Supporting Information

A Mitochondria-Targeting Self-assembly Carrier-free Lonidamine Nanodrug for Redox-activated Drug Release to Enhance Cancer Chemotherapy

Ting Yang,†[a, b] Xianfen Zhang,†[a, c] Xing Yang,[a] Ying Li,[a] Jingjing Xiang,[a] Chunbai Xiang,[a] Zhongke Liu,[a, d] Luo Hai,[e] Zhen Xu,[a] Saipeng Huang,*[c] Lihua Zhou,*[f] Ruijing Liang*[a] and Ping Gong*[a]

a. Guangdong Key Laboratory of Nanomedicine, CAS-HK Joint Lab for Biomaterials, Shenzhen Institutes of Advanced Technology, Chinese Academy of Sciences, Shenzhen, 518055, P. R. China.

b. University of Chinese Academy of Sciences, Beijing, 100049, P. R. China.

c. School of Chemical Engineering, Northwest University, Xi'an, 710069, P. R. China.

d. Nano Science and Technology Institute, University of Science & Technology of China, Suzhou, 215123, P. R. China.

e. Central Laboratory, National Cancer Center/National Clinical Research Center for Cancer/Cancer Hospital & Shenzhen Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Shenzhen, 518116, P. R. China.

f. School of Applied Biology, Shenzhen Institute of Technology, No. 1 Jiangjunmao, Shenzhen 518116, P. R. China

† These authors contributed equally to this work.

*Corresponding authors, E-mail: ping.gong@siat.ac.cn (P. Gong), rj.liang@siat.ac.cn (R. Liang), zhoulihuasit@163.com (L. Zhou), huangsaipeng@nwu.edu.cn (S. Huang)

Experiments section

Materials

Heptamethine cyanine dye IR780 and Lonidamine (LND) were purchased from sigma company (USA). N, N-Dimethylformamide (DMF), N, N'-Dicyclohexyl carbodiimide (DCC), Triethylamine (TEA), N-Hydroxysuccinimide (NHS) were purchased from Shanghai Aladdin Bio-Chem Technology Co., Ltd. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and penicillin-streptomycin were obtained from Gibco. Cell Counting Kit-8, ATP Assay Kit, and mitochondrial membrane potential kit were purchased from Beyotime Biotech Co., Ltd. MitoTracker™ Red FM, LysoTracker™ Green DND-26, and ER-Tracker™ Green were purchased from Thermo-Fisher Scientific.

Synthesis of Cy-TK

Compound 3 (100 mg,0.515 mmol) was dissolved in anhydrous DMF (2 ml) with TEA (100 μL). Then, Cy-Cl (70 mg,0.105 mmol) dissolved in anhydrous DMF (1 mL) was drop-wise added to the above solution under N_2 atmosphere at room temperature. The solvent was evaporated under reduced pressure to get crude product Cy-TK 6 h later. The product was further purified by silica gel column chromatography with dichloromethane and methanol. Yield: 36 mg (40%). ^{1}H NMR (400 MHz, Chloroform-d) δ 7.75 (d, J = 12.9 Hz, 2H), 7.34 (d, J = 7.4 Hz, 2H), 7.07 (t, J = 7.5 Hz, 2H), 6.86 (d, J = 7.9 Hz, 2H), 5.65 (d, J = 12.9 Hz, 2H), 4.08 (t, J = 6.5 Hz, 2H), 3.79 (t, J = 7.0 Hz, 4H), 3.24 (t, J = 6.5 Hz, 2H), 3.14 (t, J = 6.7 Hz, 2H), 2.99 (t, J = 6.7 Hz, 2H), 2.46 (t, J = 6.4 Hz, 4H), 1.82 (q, J = 7.4 Hz, 6H), 1.73 (s, 12H), 1.66 (s, 6H), 1.03 (t, J = 7.4 Hz, 6H).

Synthesis of Cy-TK-LND

Under N_2 atmosphere, the LND (20 mg,0.062 mmol) activated by NHS (7 mg,0.06 mmol), DCC (11.5 mg,0.6 mmol), and TEA (60 μL), were added to Cy-TK (30 mg,0.043 mmol) dissolved in 2 mL DMF. The reaction was going overnight at room temperature. Finally, the Cy-TK-LND was purified by silica gel column chromatography with dichloromethane and methanol. Yield: 24 mg (55%). ^{1}H NMR (400 MHz, CDCl3) δ 8.36 (d, J = 14.0 Hz, 1H), 7.73 (d, J = 15.2 Hz, 2H), 7.41 – 7.31 (m, 5H), 7.07 – 6.97 (m, 4H), 6.79 (d, J = 7.9 Hz, 2H), 6.71 (d, J = 8.3 Hz, 1H), 5.59 (t, J = 6.4 Hz, 4H), 4.09 (s, 2H), 3.75 – 3.66 (m, 6H), 3.30 – 3.22 (m, 2H), 2.92 (t, J = 7.1 Hz, 2H), 2.47 (d, J = 6.4 Hz, 4H), 1.83 – 1.74 (m, 10H), 1.66 (d, J = 2.5 Hz, 12H), 1.63 (s, 6H), 1.02 – 0.97 (m, 6H). ^{13}CNMR (101 MHz, Chloroform-d) δ 169.949, 167.255, 162.411, 143.234 141.022, 140.294, 138.411, 138.139, 134.440, 133.148,

132.317, 129.822, 129.367, 127.893, 127.668, 127.258, 123.030, 122.965, 122.462, 122.081, 122.321, 109.348, 108.197, 56.355, 53.450, 49.958, 49.527, 47.741, 39.006, 31.854, 31.598, 31.271, 30.442, 28.984, 25.204, 22.667, 21.592, 14.138, 11.741.

Cell culture

4T1 mouse breast cancer cells, Hela human cervical cancer cells, and A549 human adenocarcinoma alveolar basal epithelial cell line were purchased from ATCC. These cell lines were cultured in DMEM with 10% FBS and 1% penicillin-streptomycin at 37℃ under humidified incubator with 5% CO_2.

In vitro responsiveness

10 μM Cy-TK-LND NPs mixed with 10 mM H_2O_2 solution (with 3.2 μM $CuCl_2$, DMSO/PBS = 5/2, v/v, PH=7.4) or GSH solution (DMSO/PBS = 5/2, v/v, PH=7.4), and gently shaken in a 37℃ temperature-controlled incubator. At predetermined time points, different samples were extracted and detected by using the spectrophotometer and fluorescence spectrometer (λ_{ex} = 620 nm, λ_{em} = 780 nm; λ_{ex} =650 nm, λ_{em} = 760 nm;). The size of nanoparticles was determined with dynamic laser scattering (DLS) measurements.

Determination of ROS generation

The ROS generation measurements of Cy-TK-LND NPs were evaluated using 2,7-dichlorodihydrofluorescein (DCFH) as the indicator. For activation of DCFH, DCFH-DA (0.5 mL, 1mM) with NaOH solution (2 mL, 10 mM) were mixed and reacted in the dark for 30 min, then the cold PBS was added into the above solution to obtain DCFH solution with a concentration of 40 μM. Thereafter, the DCFH solution (10μM) and DMSO solution of the Cy-TK-LND NPs (20μM) were added to the PBS and mixed. The mixture was irradiated with 660nm laser at equal time intervals to obtain the fluorescence intensity at 525 nm for 488 nm excitation.

The role of OATP transporters

4T1 cells were seeded in six-well plates or confocal dedicated eight-well plates and incubated at 37℃ for 24 hours. Then, BSP (250 μM) was added and pretreated for 5min, and Pravastatin (5 μM), Vincristine (10 μM), and Itraconazole (5 μM) for 30 min. 10 μM Cy-TK-LND NPs was added and incubated with cells for 30 min. Next, the supernatant was removed and washed three times with PBS, and flow cytometry and confocal imaging were performed.

In vitro cytotoxicity assay

The cytotoxicity of LND, Cy, and Cy-TK-LND NPs was assessed by the Cell Counting Kit-8 assay. Briefly, 4T1 cells and A549 cells respectively seeded in a 96-well plate at a density of 1×10^4 per cell and incubated at 37℃ for 24 hours. Then, DMEM was replaced with a fresh medium, and the cells were treated with different concentrations of LND or Cy or Cy-TK-LND NPs (0, 2, 4, 6, 10, 20, and 50 μM). After incubation for 6 hours, the light groups were irradiated by laser for 20 min. After another 24 hours of incubation, the cells were washed twice with PBS, and the CCK-8 reagent was added to each well. Finally, the absorbance of each well was measured at 450 nm by a microplate reader (BioTek).

Intracellular ROS generation

The intracellular generation of ROS was assessed by DCFH-DA. 4T1 cells were seeded and cultured in a humidified environment with 5% CO_2 at 37 °C overnight. The cells were incubated with LND and Cy-TK-LND NPs (10μM) for 3 hours. Then, the cells were washed with PBS, and DCFH-DA (10μM) was added into the fresh medium and incubated for 20 min. Afterward, cells of the light group were irradiated by a 660 nm laser for 10min. After incubation for 0.5 hours, the cells were washed and collected to analyze the intracellular ROS level using flow cytometry.

Colocalization analysis of Cy-TK-LND

Hela cells were seeded in the 35 mm confocal dish to obtain the CLSM images. After incubation for 24 hours, 10μM Cy-TK-LND NPs were added to the medium. Then cells were washed twice with cold PBS and respectively incubated with Mitotracker Red, lysotracker Green, and ER-Tracker Green for 15min at 37℃. Colocalization analysis of cells and mitochondria acquired by CLSM imaging.

Measurement of ATP content level

4T1 cells were seeded at a density of 2×10^5 per well and incubated at 37℃ for 24 hours, then 10 μM LND and Cy-TK-LND NPs were respectively added for treatment. After 4 hours, the light groups were processed with 660 nm laser irradiation for 10min. Thereafter the cells were washed with PBS, digested by trypsin, and collected by centrifugation. After that, the cells were added lysate and centrifuged at 12000g for 5 min at 4℃ to obtain the ATP supernatant. Finally, the ATP content level was assessed using the ATP Assay Kit.

Mitochondrial membrane potentials assay

The mitochondrial membrane potential kit was used to evaluate the mitochondrial depolarization in 4T1 cells treated with Cy-TK-LND NPs. Specifically, 4T1 cells were seeded in an eight-well plate and incubated for 24 hours, then 10 μM LND and Cy-TK-LND NPs were added for treatment. After 4 hours, the light groups were irradiated with the 660 nm laser for 10min, then after another 24 hours of incubation, follow-up treatment was performed according to the instructions. The cells were rinsed twice with PBS, then incubated with an equal volume of serum-free medium containing JC-1 dye (5 mg/L) at 37 °C for 20 min, the supernatant was removed, and washed twice with buffer. Finally, fluorescence images of the green and red channels were taken by CLSM imaging. The images of JC-1 monomers (green) were taken at 488 nm excitation and 530 nm emission and JC-1 aggregates (red) were taken at 543 nm excitation and 590 nm emission.

In vivo biodistribution analysis

BALB/C mice were obtained from Vital River Laboratory Animal Technology Co. Ltd. Animals received care by the Guidance Suggestions for the Care and Use of Laboratory Animals. The procedures were approved by the Animal Care and Use Committee (Shenzhen Institutes of Advanced Technology, Chinese Academy of Sciences; SIAT-IACUC-210304-YYS-GP-A1708). The 4T1 tumor-bearing BALB/C mice were intravenously injected with 100 μl Cy-TK-LND NPs (50 μM) (n=3). Fluorescence images were captured at 0.5, 3, 9, 24, 48, 56, and 72h post-injected using the NIR-II fluorescence in vivo small animal imaging system, and excited at 640 nm. After 72 hours, the mice were sacrificed and organs including heart, liver, spleen, lung, kidneys, and tumor were collected for biodistribution analysis and imaging.

In vivo anti-tumor effect

6–8-week female BALB/C mice were subcutaneously injected with 4T1 cells (1×10^7) in the flank region. The tumor volume of tumor-bearing mice grew up to 80-100 cm^3 and was injected with PBS, LND, and Cy-TK-LND NPs (50 μM) via the tail vein (n=3). At 48 h after administration, the tumor region was irradiated by laser for 10 min. Subsequently, the tumor volume and mice body weight changes were monitored.

In vivo biosafety

The mice treated with PBS, LND, LND +Laser, Cy-TK-LND NPs, or Cy-TK-LND NPs +Laser were sacrificed. After that, the main organs and tumors of the mice were collected and fixed in 10% buffered formalin for 1 day.

Then, processed routinely into the paraffin and stained with hematoxylin and eosin (H&E), followed by imaging with optical microscopy. The blood samples were collected for biosafety, and hematological data and blood biochemical analysis were measured by Servicebio Company (Wuhan, China).

Statistical Analysis

The data are reported as mean ± standard deviation (SD). The differences among groups were assessed using one-way ANOVA analysis and student's t-test; *P < 0.05, **P < 0.01, ***P < 0.001

Figures

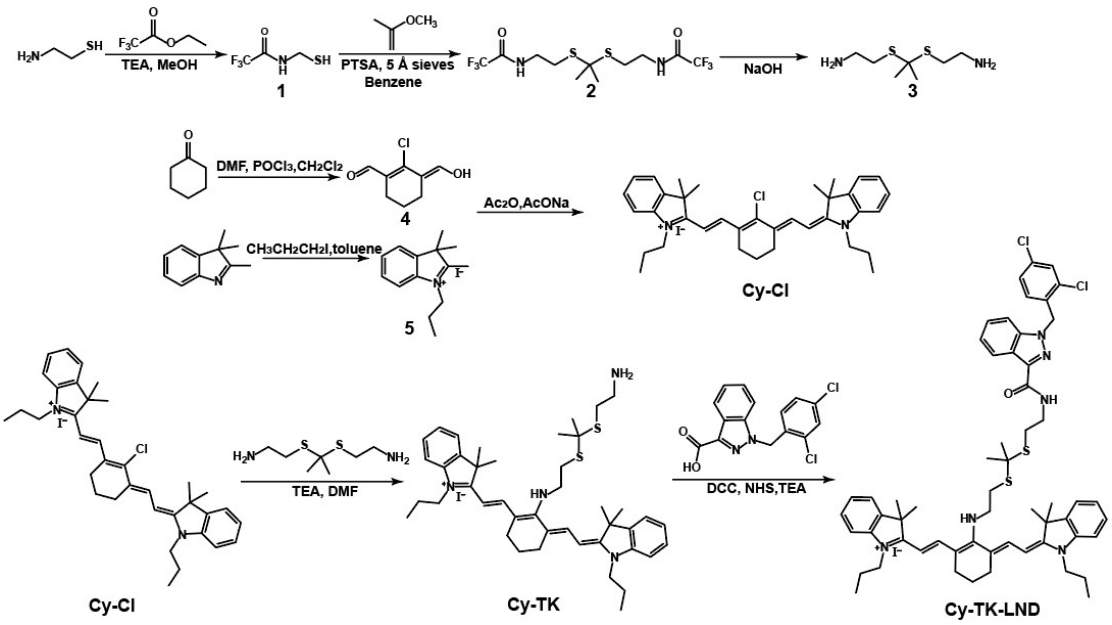


Figure S1. Schematic of the synthetic route of Cy-TK-LND.

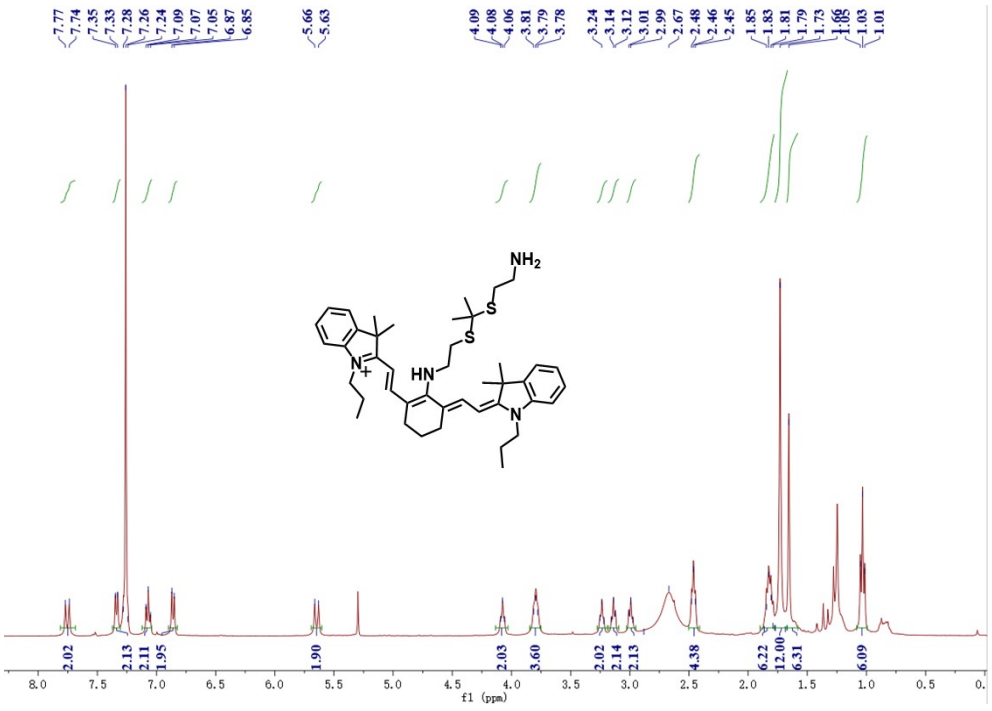


Figure S2. ^{1}H NMR spectrum of Cy-TK.

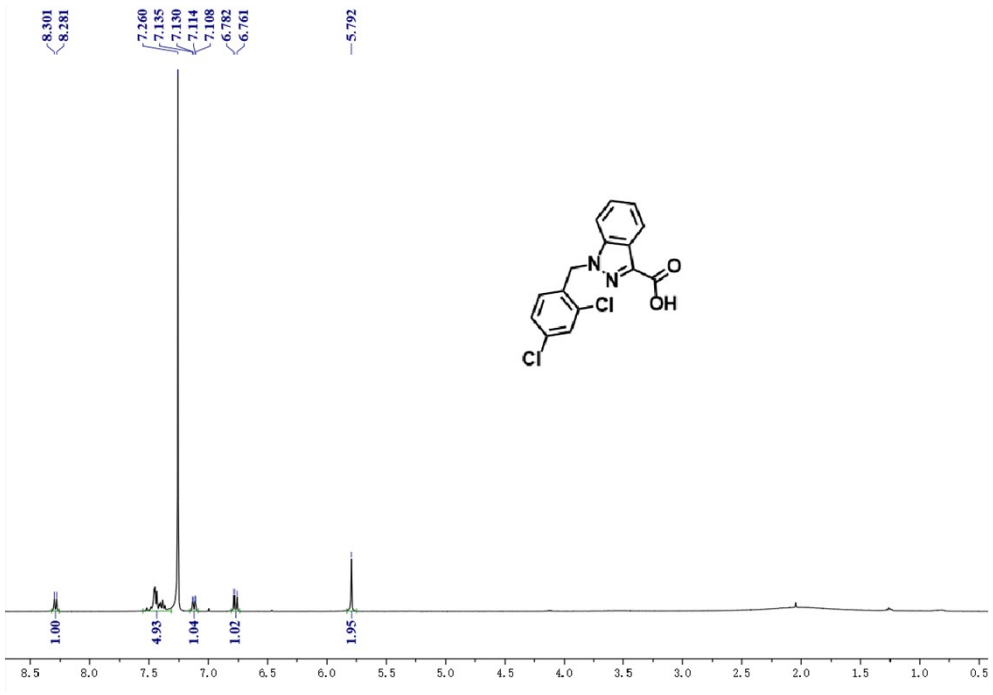
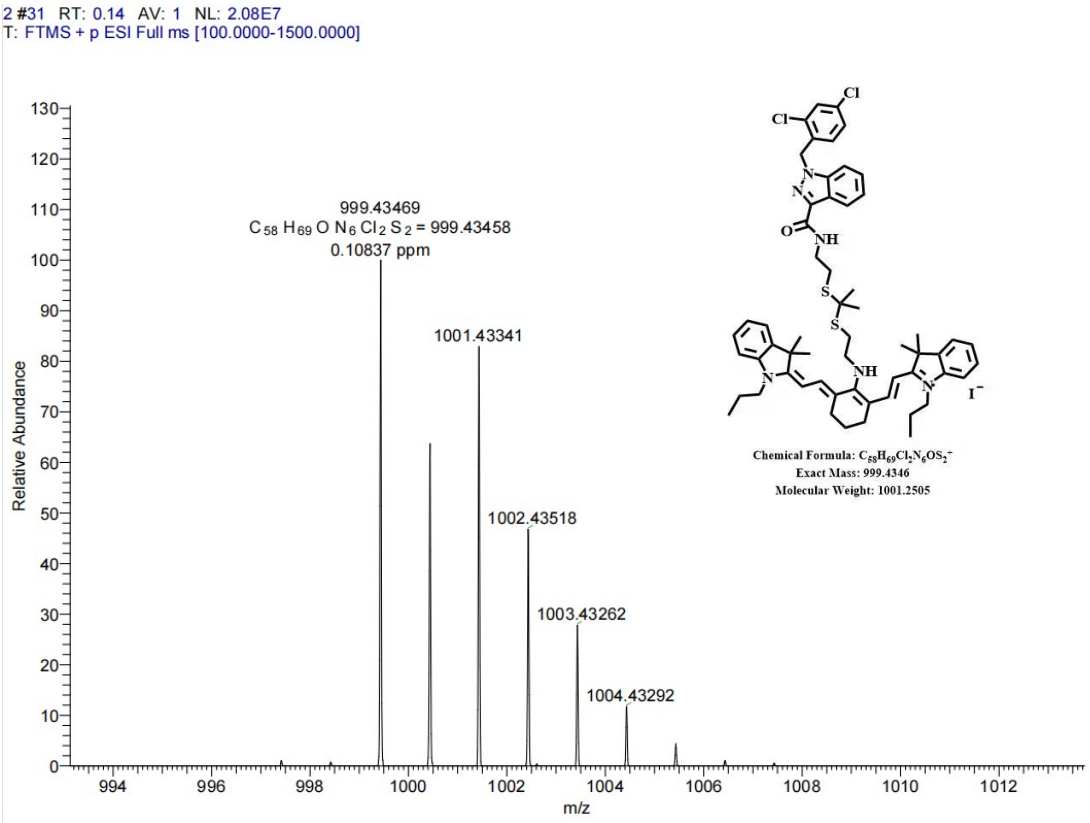


Figure S3. [1]H NMR spectrum of LND.



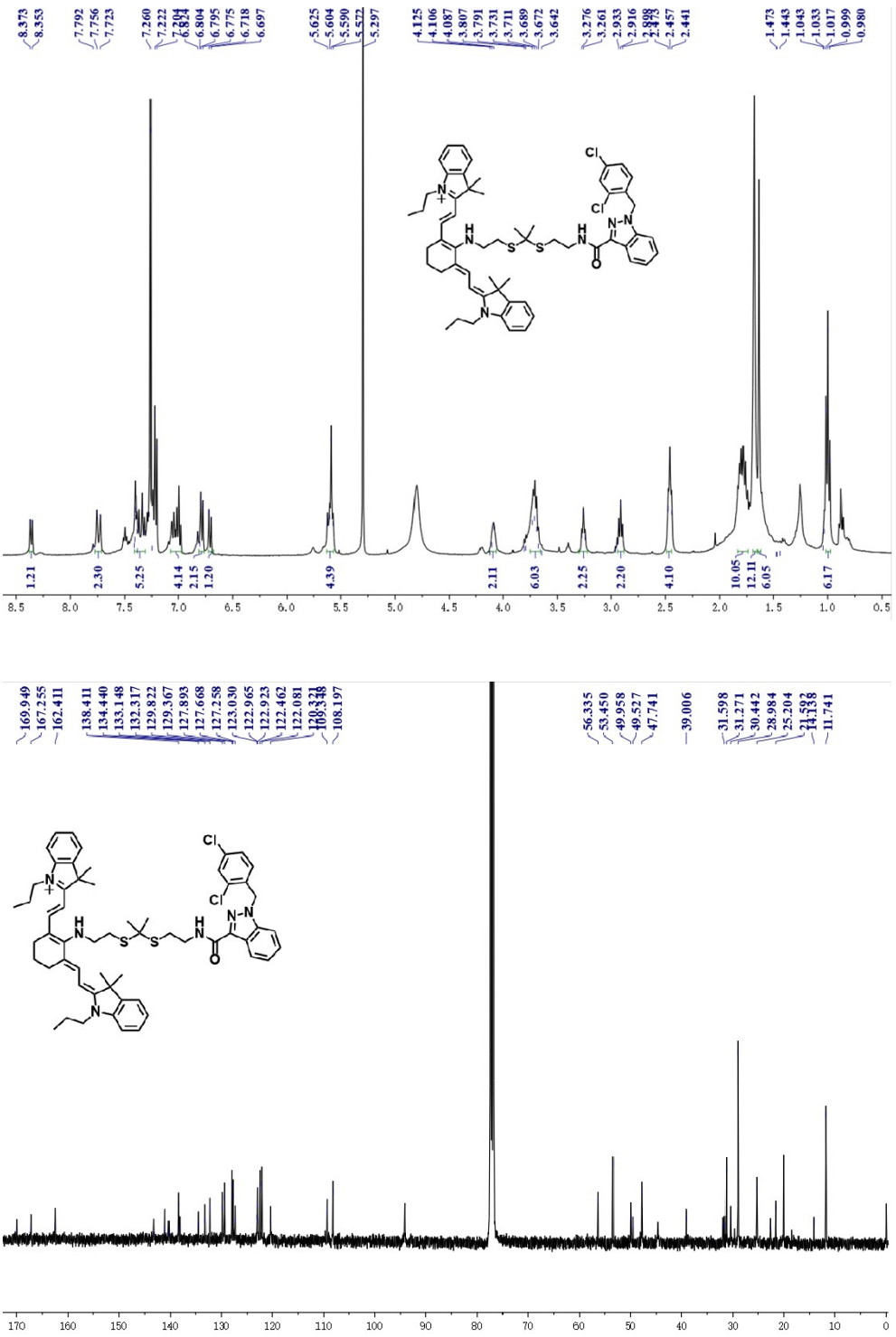


Figure S4. HRMS, ^{1}H NMR, and ^{13}C NMR spectra of Cy-TK-LND.

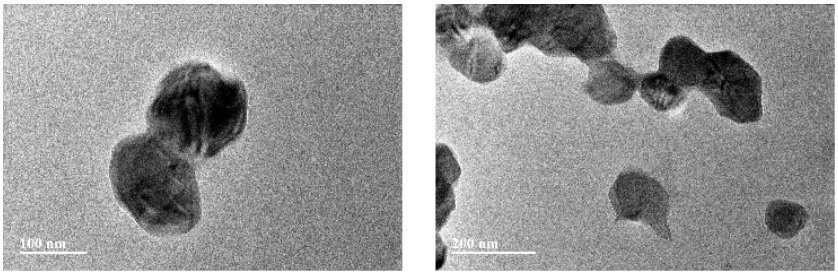


Figure S5. The TEM of Cy-TK-LND NPs in various fields.

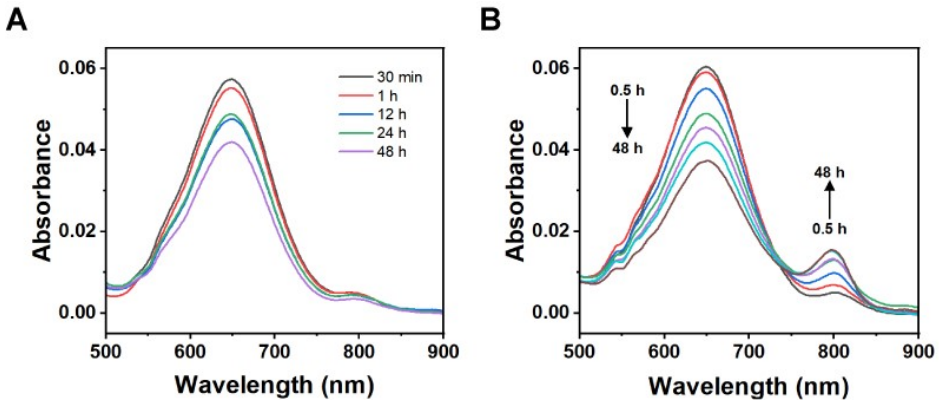


Figure S6. (A) The UV absorption changes of Cy-TK-LND NPs treated with 10 mM H_2O_2 solution in presence of 3.2 μM $CuCl_2$ (DMSO/PBS = 5/2, v/v, pH = 7.4). (B) UV-Vis absorption changes of Cy-TK-LND NPs in 10 mM GSH solution (DMSO/PBS = 5/2, v/v, pH = 7.4) at different times.

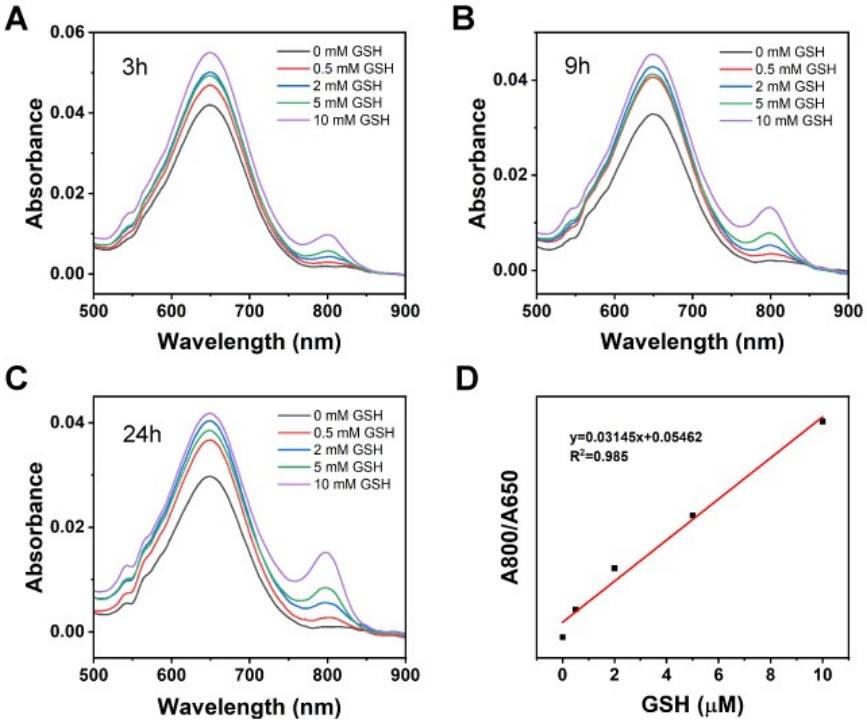


Figure S7. GSH concentration-dependent UV-vis absorption spectra of Cy-TK-LND NPs at different times (A), (B), and (C) and the absorption ratio of 800 nm compared to 650 nm for Cy-TK-LND NPs (D).

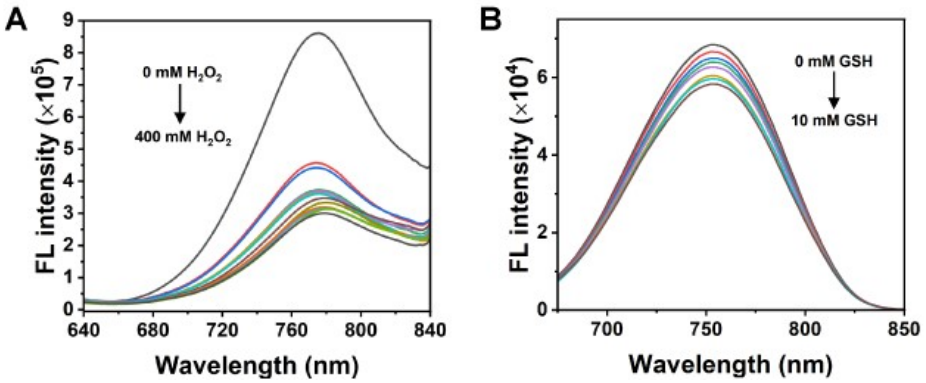


Figure S8. (A) Fluorescence emission spectra of Cy-TK-LND NPs in response to different concentrations of H_2O_2 in presence of 3.2 μM $CuCl_2$ (λex= 620 nm, λem= 780 nm). (B) Fluorescence spectra response of Cy-TK-LND NPs to GSH solution. (λex= 650 nm, λem= 760 nm).

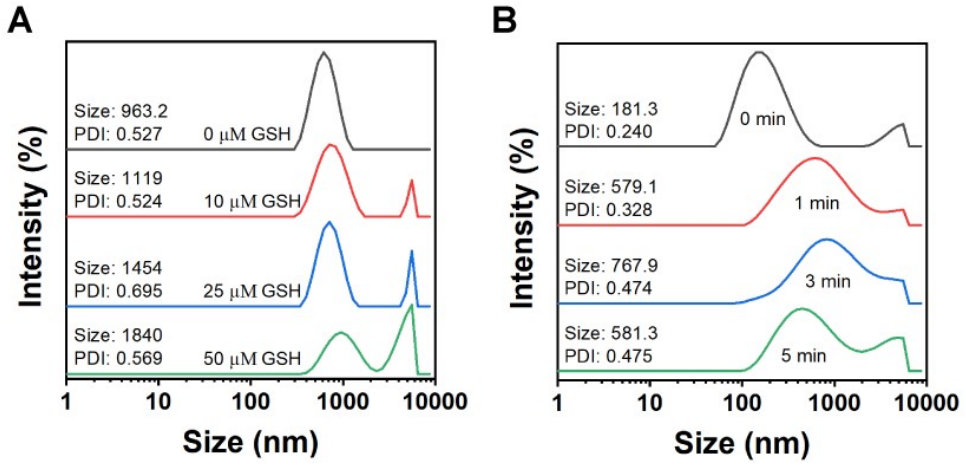


Figure S9. The dynamic laser scattering (DLS) size of Cy-TK-LND NPs in different concentrations of GSH solution (A) and under different irradiation times (B).

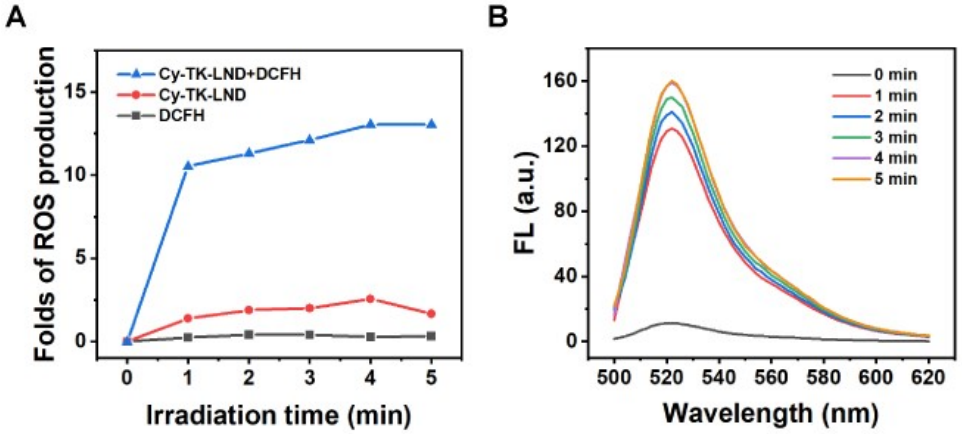


Figure S10. ROS generation of Cy-TK-LND NPs upon 660 nm laser irradiation.

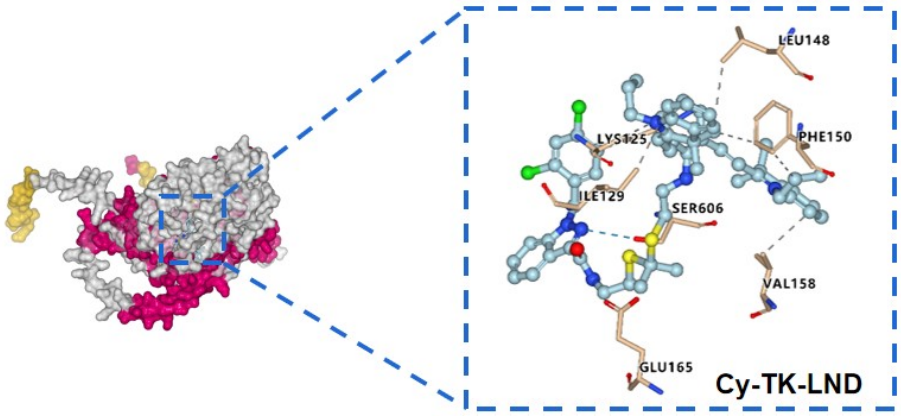


Figure S11. Molecular docking of Cy-TK-LND NPs with OATP1B3. The magnification on the right is the ligand molecules and amino acid residues.

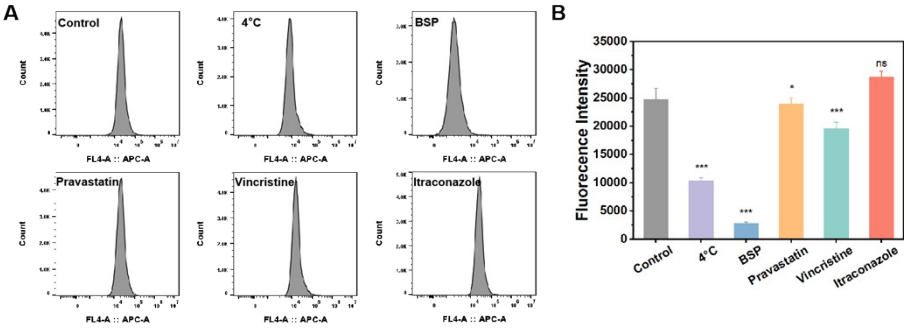


Figure S12. (A) Flow cytometry of Cy-TK-LND NPs uptake by 4T1 after pretreatment with 4°C and OATPs inhibitors. (B) Statistical results of the mean fluorescence intensity. *P < 0.05, **P < 0.01, ***P < 0.001.

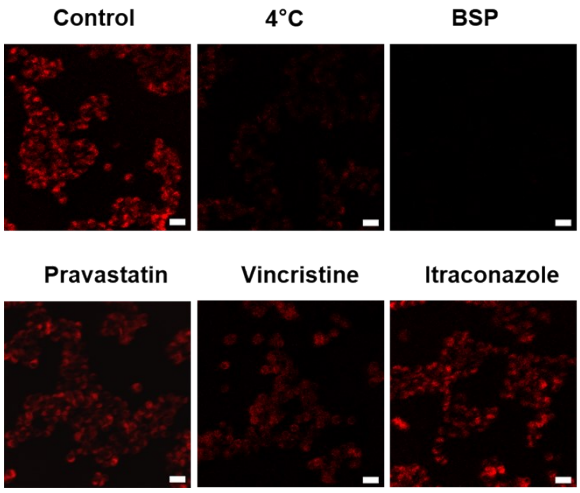


Figure S13. Confocal imaging of Cy-TK-LND NPs uptake by 4T1 after various pretreatment, scale bar= 25 μm.

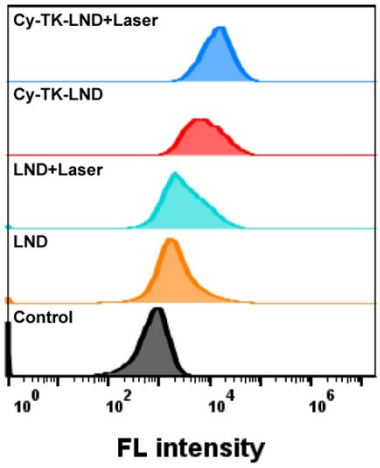


Figure S14. The relative level of ROS content in 4T1 cells after various treatments was determined by Flow cytometry. The untreated cells were used as a control.

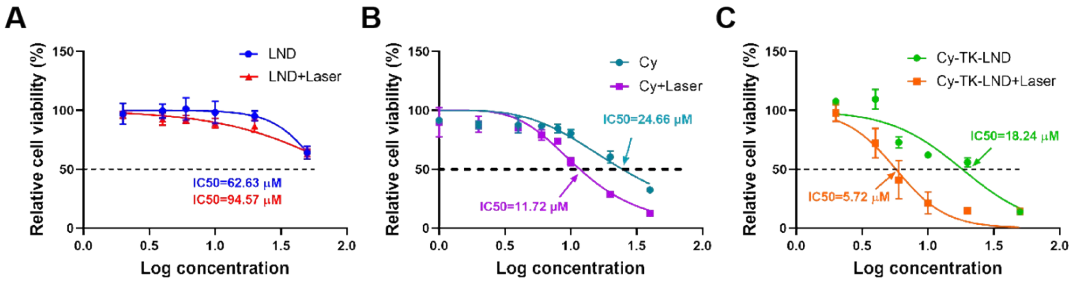


Figure S15. The cell viability of A549 cells treated with LND, Cy, and Cy-TK-LND NPs with or without laser irradiation.

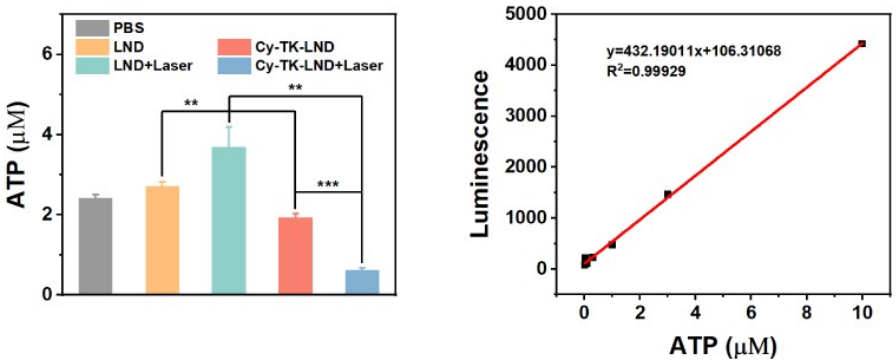


Figure S16. Measurement of ATP levels in 4T1 cells treated with LND and Cy-TK-LND NPs without or with laser irradiation. *P < 0.05, **P < 0.01, ***P < 0.001.

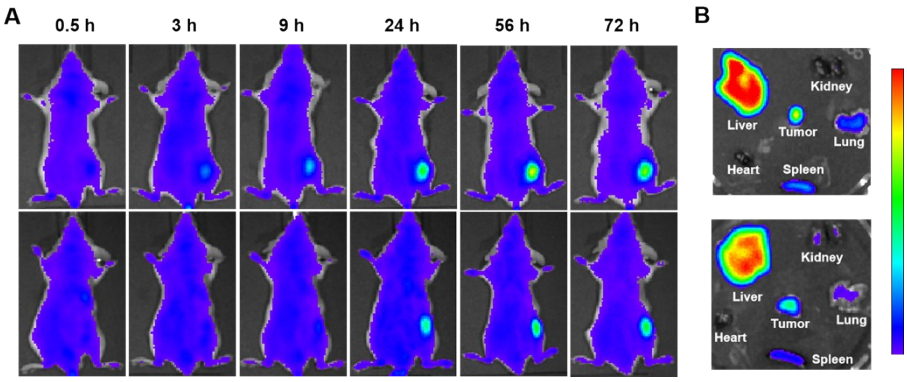


Figure S17. (A) In vivo biodistribution of Cy-TK-LND NPs in the remaining two mice. (B) Fluorescence images of major organs and tumor of the mice upon 72h post-injection.

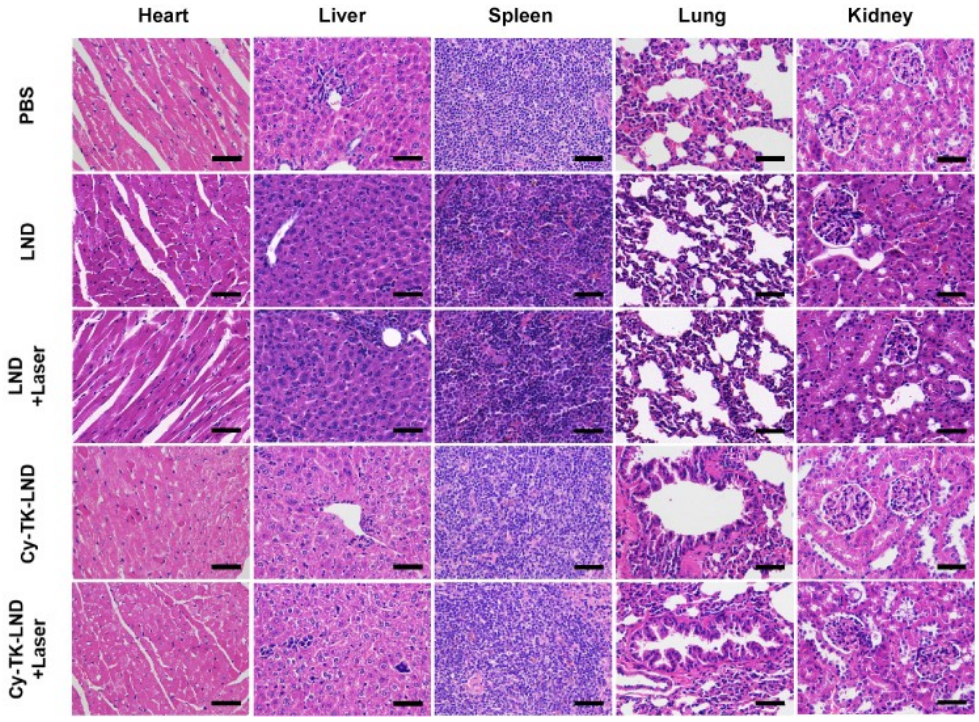


Figure S18. H&E staining of major organs in the mice under different treatments (scale bar, 50 μm).

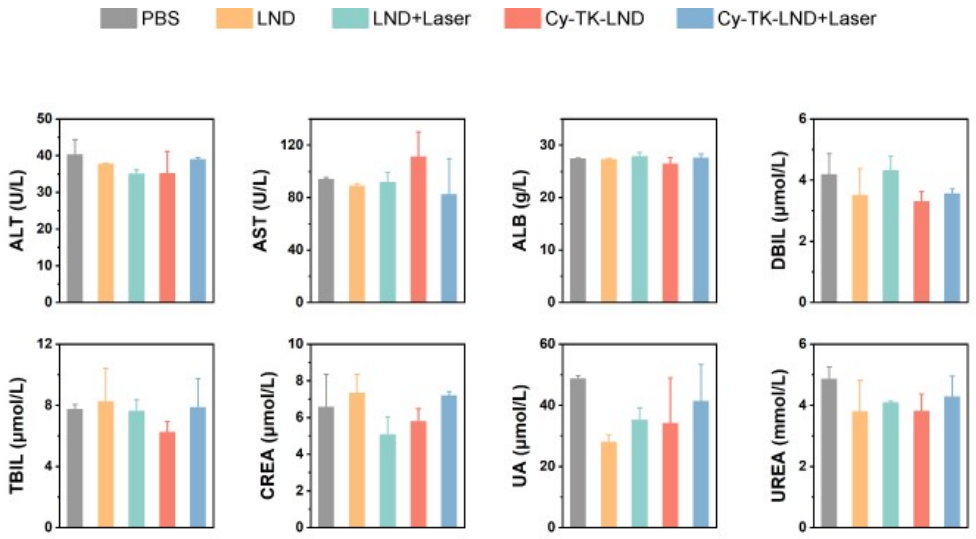


Figure S19. Serum biochemical data analysis of the mice under different treatments. The terms are the following: alanine transaminase (ALT), aspartate transaminase (AST), albumin (ALB), direct bilirubin (DBIL), total bilirubin (TBIL), creatinine (CREA), uric acid (UA), urea (UREA).

Table S1. The reference ranges of blood analysis of the mice.

Parameter	Unit	Reference range
WBC	10^9/L	0.8-6.8
Lymph	10^9/L	0.7-5.7
RBC	10^12/L	6.36-9.42
Gran	%	8.6-38.9
HGB	g/L	110-143
HCT	%	34.6-44.6
MCV	fL	48.2-58.3
MCH	pg	15.8-19
ALT	U/L	10.06-96.47
AST	U/L	36.31-235.48
ALB	g/L	21.22-39.15
DBIL	umol/L	0.45-33.89
TBIL	umol/L	6.09-53.06
CREA	umol/L	10.91-85.09
UA	umol/L	44.42-224.77