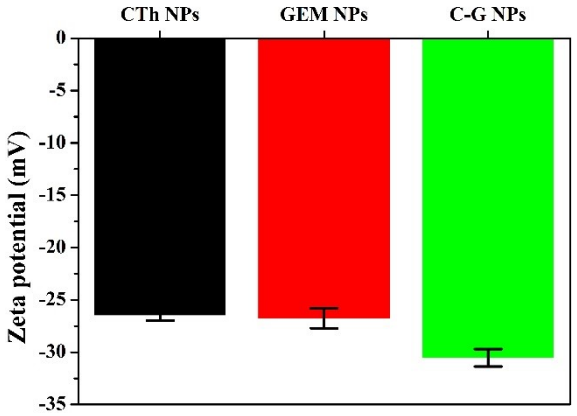


Figure S3. Zeta potential of **CTh** NPs, GEM NPs and **C-G** NPs in aqueous solution

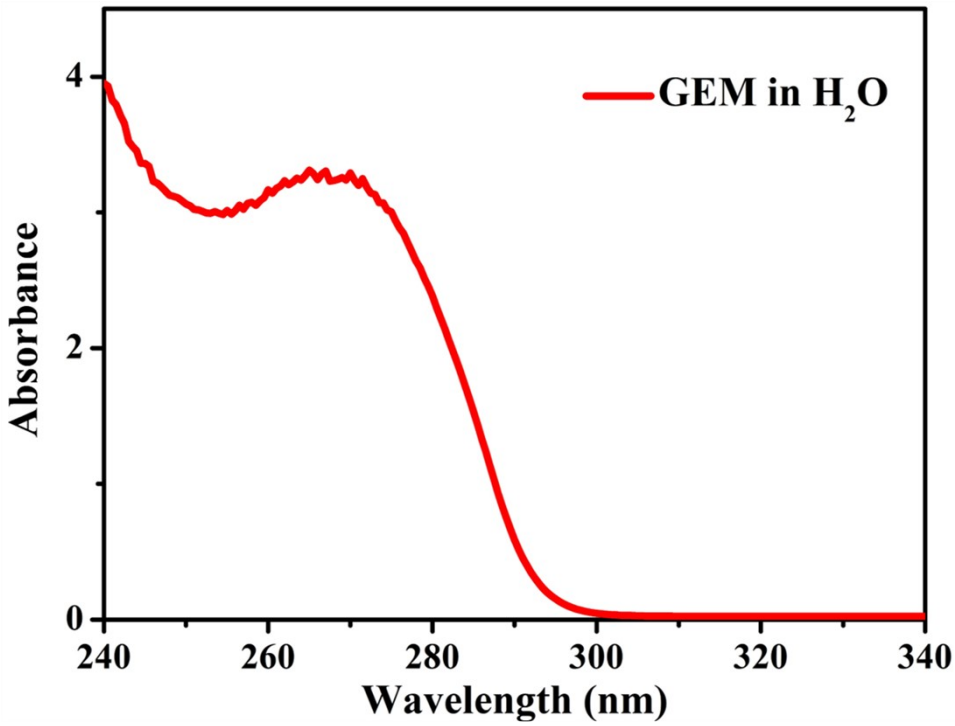


Figure S4. UV–vis absorption spectra of GEM in aqueous solution.

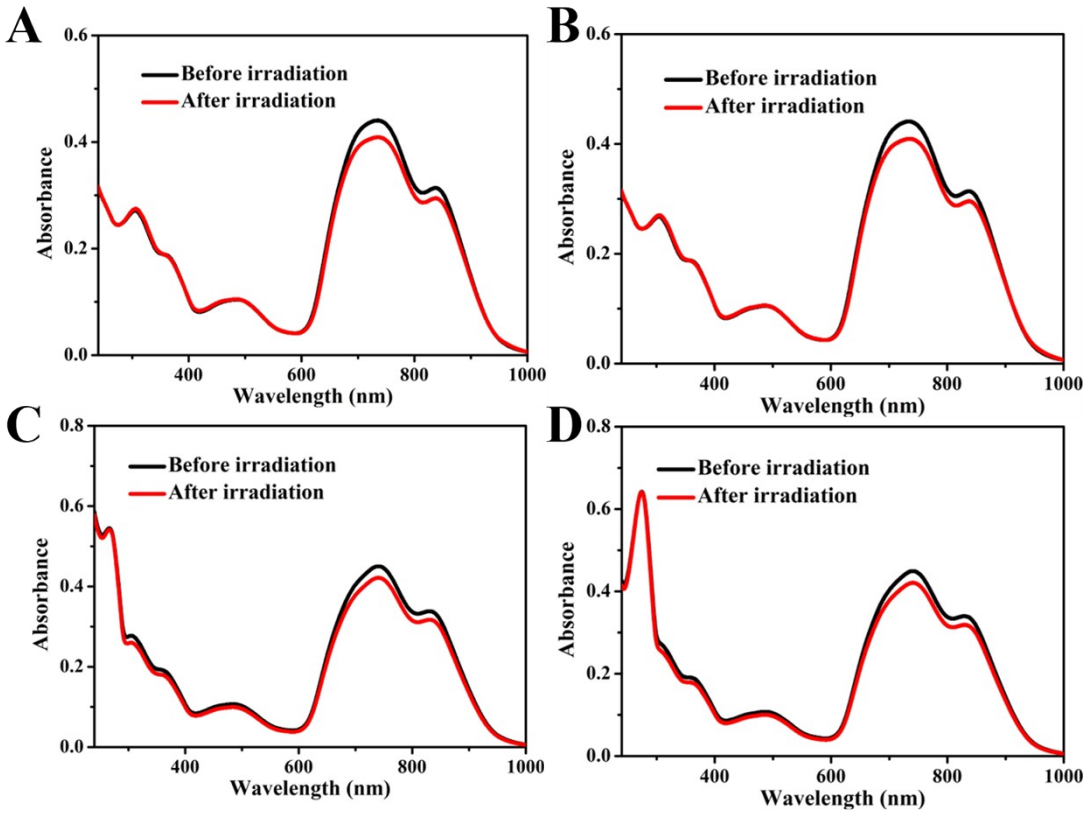


Figure S5. UV-vis-NIR absorption spectra of **CTh** NPs at pH 7.4 (A), pH 6.0 and **C-G** NPs at pH 7.4 (C), pH 6.0 (D)with the same **CTh** concentration of 0.005 mg mL^{-1} before or after 808 nm laser irradiation for 4 heating and cooling cycles.

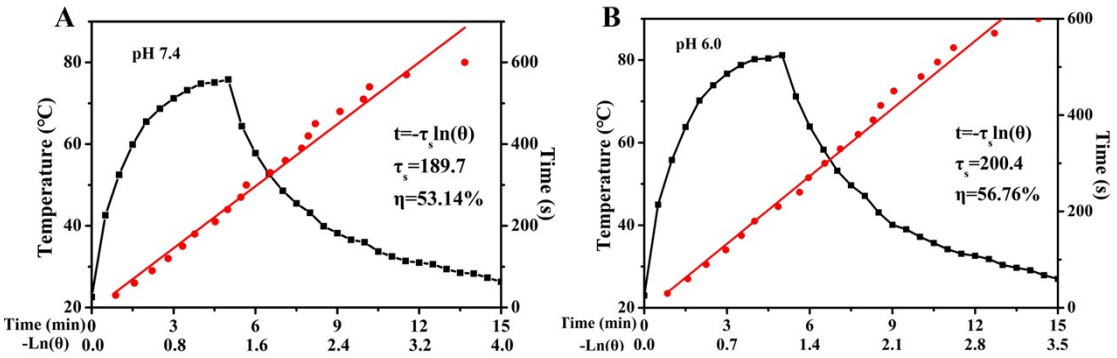


Figure S6. The calculated photothermal conversion efficiency (PCE) of **CTh** NPs at pH 7.4 (A) and pH 6.0 (B)

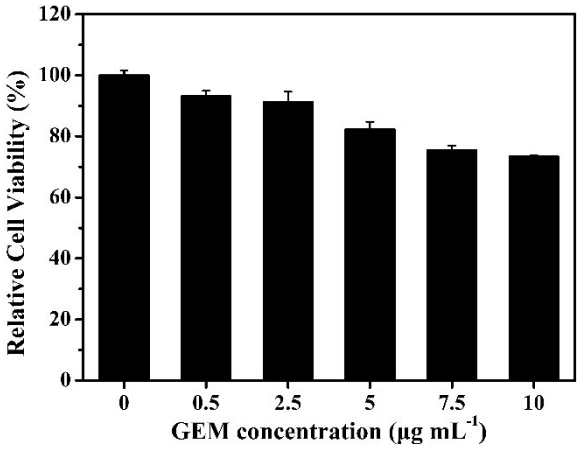


Figure S7. Relative PANC-1 cells viability treated with different concentrations of GEM NPs

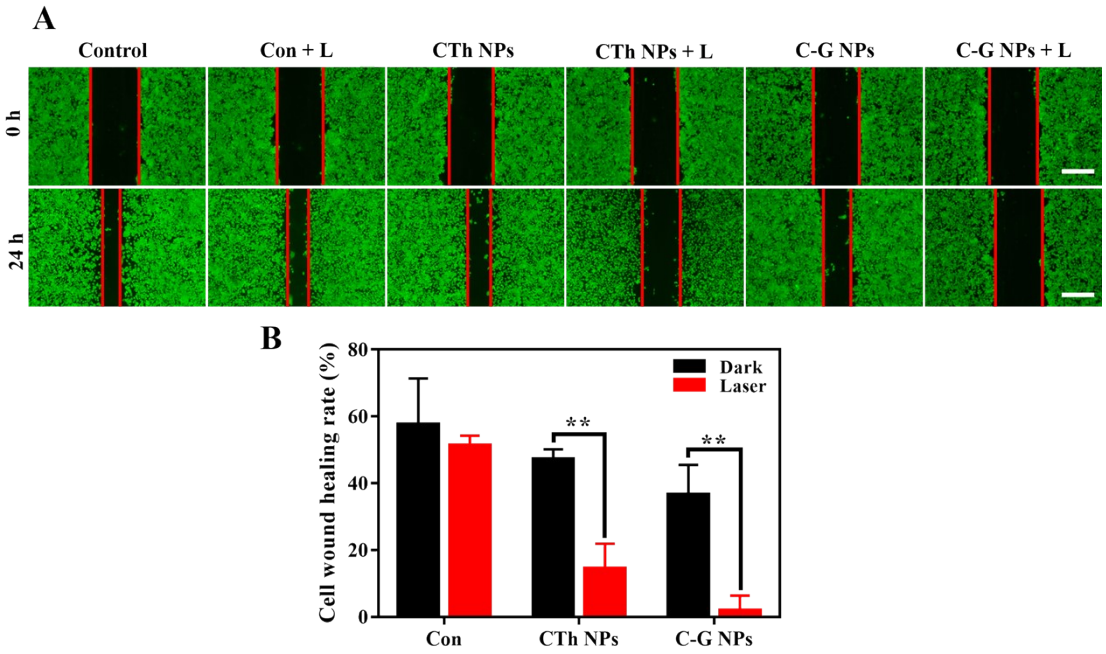


Figure S8. Calcein-AM staining of Scratch healing assay of PANC-1 cells upon different treatments and quantitative analysis of healing rate from Calcein-AM staining. (Scale bar, 100 μm) (**p<0.01)

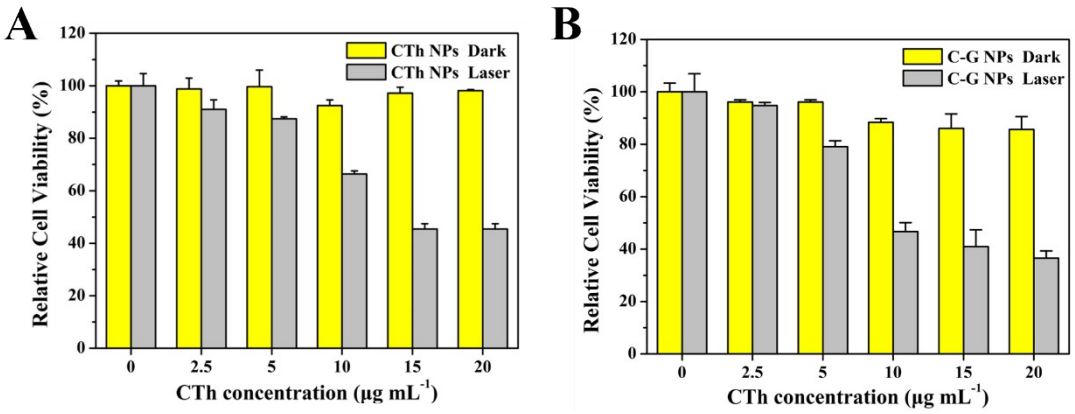


Figure S9. Relative PSCs viability treated with **CTh** NPs (A) and **C-G** NPs (B) with or without NIR irradiation.

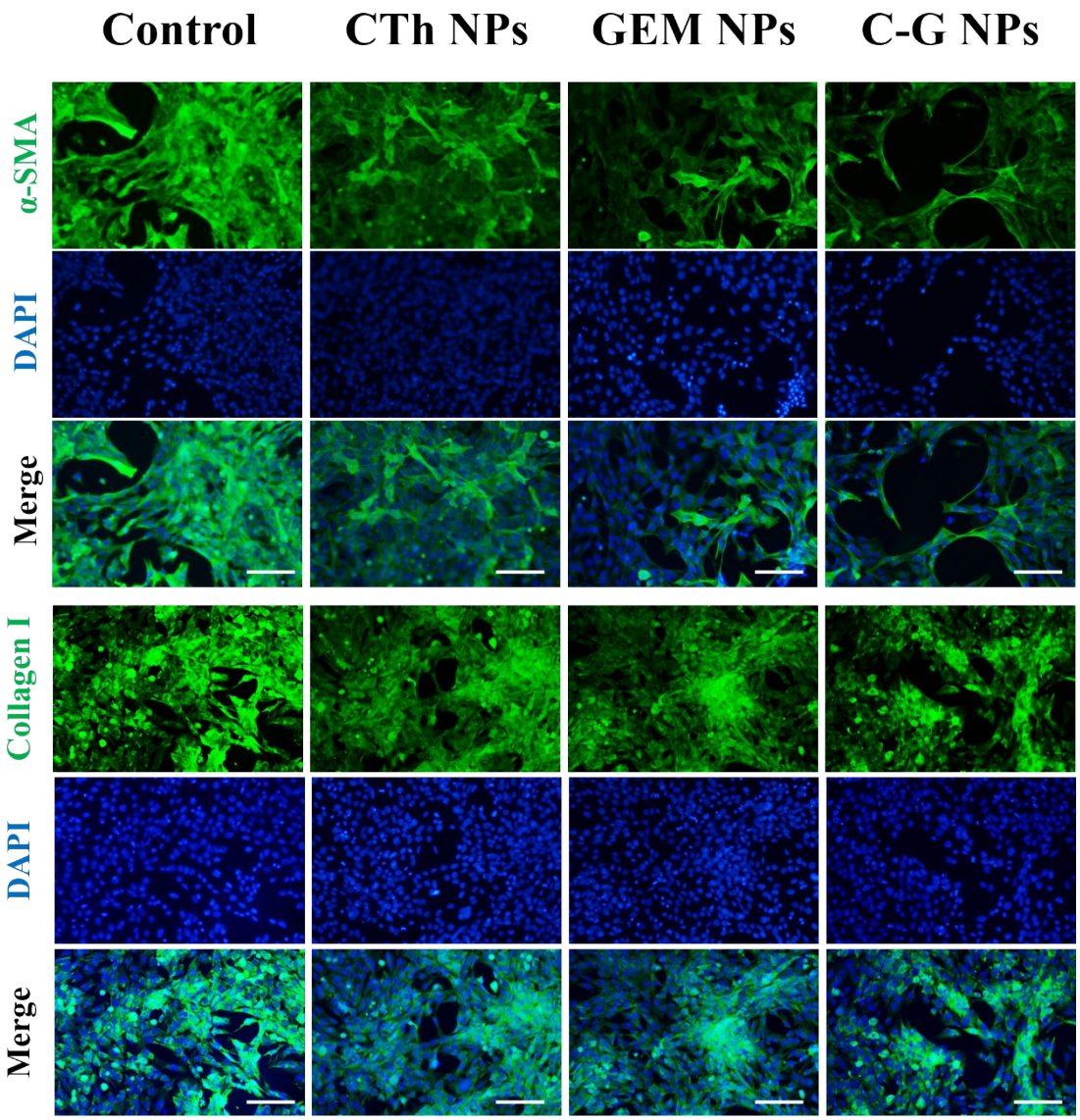


Figure S10. Immunofluorescence staining of α-SMA and Collagen I of PSCs after receiving different treatments. (Scale bar, 50 μm)

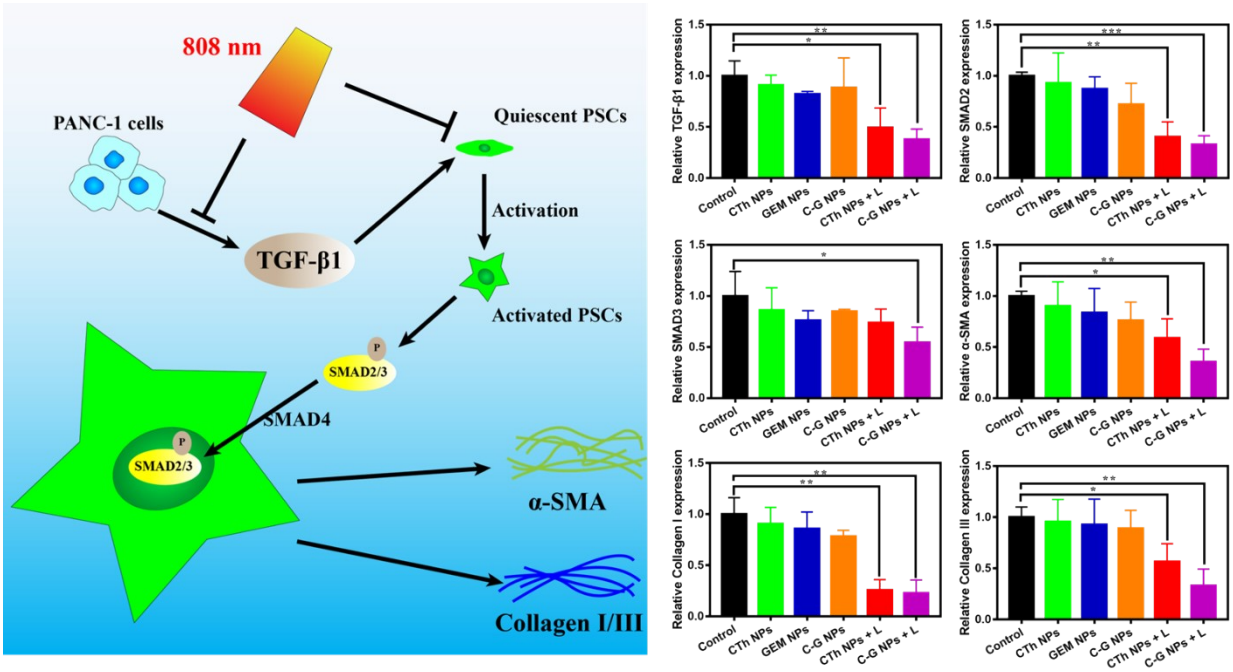


Figure S11. (A) Schematic illustration of PSCs activated by TGF-β/SMAD signaling network. (B) The expression level of TGF-β1 in PANC-1 cell under different treatments. The expression level of SMAD2 (C), SMAD3 (D), α-SMA (E), Collagen I (F) and Collagen III (G) in PSCs under different treatments.

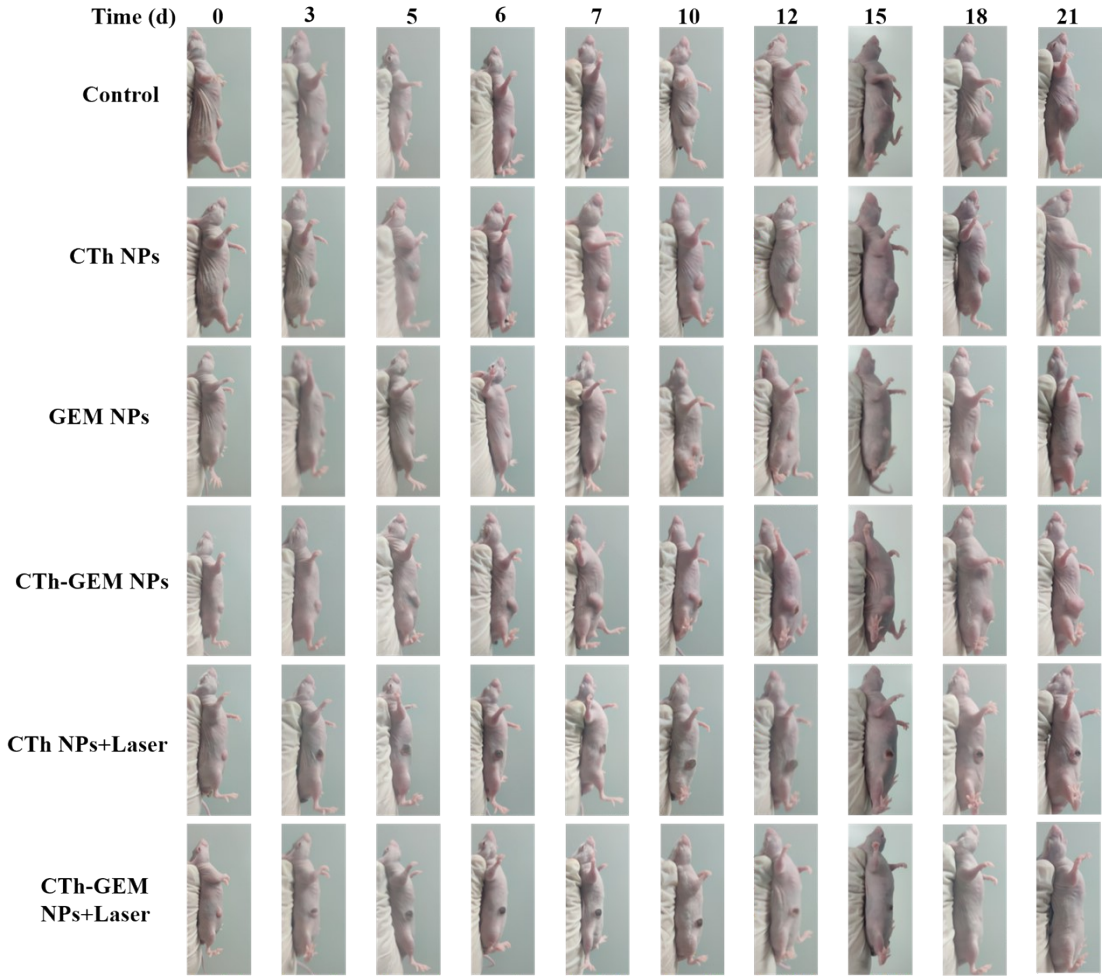


Figure S12. Photographs of for PANC-1 subcutaneous xenografts borne by nude mice upon different treatments

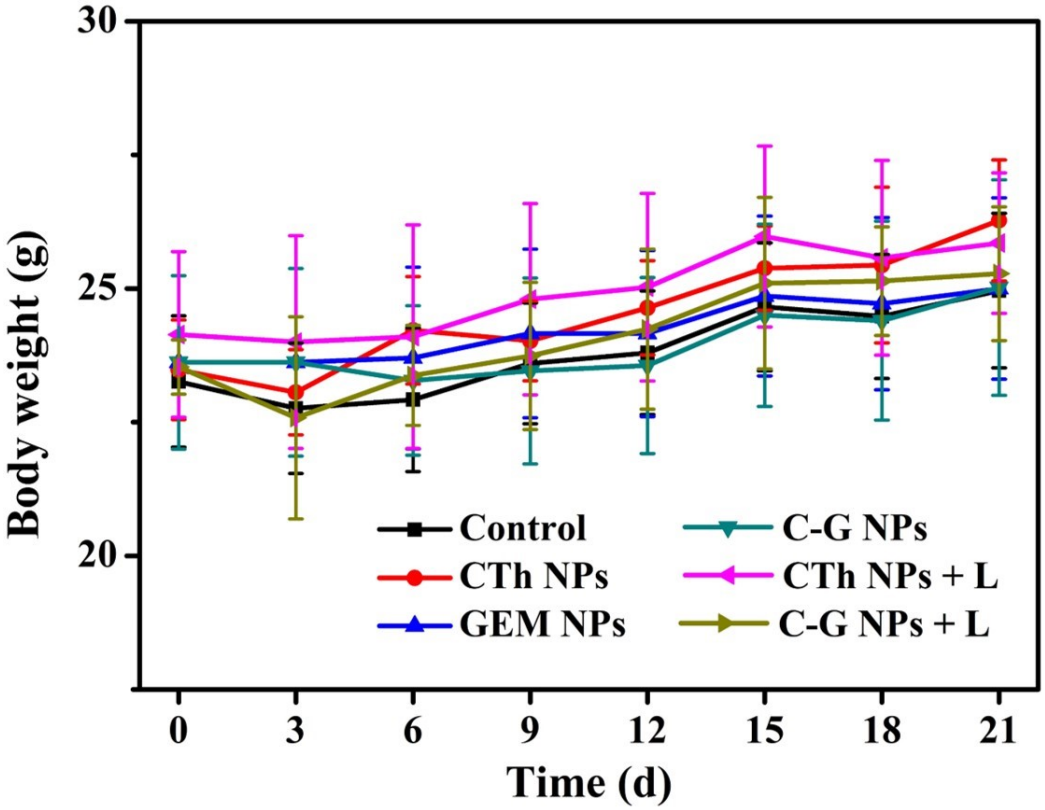


Figure S13. Mice body weight changes upon different treatments.

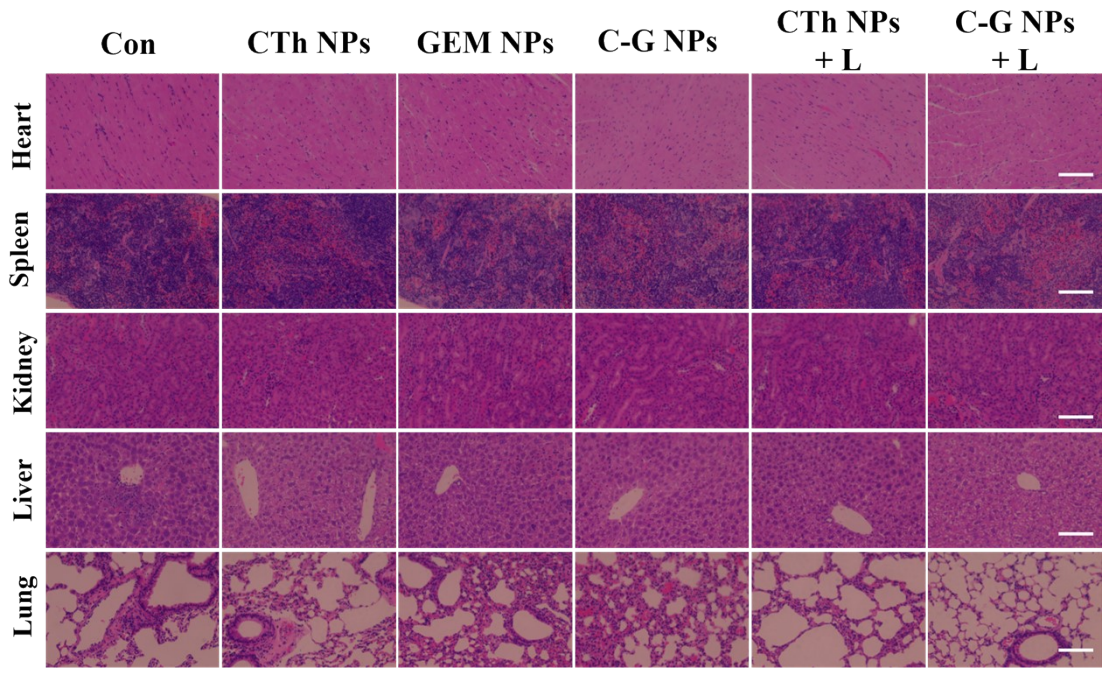


Figure S14. H&E staining of heart, spleen, kidney, liver and lung after receiving different treatment (Scale bar, 50 μm)

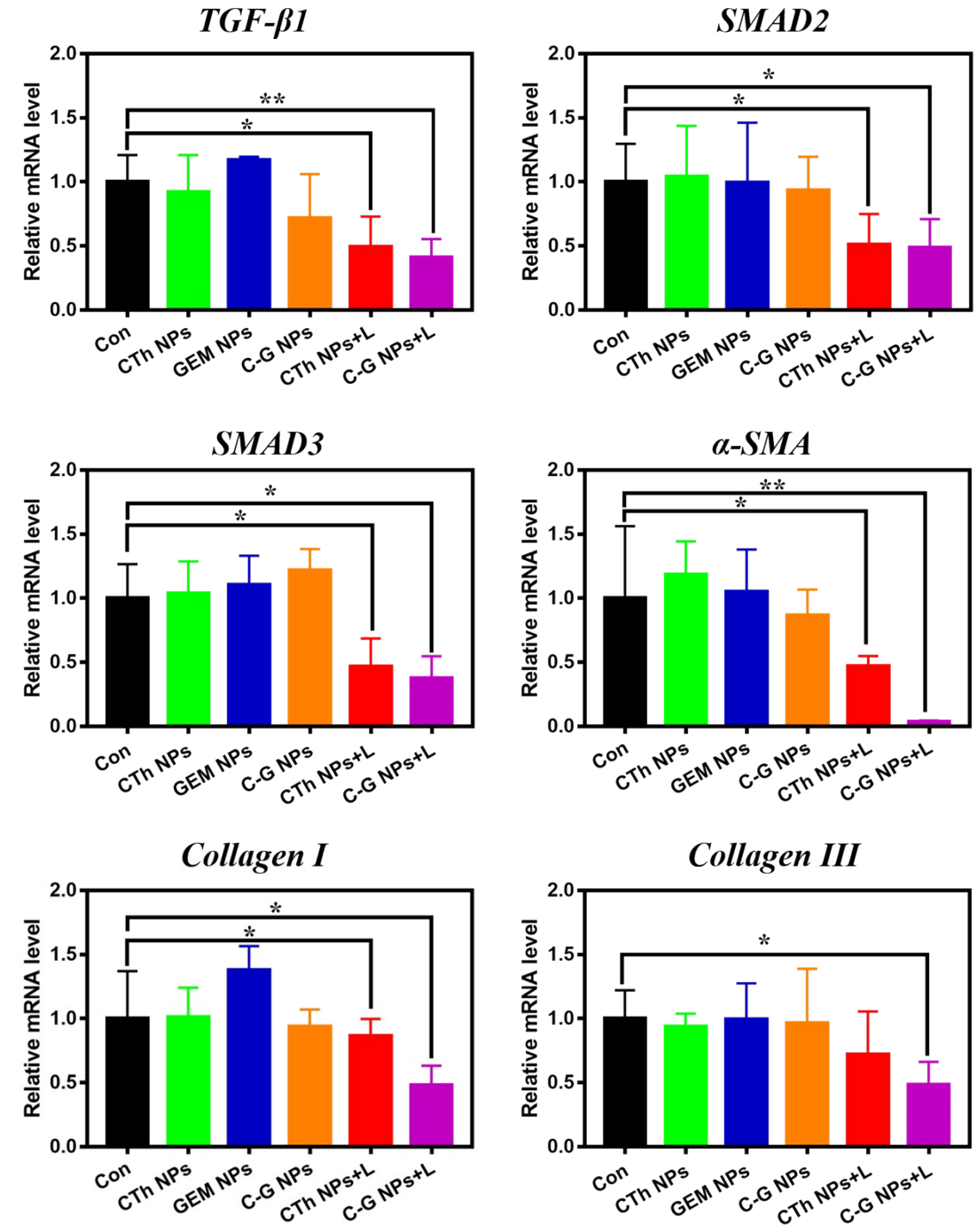


Figure S15. The mRNA expression level of *TGF-β1*, *SMAD2*, *SMAD3*, *α-SMA*, *Collagen I* and *Collagen III* of tumor tissue after receiving different treatment. (*p<0.05, **p<0.01)