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# Supplementary Information

## A nitric oxide-boosting molecular agent to enhance mild-temperature photothermal therapy

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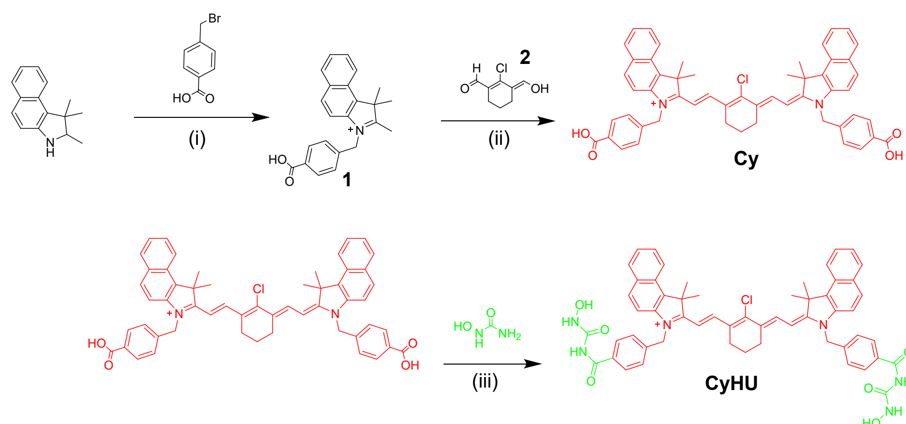
**Experimental section**

**Reagents.** Alpha-bromo-*p*-toluic-acid, 2,3,3-trimethyl-4,5-benzo-3H-indole, hydroxyurea, cyclohexanone, phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide, ethyl 2-ethoxyquinoline-1(2H)-carboxylate and sodium acetate (NaOAc) were obtained from Tianjin Heowns Biochemical Technology Co., Ltd. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2h-tetrazoliubromide (MTT) was obtained from Beijing Solarbio Science & Technology Co., Ltd. Annexin v-alexa fluor 488/PI apoptosis detection kit was purchased from Yeasen (Shanghai, China). Antibody against HSP60/70/27/90 was purchased from Boster Biological Technology Co., Ltd. Acetonitrile, absolute ether, dichloromethane (DCM) and acetic anhydride were purchased from China National Pharmaceutical (Shanghai, China).

**Instruments**: Fluorescence spectra were acquired with fluorescence spectrometer (FLS-980, Edinburgh, UK). UV-vis absorption spectra were measured on a pharmaspec UV-1700 UV-Visible spectrophotometer (Shimadzu, Japan). Confocal fluorescence imaging studies were performed with a TCS SP8 confocal laser scanning microscopy (Leica, Germany). Absorbance was measured in a microplate reader (Synergy 2, Biotek, USA) in the MTT assay. All the nuclear magnetic resonance (NMR) spectra were recorded on a Bruker NMR spectrometer. High resolution mass spectra (HRMS) were recorded on a Bruker Daltonics maXis UHR-TOF mass spectrometer.




**Scheme S1.** Synthesis of CyHU. (i) Acetonitrile, reflux; (ii) acetic anhydride, sodium acetate, 80 °C; (iii) dichloromethane, 2-ethoxy-1-ethoxycarbonyl -1,2-dihydroquinoline, 30 °C.

**Synthesis of CyHU**

**Compound 1:** The compound **1** was carefully prepared according to the references.[1] 2,3,3-trimethyl-4,5-benzo-3H-indole (5.0 g, 24 mmol) and alpha-bromo-p-toluic acid (6.0 g, 23 mmol) were dissolved in 30 mL acetonitrile and stirred under 80 °C for 6 h. The solid crude product was then filtrated and recrystallized in methanol. The purified product as denoted compound 1 was dried under vacuum (75% yield).

**Compound 2**: The compound **2** was carefully prepared according to the references.[1] 40 mL DMF/dichloromethane mixture (v/v: 1/1) was added dropwise into 40 mL solution of phosphoryl chloride/anhydrous dichloromethane (v/v: 1/1) under stirring in ice/water bath; afterwards, 5 g cyclohexanone was added dropwise into above solution. The ice/water bath was removed and the solution was then heated and refluxed for 2 h. The mixture was poured into ice, yielding a solid product which was collected by filtration and washed with iced diethyl ether. The resulting yellow product was denoted as compound 2 and used directly (60% yield).

**Compound Cy**: **Cy** was carefully prepared according to the references.[1] Under the protection of argon, **1** (848.0 mg, 2.0 mmol), **2** (173.0 mg, 1.0 mmol), and $CH_3COONa$ (164.0 mg, 2.0 mmol) were mixed with 20mL acetic anhydride in a 100 mL reaction flask. The mixed solution was kept heating at 80 °C for 1 h. The mixture

was cooled to r.t.. The crude product was formed through removed the acetic anhydride under reduced pressure. The crude product was purified through silica gel chromatography methods, using the $CH_2Cl_2/CH_3OH$ (v/v, 5:1) as the eluent (60% yield).

**Compound CyHU**: Under the protection of argon, **Cy** (0.903 g, 1.0 mmol), $NH_2CONHOH$ (258.6 mg, 3.4 mmol), EEDQ (840.8 mg, 3.4 mmol), and 30 mL dichloromethane were added to a 100 ml reaction flask, the mixed solution was heated to 30 °C for 16 h. The dichloromethane was removed under reduced pressure to give a crude product. The crude product was purified by column chromatography over silica gel using $CH_2Cl_2/CH_3OH$ (v/v, 10:1) as the eluent (30% yield). $^1$H NMR (400 MHz, $d^6$-DMSO): $\delta$ 9.85 (s, 2H), 8.35 (t, $J$ = 12.0 Hz, 4H), 8.06 (t, $J$ = 20.0 Hz, 6H), 7.70 (t, $J$ = 28.0 Hz, 4H), 7.52 (m, $J$ = 32.0 Hz, 6H), 6.62 (s, 4H), 6.40 (d, $J$ = 12.0 Hz, 2H), 6.20 (s, 2H), 5.83 (s, 4H), 3.17 (s, 2H), 2.58 (s, 4H), 2.02 (s, 12H). HRMS (ESI): *m/z* calcd for $C_{56}H_{52}ClN_6O_6^+$ $[(M\text{-}Br)]^+$: 939.3631, found: 939.3710.

**UV-vis spectra and fluorescent excitation spectra.** The UV-vis absorption spectra of CyHU were obtained using UV-visible spectrophotometer, and fluorescent spectra of CyHU in $H_2O$ and $CH_3OH$ were acquired using fluorescence spectrometer (FLS-980, Edinburgh, UK).

**Photostability test.** Aqueous soluntion of CyHU in UV-quartz cuvette were exposed to the 808 nm laser (0.33 W/cm$^2$) for different time. UV-vis spectra of the solution before and after laser irradiation were recorded.

**Photothermal effect and photothermal conversion efficiency.** For the purpose of evaluating the photothermal ability, the CyHU solution with different concentrations (0 mM, 0.05 mM, 0.10 mM, 0.50 mM, 1.0 mM, and 2.0 mM) were irradiated using 808 nm laser (0.33 W/cm$^2$). A thermometer submerged in the solution was used to monitor the temperature during the irradiated 600 s. To measure the photothermal conversion efficiency, CyHU solution (0.50 mM) were exposed to 808 nm irradiation (0.33 W/cm$^2$) for 10 min, and then the irradiation was removed for cooling down to room temperature. The temperature of the solution was recorded with an interval of 20 s during this process.

**Calculation of photothermal conversion efficiency.** The photothermal conversion efficiencies (η) were measured according to a previously described method.[2]

$$\eta = \frac{hS(\Delta T_1 - \Delta T_2)}{I(1 - 10^{-A})} \qquad hS = \frac{mc}{\tau_S} \qquad t = -\tau_S \ln(\theta) \qquad \theta = \frac{T - T_{surr}}{T_{max} - T_{surr}}$$

Where $\Delta T_1$ and $\Delta T_2$ are the maximum temperature changes of sample and $H_2O$, respectively. I represent the laser power. A is the absorbance of CyHU (0.50 mM) at 824 nm. m and c are the mass and heat capacity of solvent, respectively. T is the temperature at moment t in the cooling process. $T_{max}$ is the maximum temperature of sample. $T_{surr}$ is the surrounding temperature.

**Cell culture.** Hepa 1-6 cells were cultured in DMEM medium. All cells were supplemented with 10% fetal bovine serum (BI) and 100 units/mL of 1% antibiotics penicillin/streptomycin (Gibco) to maintain at 37 $^{o}$C in a 100% humidified atmosphere containing 5% $CO_2$.

**Cytotoxicity assays.** Hepa 1-6 cells were seeded in 96-well plates with an amount of $5 \times 10^3$ for 24 h and incubated with CyHU (0 μM, 5 μM, 10 μM, 20 μM, 50 μM and 100 μM) for another 24 h. During which, the cells were cultured with fresh complete medium, and with or without irradiation using 808 nm laser (0.33 W/cm$^2$, 10 min). After that, 150 μL MTT solution (0.5 mg/mL) was added to each well. The remaining MTT solution was removed 4 h later, and 150 μL of DMSO was added to each well to dissolve the formazan crystals. The absorbance was measured at 490 nm with a RT 6000 microplate reader. The half-maximum inhibitory concentration ($IC_{50}$) value was calculated according to the MTT results.

For Calcein-AM and PI co-staining assay, Hepa 1-6 cells ($2.0 \times 10^5$ per dish) were seeded on 35 mm confocal dishes and allowed to stabilize for 24 h. Then, Hepa 1-6 cells incubated with with Cy (100 μM), hydroxyurea (200 μM), CyHU (100 μM) were or not exposed to an 808 nm laser with the power density of 0.33 W/cm$^2$ for 600 s. After another 24 h of incubation, the cells were stained with Calcein-AM and PI for 30 min to evaluate the PTT efficacy using TCS SP8 confocal laser scanning microscopy.

**Detection of extracellular NO.** Hepa 1-6 cells were seeded in 96-well plates with an

amount of 5 × $10^3$ for 24 h and incubated with Cy (100 μM), hydroxyurea (200 μM), CyHU (100 μM). The generation of NO was determined using the griess reagent kit assay (Beyotime Biotechnology S0021). The experiment was repeated for three times.

**Intracellular generation of NO/$ONOO^-$.** For fluorescence imaging, Hepa 1-6 cells (2 × $10^5$/well) were passed on confocal dishes and incubated for 24 h. Cells were washed three times with PBS (10 mM, pH = 7.4), and incubated with different treatment (Cy (100 μM), hydroxyurea (200 μM), and CyHU (100 μM)). Cells were incubated with materials and NO fluorescent probe (DAF-FM DA)/$ONOO^-$ fluorescent probe (CBA) for 20 min. Cells were washed three times with PBS (10 mM, pH = 7.4) and taken under a confocal microscope.

**Immunofluorescence staining of HSPs.** For fluorescence imaging, Hepa 1-6 cells (2 × $10^5$/well) were passed on confocal dishes and incubated for 24h. Cells were washed three times with PBS (10 mM, pH = 7.4), and incubated with different materials for 4 h at 37 °C (Cy (100 μM), hydroxyurea (200 μM), and CyHU (100 μM)). Then cells were treated with 4% (w/v) paraformaldehyde for 20 min at 4 °C. Cells were washed three times with PBS (10 mM, pH = 7.4) and permeabilized with 0.25% (v/v) Triton X-100 for 5 min at 25 °C. Next, cells were treated with 5% bovine serum albumin (BSA) for 60 min at 25 °C. Then cells incubated with antibody against HSP70/90/27/60 for 60 min at 25 °C. Cells were washed three times with PBS (10 mM, pH = 7.4), and incubated with FITC-labeled secondary antibody for 60 min at 25 °C. Cells were washed three times with PBS (10 mM, pH = 7.4) and taken under a confocal microscope.

**Western blotting experiment of HSPs**. The Hepa 1-6 cells were divided into 4 groups to obtain the protein sample: PBS, hydroxyurea, CyHU, Cy. The concentration of protein was quantified using BCA protein assay kit. SDA-PAGE protein loading buffer was added to the protein samples and the mixture was heated with boiling water for 10 min to make the protein fully denatured. Subsequently, samples with equal protein amount were added into the wells and proteins were separated by the 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The target protein was then transferred to a carrier (PVDF) membrane and incubated with

specific primary antibodies at 4°C overnight. The secondary antibody labeled with horseradish peroxidase was combined with the primary antibody and incubated together. Then added ECL luminescent solution for color development and finally use a gel imaging system to analyze the western blotting.

**Co-localization analysis.** Hepa 1-6 cells (2.0 × $10^5$ per dish) were seeded on 35 mm confocal dishes and allowed to stabilize for 24 h. Then, Hepa 1-6 cells incubated with with Cy (100 μM), and CyHU (100 μM) for 4 h. Then the petri dishes were washing with PBS (10 mM, pH = 7.4) for another three times, and incubating with mito-tracker green for 20 min. Finally, each dish was washed with PBS (10 mM, pH = 7.4) for three times, and analyzed with a TCS SP8 confocal laser scanning microscopy.

**Immunofluorescence staining of caspase 3.** For fluorescence imaging, Hepa 1-6 cells (2 × $10^5$/well) were passed on confocal dishes and incubated for 24h. Cells were washed three times with PBS (10 mM, pH = 7.4), and incubated with different materials for 4 h at 37 °C (Cy (100 μM), hydroxyurea (200 μM), and CyHU (100 μM)). During which, the cells were cultured with fresh complete medium, and with irradiation using 808 nm laser (0.33 W/$cm^2$, 10 min). After 2 h, the cells were treated with 4% (w/v) paraformaldehyde for 20 min at 4 °C. Cells were washed three times with PBS (10 mM, pH = 7.4) and permeabilized with 0.25% (v/v) Triton X-100 for 5 min at 25 °C. Next, cells were treated with 5% bovine serum albumin (BSA) for 60 min at 25 °C. Then cells incubated with antibody against caspase 3 for 60 min at 25 °C. Cells were washed three times with PBS (10 mM, pH = 7.4), and incubated with FITC-labeled secondary antibody for 60 min at 25 °C. Cells were washed three times with PBS (10 mM, pH = 7.4) and taken under a confocal microscope.

**Western blotting experiment of caspase 3**. The Hepa 1-6 cells were divided into 4 groups to obtain the protein sample: PBS + NIR, hydroxyurea + NIR, Cy + NIR, CyHU + NIR. The concentration of protein was quantified using BCA protein assay kit. SDA-PAGE protein loading buffer was added to the protein samples and the mixture was heated with boiling water for 10 min to make the protein fully denatured. Subsequently, samples with equal protein amount were added into the wells and proteins were separated by the 10% sodium dodecyl sulfate polyacrylamide gel

electrophoresis (SDS-PAGE). The target protein was then transferred to a carrier (PVDF) membrane and incubated with specific primary antibodies at 4°C overnight. The secondary antibody labeled with horseradish peroxidase was combined with the primary antibody and incubated together. Then added ECL luminescent solution for color development and finally use a gel imaging system to analyze the western blotting.

**Detection of mitochondrial membrane potential.** Hepa 1-6 cells (2.0 × $10^5$ per dish) were seeded on 35 mm confocal dishes and allowed to stabilize for 24 h. Then, Hepa 1-6 cells incubated with with Cy (100 μM), hydroxyurea (200 μM), and CyHU (100 μM) for 4 h. Then the petri dishes were washing with PBS (10 mM, pH = 7.4) for another three times, and incubating with JC-1 fluorescent probe for 20 min. Finally, each dish was washed with PBS (10 mM, pH = 7.4) for three times, and analyzed with a TCS SP8 confocal laser scanning microscopy.

**Inhibition of glutathione reductase.** Hepa 1-6 cells were seeded in 96-well plates with an amount of 5 × $10^3$ for 24 h and incubated with Cy (100 μM), hydroxyurea (200 μM), CyHU (100 μM). The NO scavenger phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO) was co-incubated in the control group. The inhibition of glutathione reductase was determined using the glutathione ruductase assay kit assay with DTNB (Beyotime Biotechnology S0055). The experiment was repeated for three times.

**Immunofluorescence staining of glutathione reductase.** For fluorescence imaging, Hepa 1-6 cells (2 × $10^5$/well) were passed on confocal dishes and incubated for 24h. Cells were washed three times with PBS (10 mM, pH = 7.4), and incubated with different materials for 4 h at 37 °C (Cy (100 μM), hydroxyurea (200 μM), and CyHU (100 μM)). The NO scavenger phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO) was co-incubated in the control group. Then cells were treated with 4% (w/v) paraformaldehyde for 20 min at 4 °C. Cells were washed three times with PBS (10 mM, pH = 7.4) and permeabilized with 0.25% (v/v) Triton X-100 for 5 min at 25 °C. Next, cells were treated with 5% bovine serum albumin (BSA) for 60 min at 25 °C. Then cells incubated with antibody against glutathione reductase for 60 min at 25 °C.

Cells were washed three times with PBS (10 mM, pH = 7.4), and incubated with FITC-labeled secondary antibody for 60 min at 25 °C. Cells were washed three times with PBS (10 mM, pH = 7.4) and taken under a confocal microscope.

**Western blotting experiment of glutathione reductase**. The Hepa 1-6 cells were divided into 6 groups to obtain the protein sample: PBS; Cy; hydroxyurea; CyHU; hydroxyurea + NO scavenger and CyHU + NO scavenger. The concentration of protein was quantified using BCA protein assay kit. SDA-PAGE protein loading buffer was added to the protein samples and the mixture was heated with boiling water for 10 min to make the protein fully denatured. Subsequently, samples with equal protein amount were added into the wells and proteins were separated by the 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The target protein was then transferred to a carrier (PVDF) membrane and incubated with specific primary antibodies at 4°C overnight. The secondary antibody labeled with horseradish peroxidase was combined with the primary antibody and incubated together. Then added ECL luminescent solution for color development and finally use a gel imaging system to analyze the western blotting.

**Reduction of GSH.** Hepa 1-6 cells were seeded in 96-well plates with an amount of $5 \times 10^3$ for 24 h and incubated with Cy (100 μM), hydroxyurea (200 μM), CyHU (100 μM). The NO scavenger phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO) was co-incubated in the control group. The reduction of GSH was determined using the total glutathione assay kit assay (Beyotime Biotechnology S0052). The experiment was repeated for three times.

**Animal tumor xenograft models.** Animal experiments were reviewed and approved by the Ethics Committee of Shandong Normal University, Jinan, P. R. China (approval number AEECSDNU2024067). All animal experiments were conducted and obeyed the Principles of Laboratory Animal Care (People's Republic of China) and the Guidelines of the Animal Investigation Committee, Biology Institute of Shandong Academy of Science, China. C57 mice (4-6 weeks old, male, weighing about 20 g) were fed under normal conditions with an average of 12 h of light and dark cycles per day, and given enough food and water. The establishment of mouse

tumor model: Hepa 1-6 cells were trypsin zed and redispersed in 50 μL DMEM serum-free medium (about $2 \times 10^5$ cells), and then subcutaneously inoculated into the right armpit of the mouse. By measuring the length (L) and width (W) of mouse tumors, the tumor volume (V) of tumor-bearing mice were calculated ($V = L \times W^2/2$). The calculation method of the relative tumor volume of mice is $V/V_0$ ($V_0$ is the tumor volume of mice before treatment). When the mouse tumor volume reached about 50 $mm^3$, the C57 mice were treated.

**Living tumor treatment experiment.** Six groups of tumor-bearing mice were divided at random (five in each group): (1) 50 μL PBS buffer solution, (2) 50 μL PBS buffer solution with laser irradiated, (3) 50 μL hydroxyurea solution (2.0 mM), (4) 50 μL CyHU solution (1.0 mM) with laser irradiated, (5) 50 μL Cy solution (1.0 mM) with laser irradiated and (6) 50 μL Cy (1.0 mM) + hydroxyurea solution (2.0 mM) with laser irradiated. The mice with laser groups were treated with 808 nm irradiation (0.33 $W/cm^2$) for 1 hour. Remarkably, the irradiation was performed 8 hour after intratumoral injection in the group (4 and 6). During the treatment (14 days), the mouse weight was measured with a vernier caliper with a scale and the change of tumor volume every other day.

## Supplementary figures

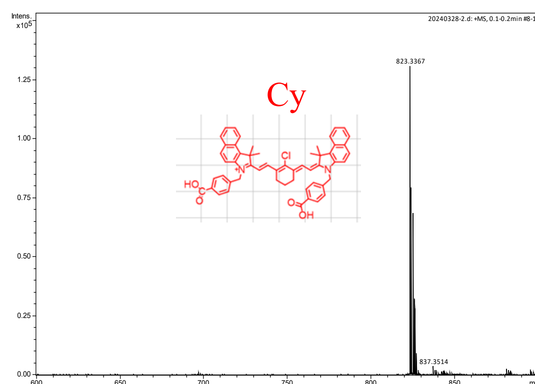



**Fig. S1** HRMS spectrum of Cy.

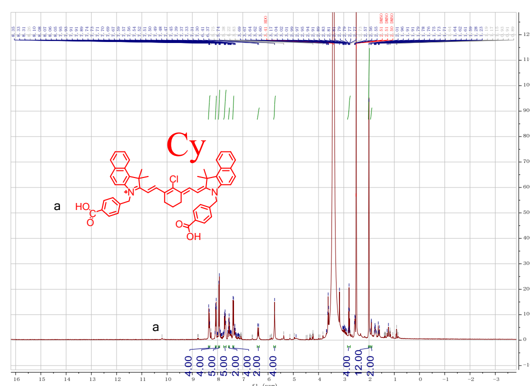



**Fig. S2** $^{1}$H-NMR spectrum of Cy ($d^{6}$-DMSO).

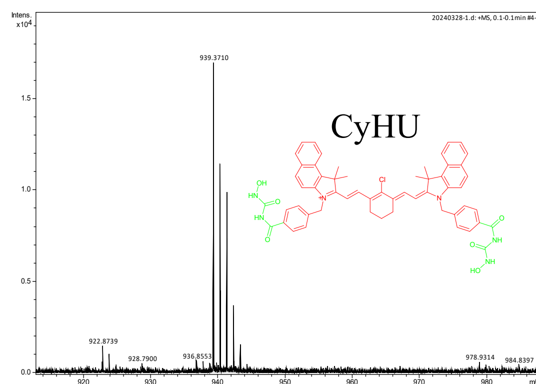



**Fig. S3** HRMS spectrum of CyHU.

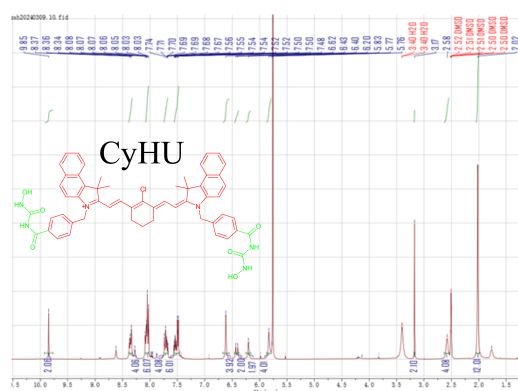



**Fig. S4** $^{1}$H-NMR spectrum of CyHU ($d^{6}$-DMSO).

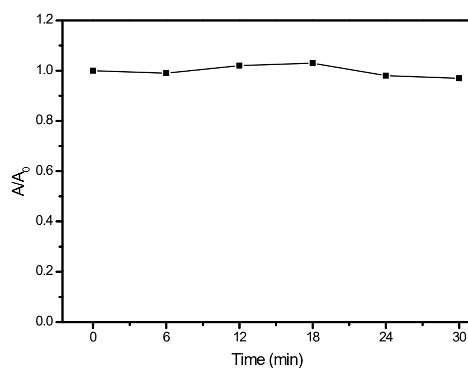



**Fig. S5** Photostability of CyHU in DMSO/water (v/v = 1:9).

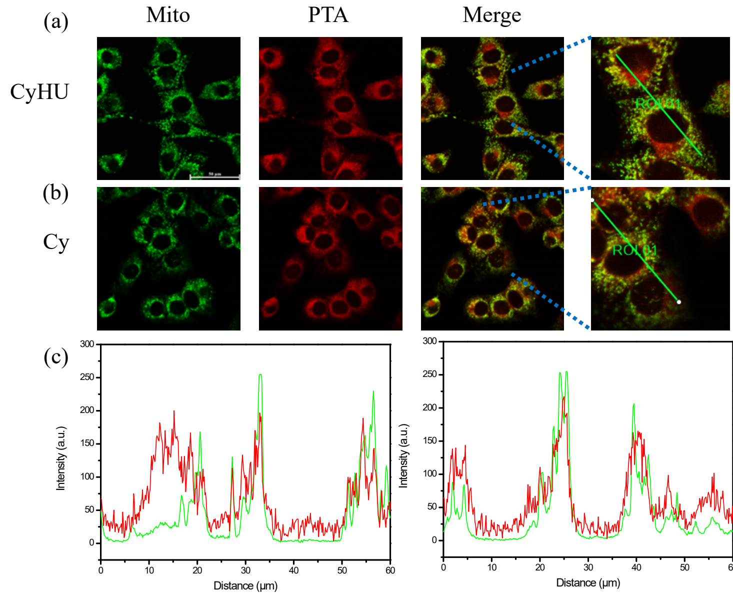



**Fig. S6** The co-localization images of (a) CyHU and (b) Cy with mitochondrial green in cells using confocal microscope. Scale bar = 50 μm. (c) And the corresponding overlapping curves of green (mitochondrial dyes) and red (left: CyHU and right: Cy) channels.

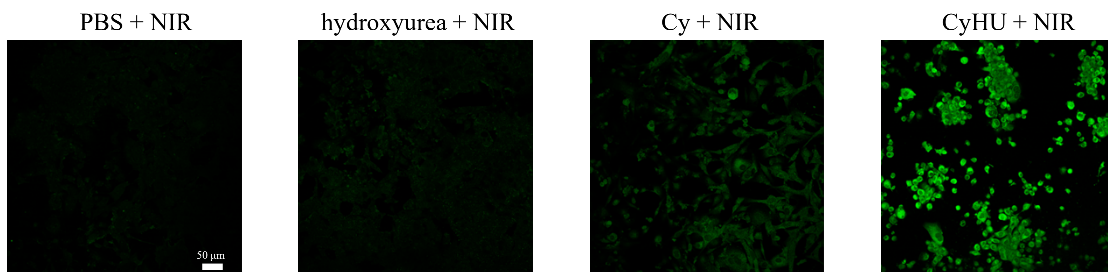



**Fig. S7**. Immunofluorescence staining of caspase 3 expression in Hepa 1-6 cells upon various treatments (Scale bar: 50 μm).

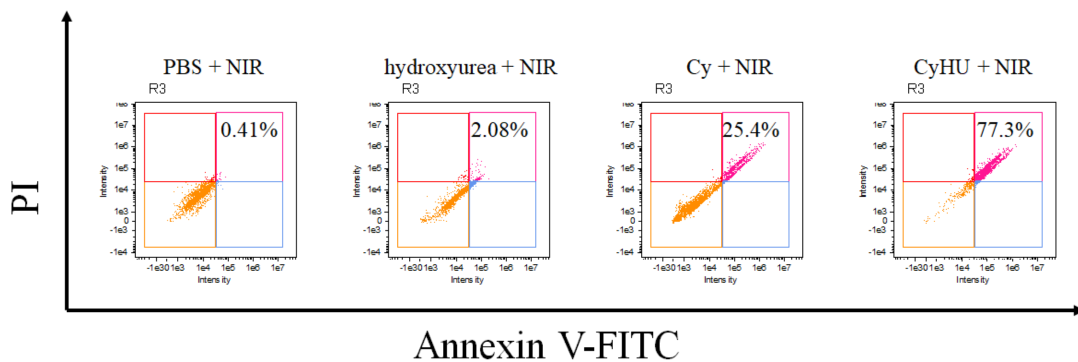



**Fig. S8**. The Apoptosis and necrosis of the Hepa 1-6 cells with different treatments by flow cytometry.

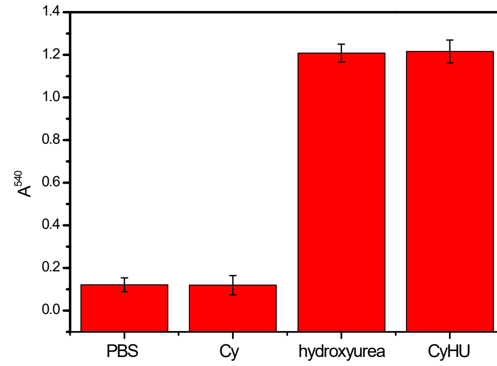



**Fig. S9.** In vitro NO generation in Hepa 1-6 cells with different treatment (determined using Griess method).

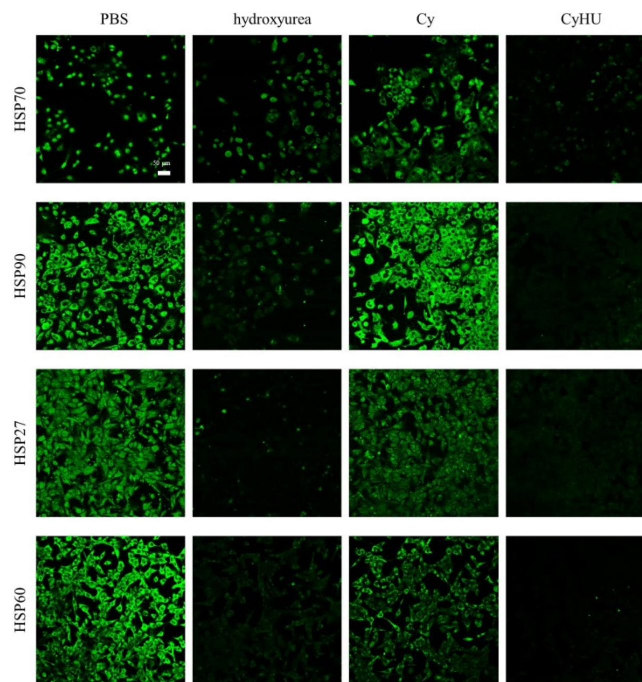



**Fig. S10.** Immunofluorescence staining of HSPs expression in Hepa 1-6 cells upon various treatments (Scale bar: 50 μm).

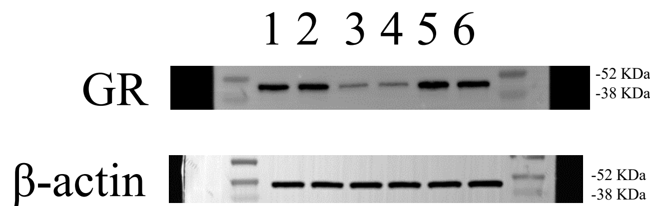



**Fig. S11.** Different groups of Western blotting analysis of GR expression in Hepa 1-6 cells upon various treatments (1: PBS; 2: Cy; 3: hydroxyurea; 4: CyHU; 5: hydroxyurea + NO scavenger and 6: CyHU + NO scavenger).

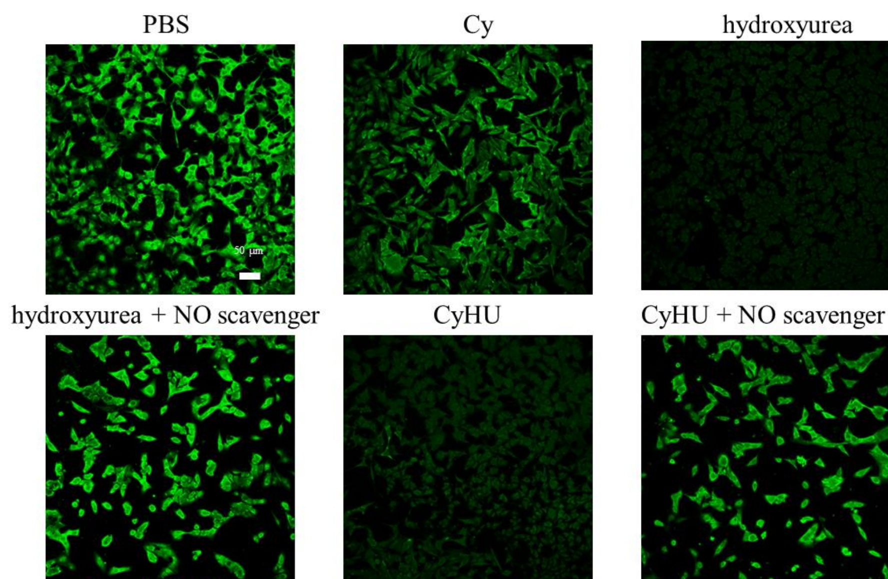



**Fig. S12.** Immunofluorescence staining of GR expression in Hepa 1-6 cells upon various treatments (Scale bar: 50 μm).

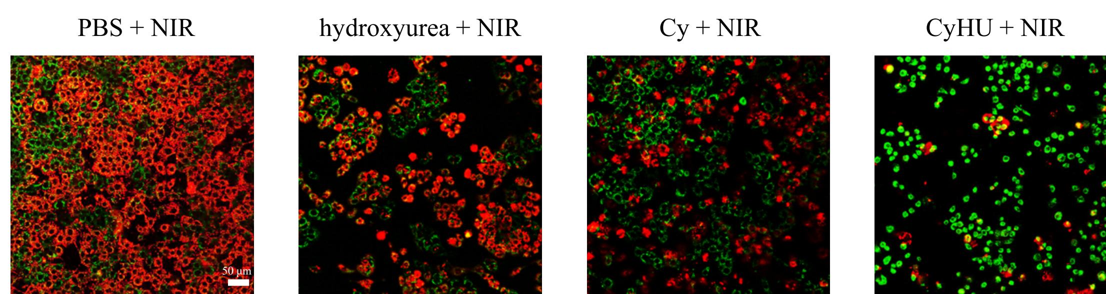



**Fig. S13.** Fluorescence microscopy images of JC-1 labeled Hepa 1-6 cells treated with different treatment.

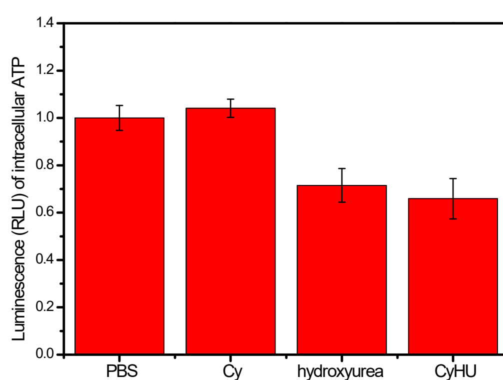



**Fig. S14.** ATP levels analysis of Hepa 1-6 cells after different treatments by ATP assay kit.

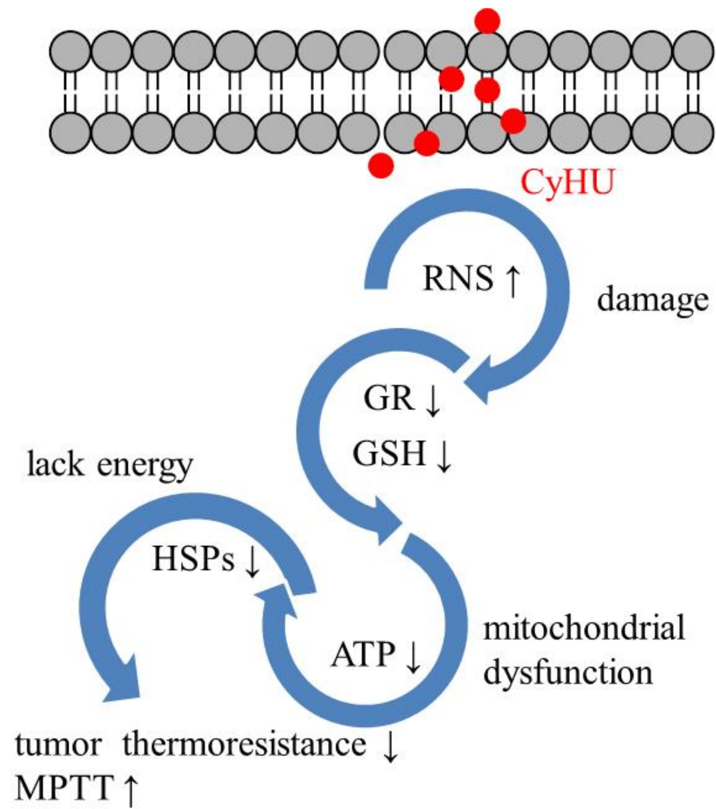



**Fig. S15.** The upstream and downstream signaling pathway after cancer cells treat with CyHU.

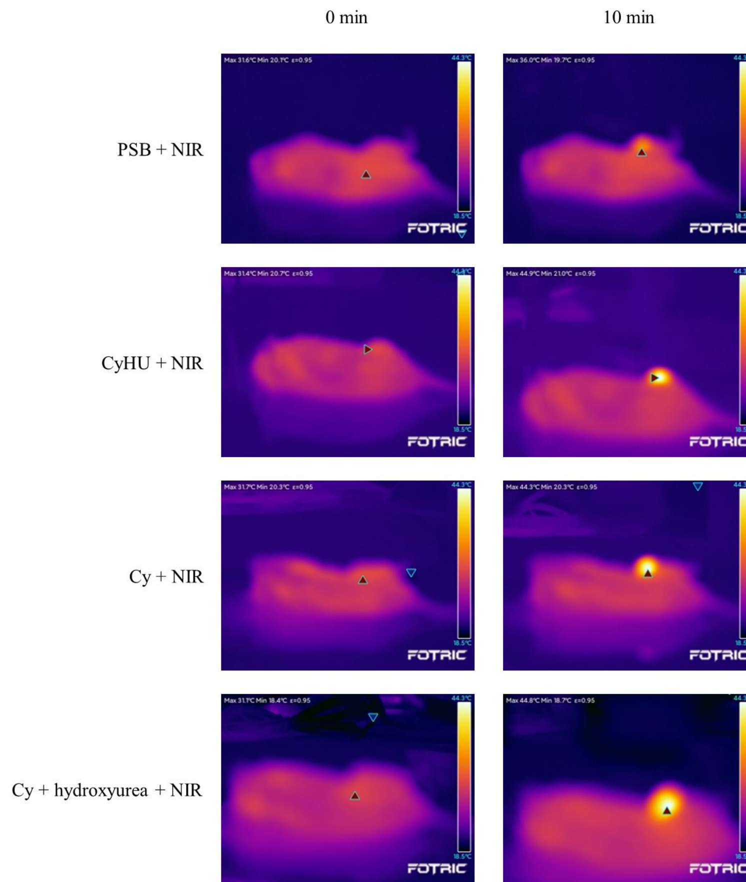



**Fig. S16** IR thermal images of Hepa 1-6 tumor-bear mice under NIR irradiation (0.33 W/cm$^2$) with different treatments.

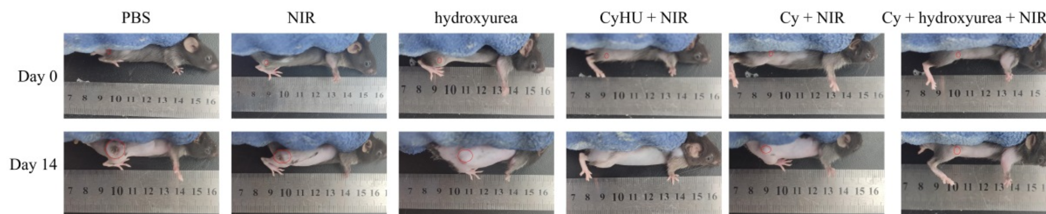



**Fig. S17.** Pictures of mice from various groups at day 0 and day 14 after different treatments.

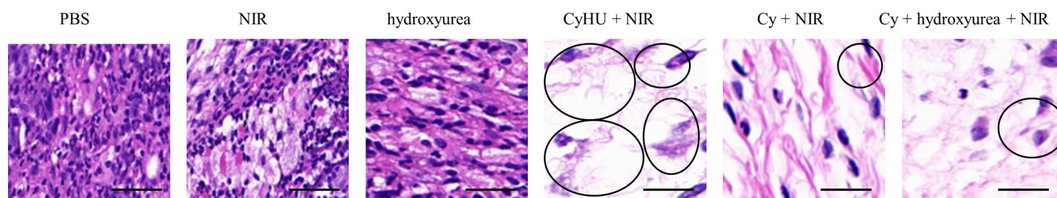



**Fig. S18.** H&E staining of tumor with different treatments (circle part: necrotic area). Scale bar = 50 μm.

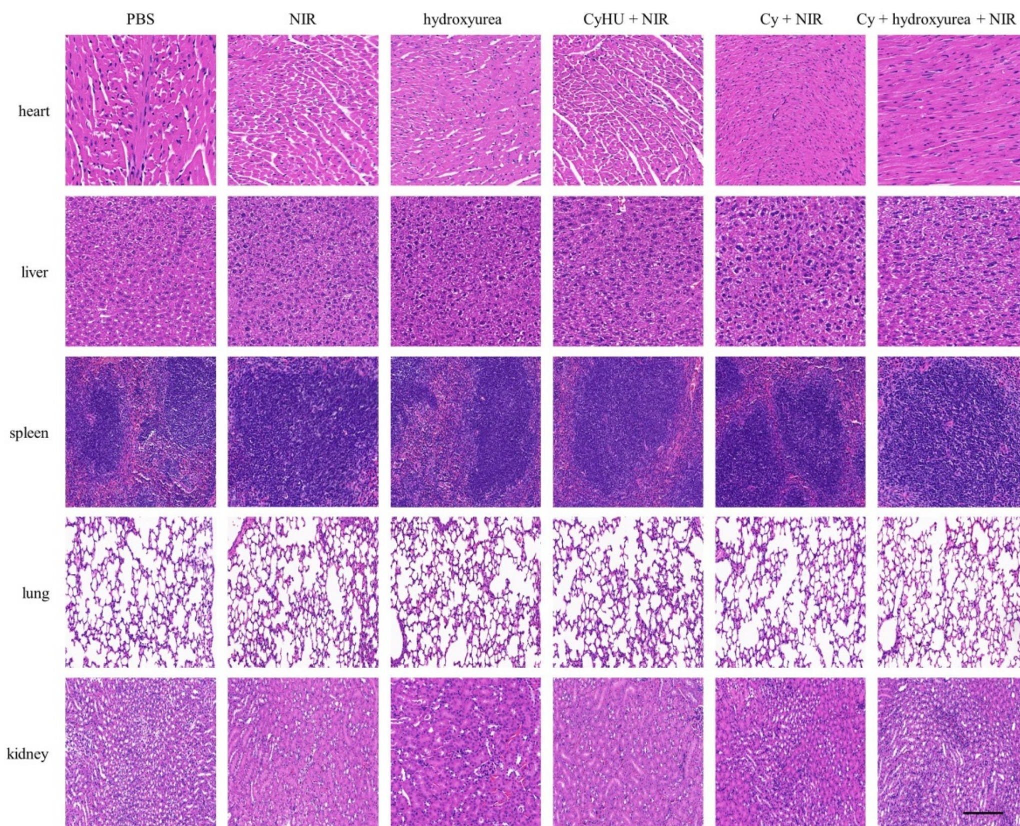



**Fig. S19.** H&E staining of major organs with different treatments. Scale bar = 200 μm.

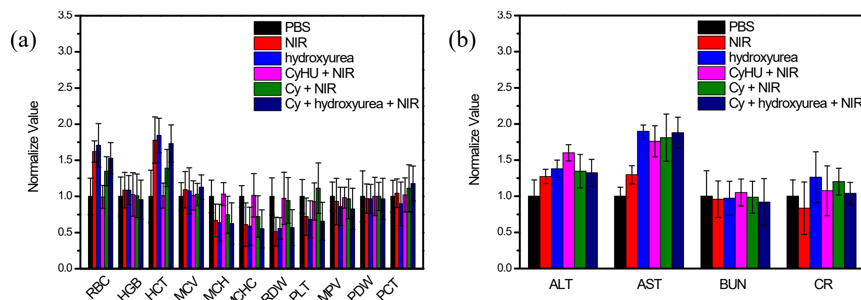



**Fig. S20.** Blood routine (a) and blood biochemistry (b) analysis of mice.

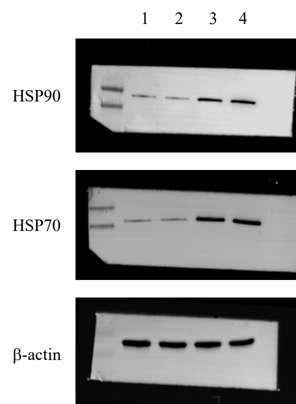



**Fig. S21.** The original pictures of the western blot of HSP90 and HSP70 (1: hydroxyurea; 2: CyHU; 3: Cy and 4: PBS).

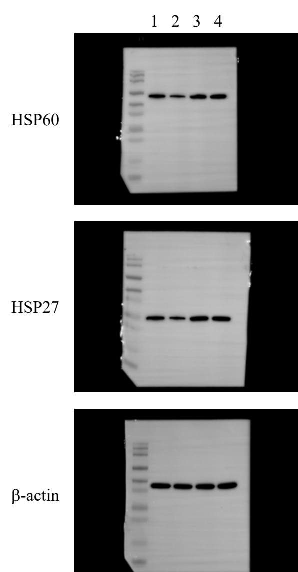



**Fig. S22.** The original pictures of the western blot of HSP60 and HSP27 (1: hydroxyurea; 2: CyHU; 3: Cy and 4: PBS).

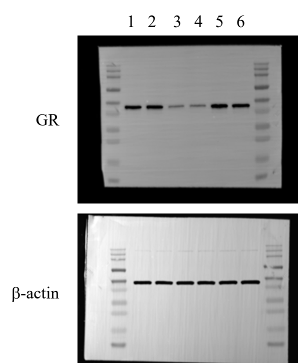



**Fig. S23.** The original pictures of the western blot of GR (1: PBS; 2: Cy; 3: hydroxyurea; 4: CyHU; 5: hydroxyurea + NO scavenger and 6: CyHU + NO scavenger).

## Reference


1. L. Cheng, W. He, H. Gong, C. Wang, Q. Chen, Z. Cheng, Z. Liu, *Adv. Funct. Mater.* 2013, **23**, 5893-5902.
2. Q. Jiang, Z. Luo, Y. Men, P. Yang, H. Peng, R. Guo, Y. Tian, Z. Pang, W. Yang, *Biomaterials* 2017, **143**, 29-45.