Enhancing photodynamic therapy effects by a Glutathione-responsive SO_2 donor as nanocarriers for cancer therapy

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Materials

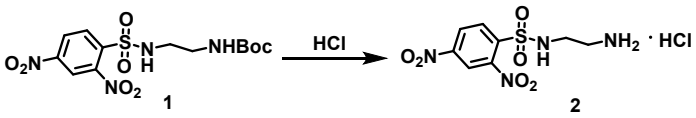
Unless stated otherwise, all the chemical reagents and solvents were obtained commercially and used without further purification. Compound 1 was synthesized by the reported method. WP5-PEG-COOH and Y6-CHO were synthesized via our previously reported method[1, 2]. Compound 1 was synthesized by the previously reported method[3]. Singlet oxygen sensor green (SOSG) and aminophenyl fluorescein (DHE) were purchased from Beyotime biotechnology Co., Ltd. ROS-ID™ hypoxia/oxidative stress detection kit was purchased from Enzo Life Sciences Inc. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from PAA Laboratories (Austria). Other organic reagents were purchased from Meryer Chemical Inc and TCI Chemical Inc. Cancer cells were ordered from Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). The Balb/c nude mice were obtained from the Chinese Academy of Sciences (Shanghai, China).

Instruments

The ^{1}H NMR and ^{13}C NMR spectra were recorded using a Bruker AVANCE 400 MHz spectrometer. High-resolution transmission electron microscopy (TEM) images were acquired using a Tecnai 20 high-resolution transmission electron microscope. Dynamic light scattering measurements were performed on a goniometer ALV/CGS-3 using a UNIPHASE He-Ne laser operating at 632.8 nm. UV-Vis spectra were recorded in a quartz cell (light path 10 mm) on a Shimadzu UV-3600 spectrophotometer. Fluorescence spectra were recorded in a conventional quartz cell (light path 10 mm) on a Varian Cary Eclipse. The output power of the laser was

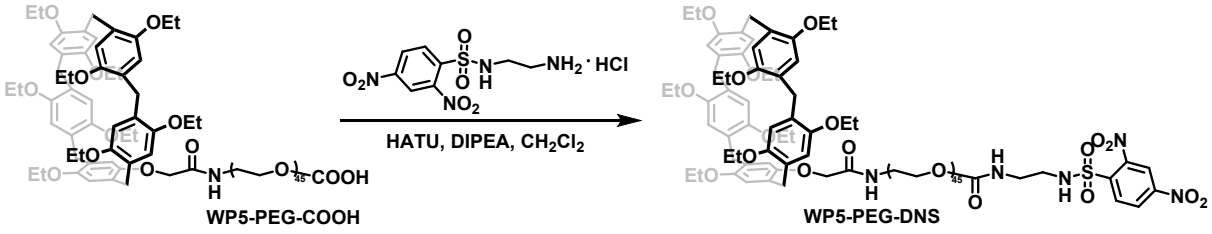
controlled by a fiber coupled laser system (LR-MFJ-660/1000mW, Changchun Lei Rui Optoelectronics Technology). The intracellular fluorescence imaging was observed using a Nexcope NIB610-FL fluorescence microscope or a confocal laser scanning microscope (microscope).

Synthesis and characterization



Scheme S1 The synthetic route for compound 2

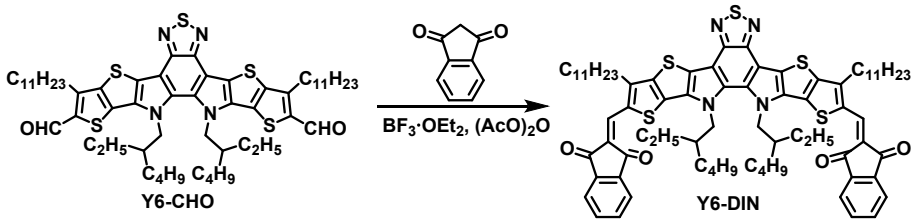
Synthesis of compound 2: To a one-necked round bottom flask were added compound 1 (117 mg, 0.30 mmol) and HCl/dioxane (4 M, 1 mL). The mixture were stirred at room temperature for 1 h. The product was obtained by removing the solvent (89 mg, 91%). ^{1}H NMR (400 MHz, D_2O) δ 8.74 (d, J = 2.2 Hz, 1H), 8.55 (dd, J = 8.7, 2.2 Hz, 1H), 8.23 (d, J = 8.7 Hz, 1H), 3.30 (t, J = 5.7 Hz, 2H), 3.08 (t, J = 5.6 Hz, 2H). ^{13}C NMR (101 MHz, D_2O) δ 150.1, 147.6, 136.7, 132.3, 127.9, 121.2, 40.3, 39.2.



Scheme S2 The synthetic route for WP5-PEG-DNS

Synthesis of WP5-PEG-DNS: To an one-necked round bottom flask were added WP5-PEG-COOH (75 mg, 0.025 mmol), DIPEA (15 μL, 0.15 mmol), HATU (11 mg, 0.03 mmol) and CH_2Cl_2 (3 mL). After the mixture was stirred for 30 min at room temperature, compound 2 (8 mg, 0.025 mmol) was added into the mixture. The

mixture was stirred at room temperature overnight. After the reaction was completed, the reaction mixture was put into the dialysis bag (MWCO: 3500). WP5-PEG-DNS was purified by dialysis in distilled water for 48 h.



Scheme S3. The synthetic route for Y6-DIN

Synthesis of Y6-DIN: Y6-CHO (51 mg, 0.05 mmol) and 1*H*-indene-1,3(*2H*)-dione (15 mg, 0.10 mmol) were dissolved in toluene (2.50 mL). To the mixture were added $BF_3{\cdot}OEt_2$ (0.06 mL) and acetic anhydride (0.05 ml). The mixture was stirred at room temperature for 30 min. After cooled down to room temperature, the crude product was obtained by column chromatography on silica gel using dichloromethane as eluent to give a dark blue solid. Then, the crude product was dissolved in dichloromethane, and added dropwise into methanol with stirring. The precipitate was collected, washed with methanol several times to give Y6-DIN (59 mg, 92%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.25 (s, 2H), 8.02 – 7.97 (m, 4H), 7.82 – 7.77 (m, 4H), 4.78 (d, *J* = 7.9 Hz, 4H), 3.22 (t, *J* = 7.6 Hz, 4H), 2.16 – 2.08 (m, 2H), 1.95 – 1.87 (m, 4H), 1.53 – 1.46 (m, 4H), 1.40 – 1.37 (m, 4H), 1.28 – 1.25 (m, *J* = 10.3 Hz, 28H), 1.09 – 0.95 (m, 12H), 0.86 (t, *J* = 6.7 Hz, 6H), 0.74 (td, *J* = 7.3, 3.3 Hz, 6H), 0.65 (td, *J* = 7.2, 2.7 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 189.5, 189.0, 149.3, 146.6, 143.1, 141.0, 139.4, 136.5, 133.8, 133.6, 132.8, 132.1, 131.7, 131.6, 127.3, 121.8, 121.6, 121.1, 112.0, 54.4, 39.2, 30.9, 29.7, 28.7, 28.6, 28.6, 28.5, 28.4, 28.3, 28.2, 26.5 (d, *J*

= 3.1 Hz), 22.1 (d, *J* = 1.9 Hz), 21.8, 21.7, 13.1, 12.7, 9.20 (d, *J* = 4.5 Hz).

Preparation of nanoparticle

Y6-DIN (1 mg) and WP5-PEG-COOH or WP5-PEG-DNS (5 mg) were dissolved into THF (1 mL). Then the mixed solution was dropwise injected into 10 mL of deionized water under vigorous stirring. After the mixture was stirred for 2 h, the prepared nanoparticles were purified by dialysis (molecular weight cutoff 10000) in distilled water for 48 h.

SO_2 release experiments

WP5-PEG-DNS with different concentrations was added to the probe I solution (PBS, pH 7.4, 10 × 10^{-3} M, 30% DMF), then GSH was added to investigate the generation of SO_2 from WP5-PEG-DNS. In this study, different concentrations of $NaHSO_3$ standard solution (10, 20, 30, 40 μM) were prepared to obtain the relationship between the absorbance of the probe and the concentration of SO_2.

The calculation method for the molar concentration of Y6-DIN in nanoparticle

Nanoparticles were frozen dehydrated and then dissolved into $CHCl_3$. Then the UV absorbance at 630 nm can be obtained. The molar concentration of Y6-DIN can be calculated through this absorbance at 630 nm and the standard curves of Y6-DIN versus concentrations.

Photodynamic experiments

Total ROS detection: Sample solution (2.97 mL) in quartz cuvettes were mixed with DPBF in ethanol (10.0 mM, 30 μL), which was then irradiated by lasers (660 nm, 0.5 W/cm^2) for a period of 30 s. The absorbance at 415 nm of the solution was

recorded at the pre-set time points during the process.

ROS quantum yield: Y6-DIN in CH_2Cl_2 (2.97 mL) in quartz cuvettes were mixed with DPBF in ethanol (10.0 mM, 30 μL), which was then irradiated by lasers (660 nm, 0.5 W/cm^2) for a period of 30 s. The absorbance at 415 nm of the solution was recorded at the pre-set time points during the process. The absorbance at 415 nm of sample solution without DPBF was also recorded, which was subtracted from the absorbance of the mixture to give the absorbance at 415 nm of DPBF. The photodynamic experiments of MB in CH_2Cl_2 were carried out following the same method. Then the ROS quantum yield was calculated according to *eq* (1)

$$\Phi_{Y6\text{-}DIN} = \Phi_{MB} \text{ x } (S_{Y6\text{-}DIN} / S_{MB}) \text{ x } (F_{ICG} / F_{Y6}) \quad (1)$$

Where S is the slope of a plot of the absorbance of DPBF (at 415 nm) versus irradiation time, and F is calculated by $F = 1\text{-}10^{-OD}$, where OD represents the absorbance of Y6-DIN and MB at 808 nm. $\Phi_{MB} = 57\%$

$^1O_2$ detection: Y6-DIN in CH_2Cl_2 were mixed with SOSG in methanol (100 μM), which was then irradiated by laser (660 nm, 0.5 W/cm^2) for a period of 120 s. The fluorescence intensity at 530 nm of the solution was recorded at 0, 30, 60, 90 and 120 s respectively (Excitation wavelength: 488 nm).

$O_2^{\bullet-}$ detection: Y6-DIN in CH_2Cl_2 were mixed with DHE in DMSO (40 μM) and ctDNA in H_2O (500 μg/mL), which was then irradiated by laser (660 nm, 0.5 W/cm^2) for a period of time. The fluorescence intensity at 580 nm of the solution was recorded at 0, 30, 60, 90, 120 and 180 s respectively (Excitation wavelength: 510 nm).

***In vitro* experiments**

Cellular Uptake: Cells were incubated in DMEM. The medium was supplemented with 10% FBS and 1% Penicillin-Streptomycin. HeLa cells were seeded in 6-well plates (1×10^5 cell mL^{-1},1.0 mL per well) for 24 h at 37 °C in 5% CO_2. Then DMEM containing DNS/Y6-DIN (350 μg/mL), Hoechst 33342 and Lysotracker green was introduced to replace the original medium. The fluorescence was observed using fluorescence microscopy at the pre-set time points during the process.

Intracellular $^1O_2$ detection: HeLa cells were incubated with DNS/Y6-DIN (350 μg/mL) for 4 h followed by incubation with 2 μM SOSG for 15 min. After being washed by PBS buffer for three times, cells were irradiated with 660 nm laser at a power density of 0.5 W/cm^2 for 15 min. Then, the fluorescence was immediately observed using confocal laser scanning microscopy (λ_{ex}: 460 – 495 nm, $\lambda_{em:}$ > 510 nm).

Intracellular $O_2^{\bullet-}$ detection: HeLa cells were incubated with DNS/Y6-DIN (350 μg/mL) for 4 h followed by incubation with 10 μM DHE for 15 min. After being washed by PBS buffer for three times, cells were irradiated with 660 nm laser at a power density of 0.5 W/cm^2 for 15 min. Then, the fluorescence was immediately observed using confocal laser scanning microscopy. Then, the fluorescence was immediately observed using confocal laser scanning microscopy (λ_{ex}: 510 – 550 nm, $\lambda_{em:}$ > 575 nm).

Cell viability test: Cells were incubated in DMEM. The medium was supplemented with 10% FBS and 1% Penicillin-Streptomycin. Cells were seeded in 96-well plates (5×10^4 cell mL^{-1}, 0.2 mL per well) for 24 h at 37 °C in 5% CO_2. Then DMEM containing different concentrations of nanodrugs was introduced to replace

the original medium. Four hours later, the cells were treated with or without a 660 nm laser (0.5 W/cm^2). After 20 min irradiation, HeLa cells were cultured for the next 24 h. The relative cellular viability was determined by the MTT assay.

Live-dead cell staining: The same density of cancer cells (3×10^5 cell mL^{-1}) were distributed into two confocal dishes (35 mm) for 12 h. Then they were cultured with new DMEM containing DNS/Y6-DIN or WP5/Y6-DIN (10 μM), respectively. After 48 h, the cells were stained with a calcein AM/propidium iodide mixture for 30 min and washed twice using PBS. The fluorescence images eventually acquired via a confocal laser scanning microscope (AM: λ_{ex}: 460 – 495 nm, $\lambda_{em:}$ > 510 nm; propidium iodide: λ_{ex}: 510 – 550 nm, $\lambda_{em:}$ > 575 nm).

Intracellular hypoxia detection: HeLa cells were incubated in DMEM with 10% FBS and 1% Penicillin-Streptomycin. For hypoxia, the original culture medium was replaced by 1 mL fresh medium containing DFO (100 μM). Then the cells were returned to incubator (5% CO_2, 20% O_2 at 37 °C) and incubated for 8 h. Next, the cells were washed thoroughly with PBS three times. The ROS-ID™ hypoxia/oxidative stress detection kit was used to indicate the intracellular hypoxic condition according to the product manual. Other operations were same to that in normoxic environment.

Hemolysis test: The red blood cells (1 mL) of healthy mice were collected and centrifuged (3500 rpm, 15 min). The supernatant was removed, and the pellet was washed with PBS five times. Finally, the red blood cell (RBC) pellet was re-suspended in PBS (5 ml) for further uses. In the hemolysis experiment, the RBC

suspensions (200 μL) were treated with DNS/Y6-DIN with different concentration, PBS, and DI Water. After incubation at 37 °C for 3 h, all the samples were centrifuged, and the absorbance of the supernatant was measured at 540 nm by microplate reader. The percentage of the sample-induced hemolysis was calculated by the following *eq* 2:

$$\text{Hemolysis}\ (\%) = \frac{\text{Asample} - \text{APBS}}{\text{Awater} - \text{APBS}} \text{x}\ 100 \quad (2)$$

A_{sample}, A_{PBS} and A_{water} refer to the absorbance of the supernatant, respectively.

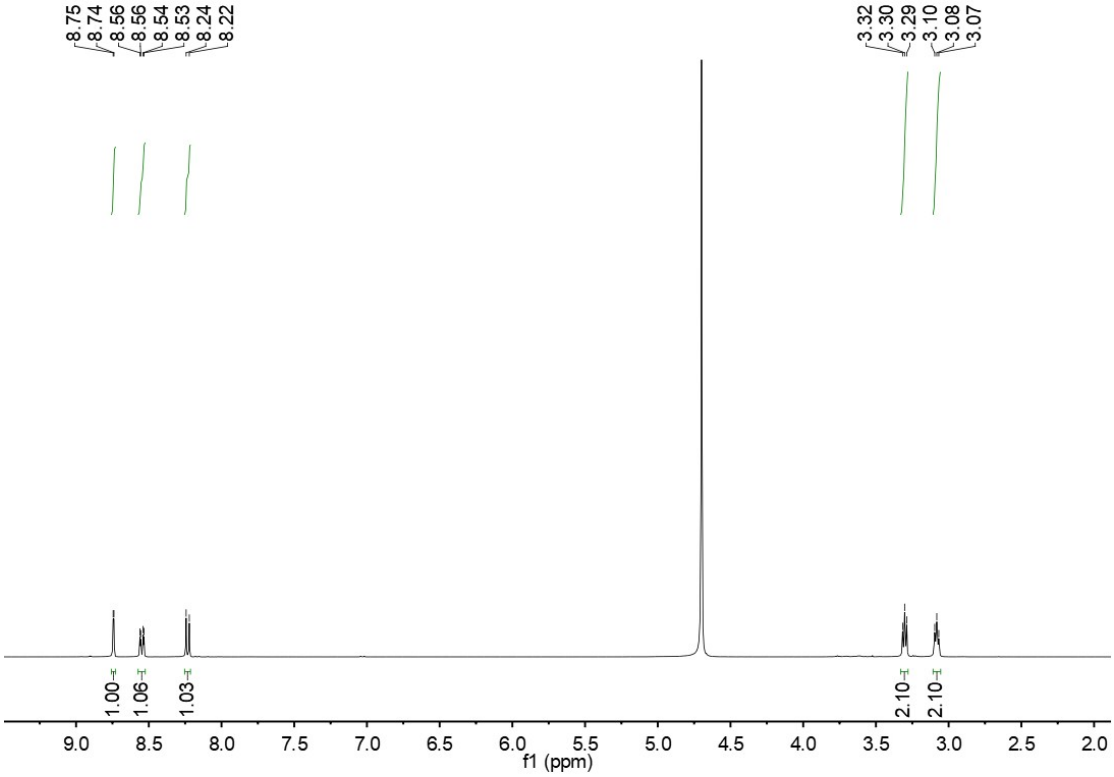
Flow cytometry: HeLa cells were seeded in 6-well plates and pre-cultured for 24 h, and then medium was replaced by DNS/Y6-DIN or WP5/Y6-DIN with Y6-DIN (10.0 μM). After 4-h incubation, the cells were gently washed by PBS buffer for three times to remove the noninternalized drugs. Next, the cells were incubated with DCFH-DA for 15 min and then applied to 660 nm laser irradiation (0.5 W/cm^2, 30 min) Finally, the cells were collected for flow cytometry analysis.

***In vivo* antitumor**

All animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals (Chinese Association for Laboratory Animal Science, 2018) and approved by the Laboratory Animal Ethics Committee of the Nantong University.

After being acclimated and tested for infectious diseases for 1 week, 4-week-old BALB/c mice were subcutaneously injected with HeLa cells (1×10^7 cells each mouse) at the flank region. When the tumor volume reached approximately 80 mm^3, the HeLa tumor-bearing BALB/c nude mice were stochastically assigned to four groups (n = 5)

for different treatments: group i, PBS injection; group ii, PBS injection and 660 nm laser irradiation (0.5 W/cm^2, 15 min); group iii, only DNS/Y6-DIN (1 mg/mL) injection; group iv, DNS/Y6-DIN (1 mg/mL) injection and 660 nm irradiation (0.5 W/cm^2, 15 min). At 24 h postinjection, the tumor sites were irradiated by 660 nm laser. The tumor volumes of mice from different groups were continually measured and recorded up to 20 days. After 20 days, the tumors were dissected for TUNEL staining.



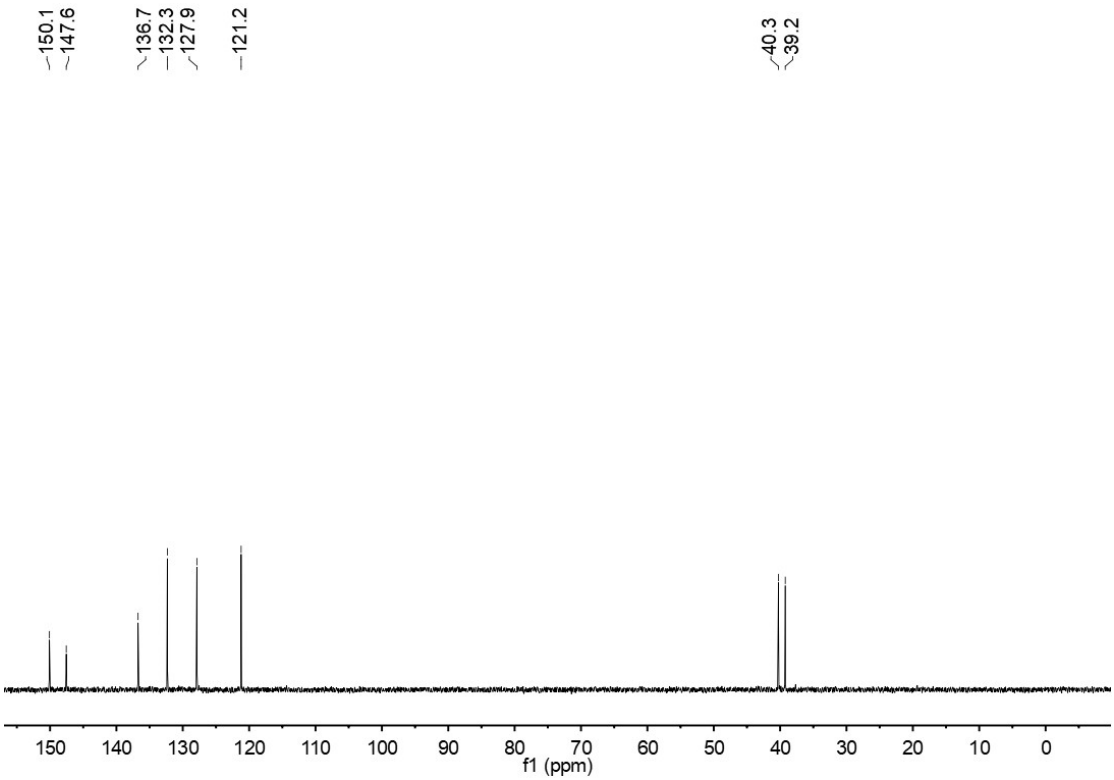


Fig. S1 The ^{1}H NMR and ^{13}C NMR spectra (D_2O) for compound 2.

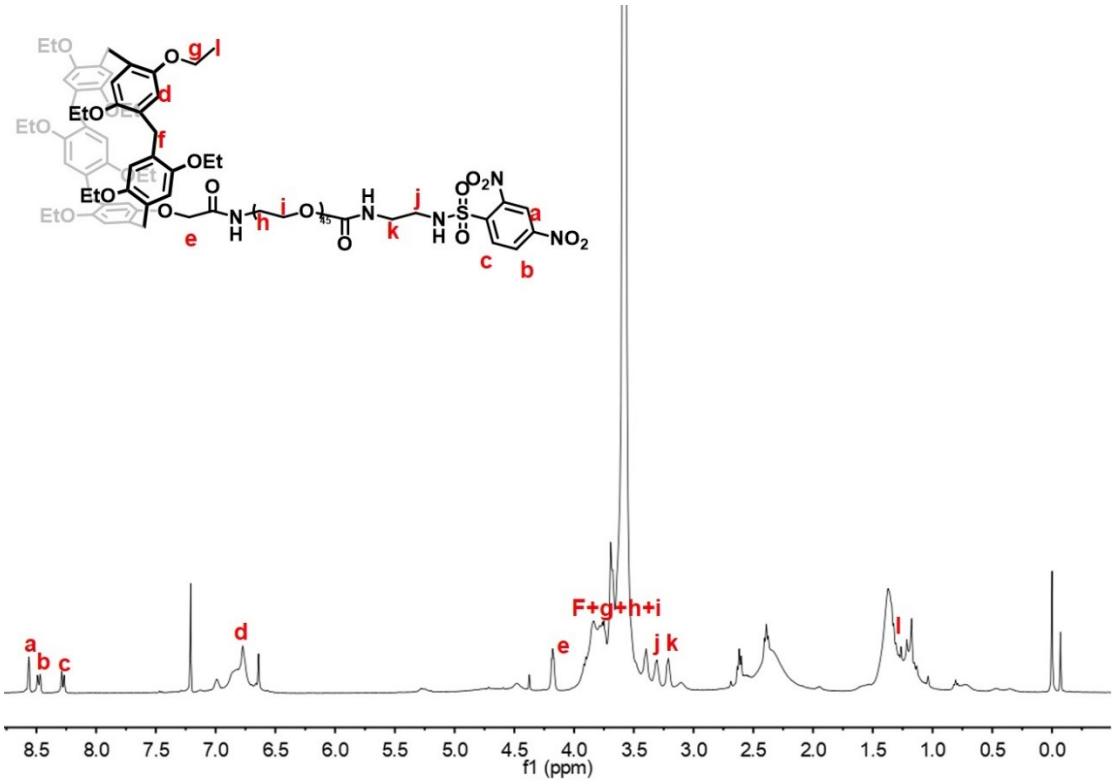


Fig. S2 The ^{1}H NMR and ^{13}C NMR spectra ($CDCl_3$) for WP5-PEG-DNS.

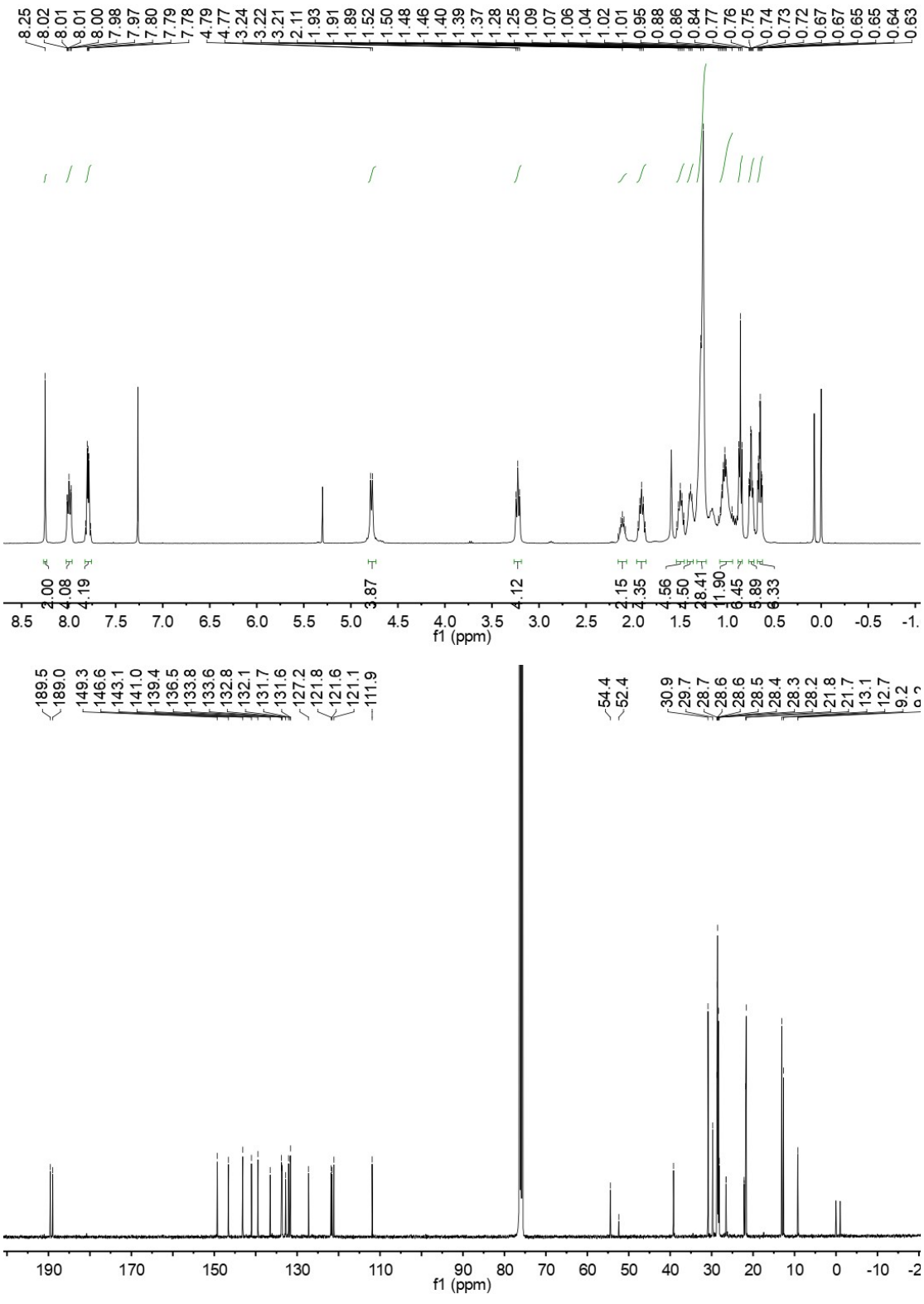


Fig. S3 The ^{1}H NMR and ^{13}C NMR spectra ($CDCl_3$) for Y6-DIN

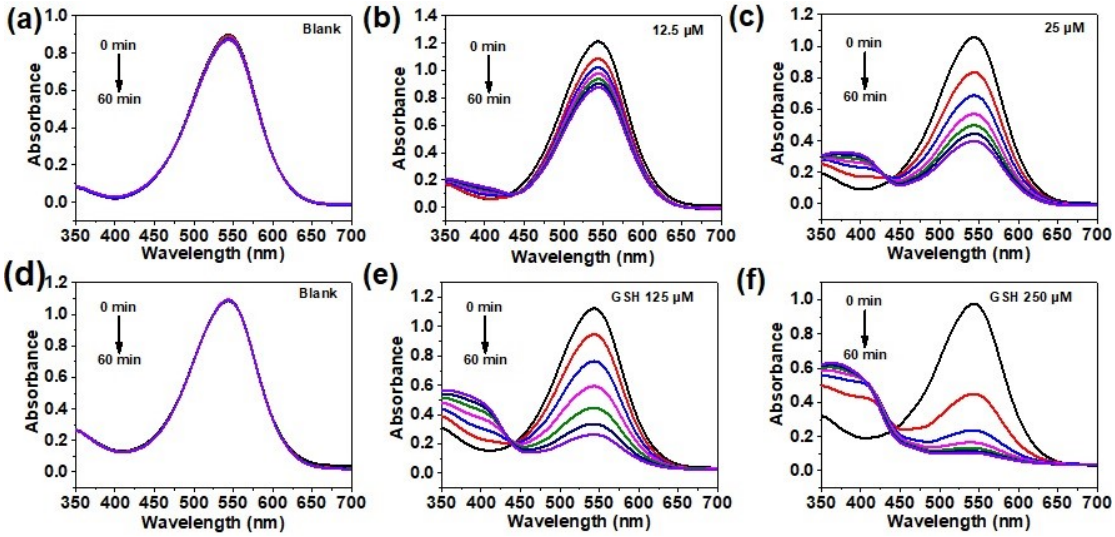


Fig. S4 The changes of UV-vis absorption spectra of probe I solution in the presence of GSH (500 μM) upon addition of different concentrations of WP5-PEG-DNS: (a): 0 μM, (b): 12.5 μM, (c): 25 μM. The changes of UV-vis absorption spectra of probe I solution in the presence of WP5-PEG-DNS (50 μM) upon addition of different concentrations of GSH: (a): 0 μM, (b): 125 μM, (c): 250 μM.

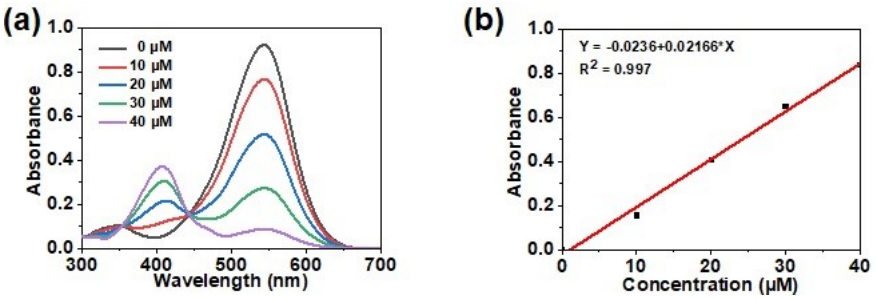


Fig. S5 (a) The changes of UV-vis absorption spectra of probe I solution upon addition of different concentrations of $NaHSO_3$. (b) The fitting line of the absorbance at 410 nm of probe I versus the concentration of $NaHSO_3$.

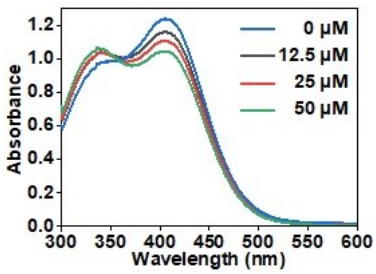


Fig. S6 The GSH depletion abilities of WP5-PEG-DNS aqueous solution of different concentrations by using DTNB as the indicator.

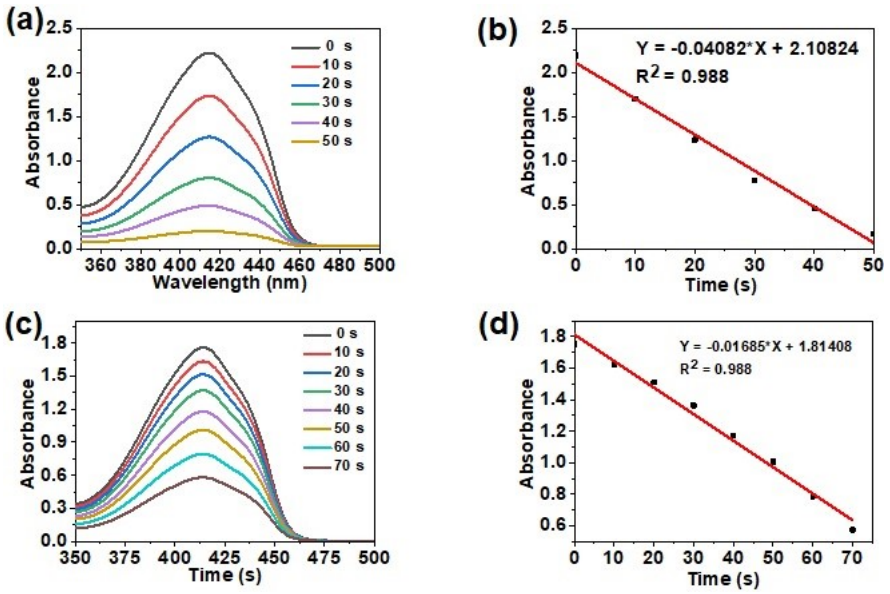


Fig. S7 (a) The absorption spectra of the mixture of DPBF and MB with laser irradiation at different time intervals. (b) The fitting lines of the absorbance at 415 nm of DPBF. (c) The absorption spectra of the mixture of DPBF and Y6-DIN with laser irradiation at different time intervals. (d) The fitting lines of the absorbance at 415 nm of DPBF.

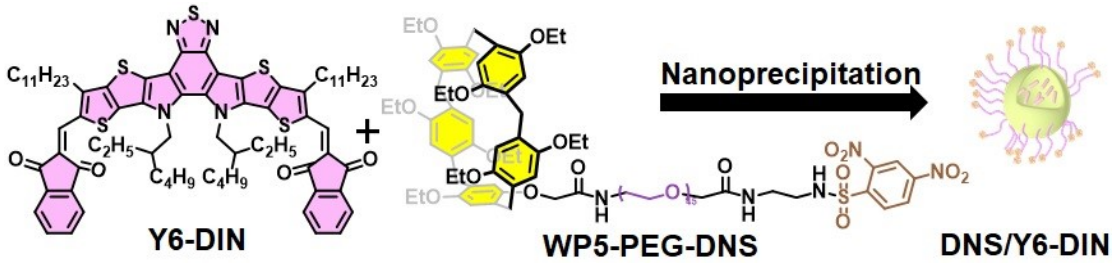


Fig. S8 Preparation of DNS/Y6-DIN.

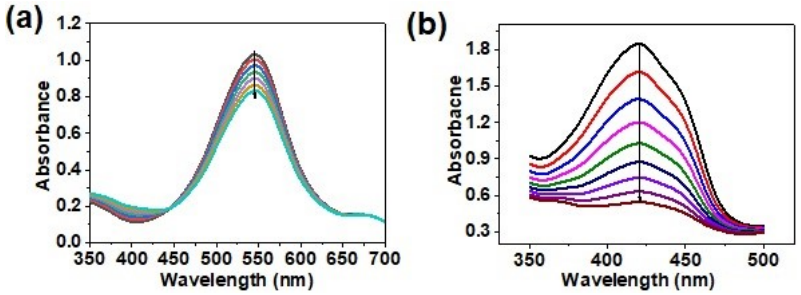


Fig. S9 (a) The UV-vis absorption spectra of probe I solution in the presence of DNS/Y6-DIN (100 μg/mL) upon addition of GSH (500 μM) at different time. (b) The absorption spectra of the mixture of DPBF and DNS/Y6-DIN (100 μg/mL) with laser irradiation at different time. intervals.

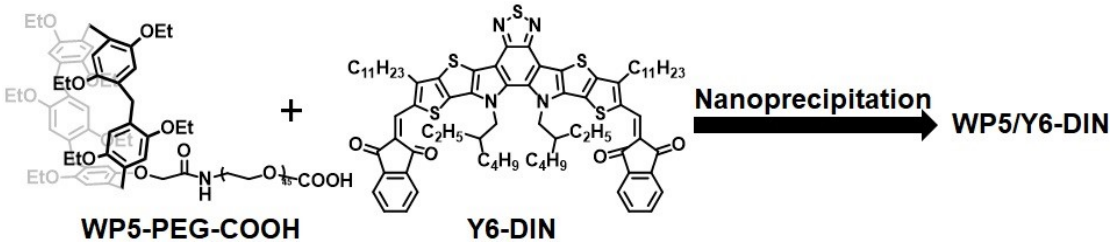


Fig. S10 Preparation of WP5/Y6-DIN.

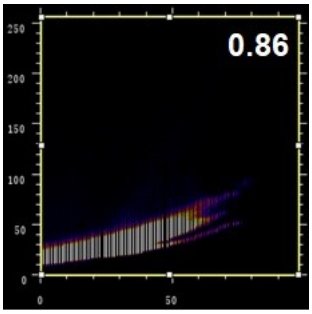


Fig. S11 The calculated Pearson correlation coefficient

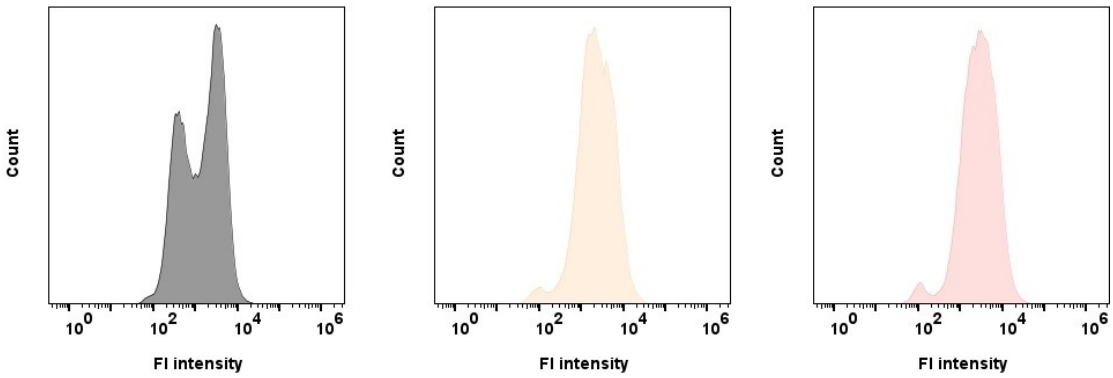


Fig. S12 The fluorescence intensities of DCFH-DA in HeLa cells after received

different treatments.

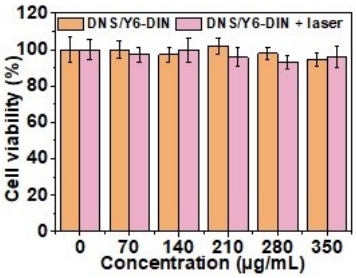


Fig. S13 Viability of HL-7702 cell incubated with different concentration of DNS/Y6-DIN with and without laser irradiation, respectively. All the viabiality data are presented as the average ± standard deviation (n = 6, mean ± SD).

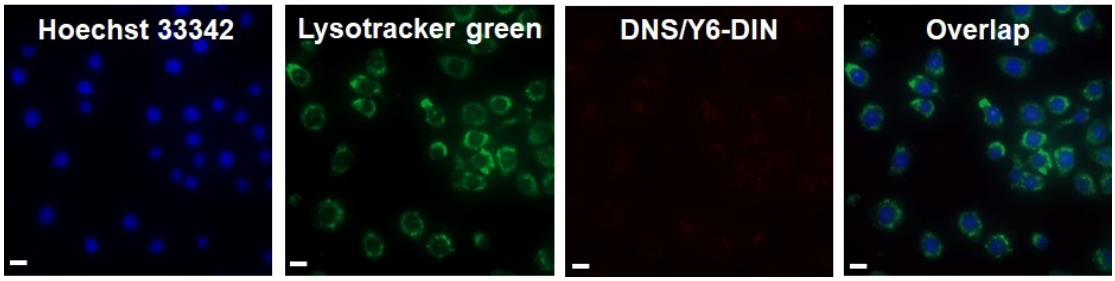


Fig. S14 Fluorescence imaging of HL-7702 cells after stained by Hoechst 33342, Lysotracker green and DNS/Y6-DIN, respectively. Scale bar: 50 μm

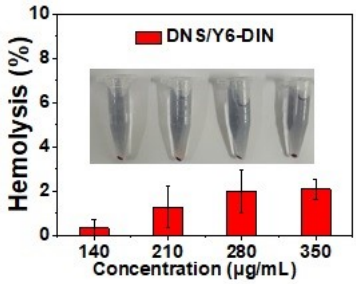


Fig. S15 Percentage of hemolysis of the red blood cells incubated with DNS/Y6-DIN with different concentrations, respectively. Data are presented as the average ± standard deviation (n = 6).

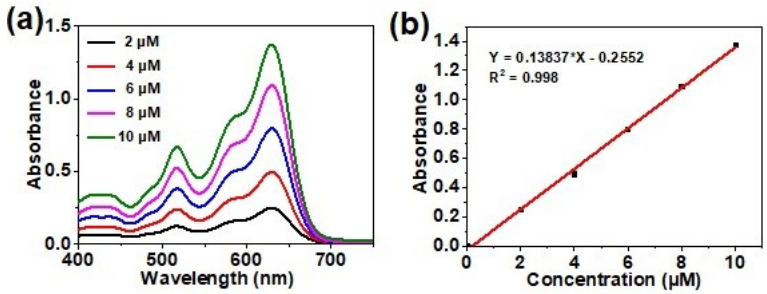


Fig. S16 (a) UV-vis absorption spectra of Y6-DIN of different concentrations in $CHCl_3$. (b) The standard curves of Y6-DIN versus concentrations.

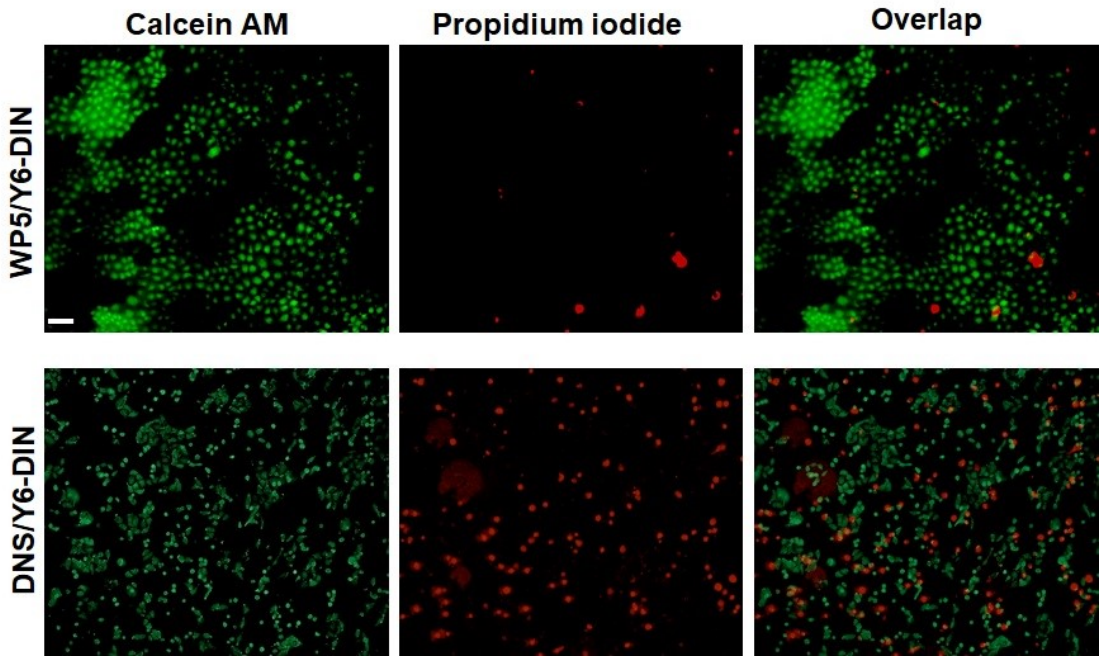


Fig. S17 The staining images of live/head cells using calcein AM (green, live cells) and propidium iodide (red, dead cells) as co-staining dyes (Scale bar: 50 μm).

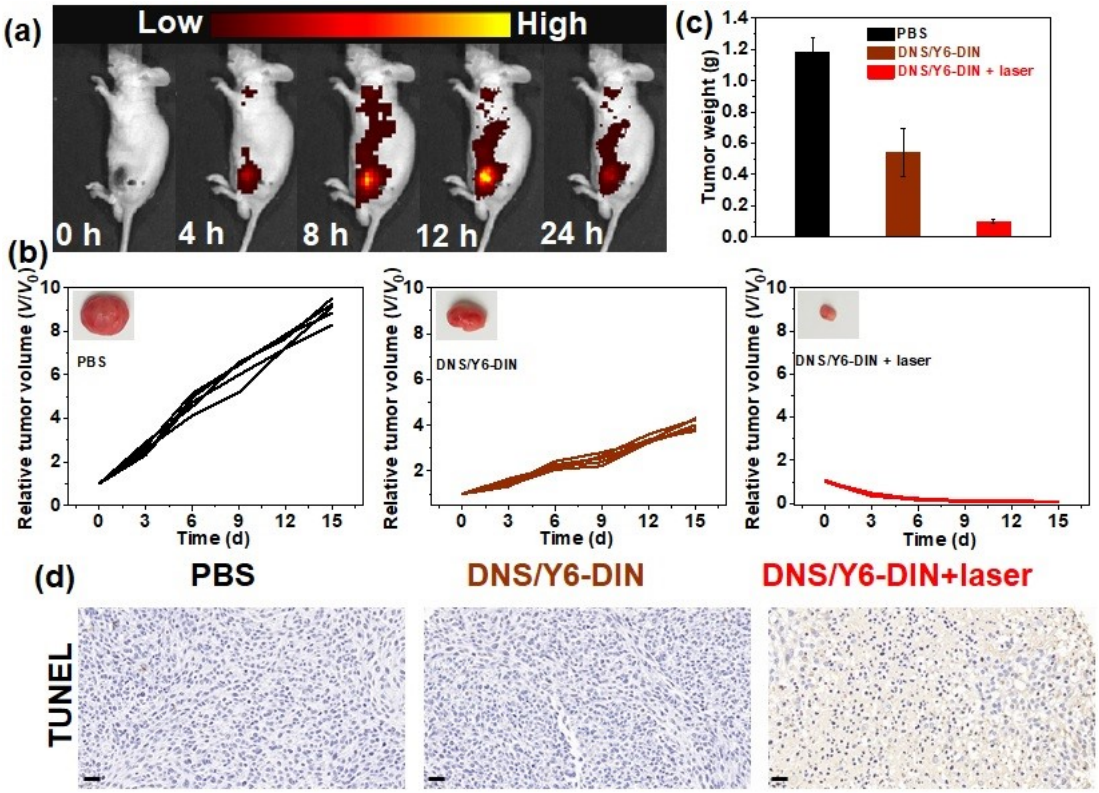


Fig. S18 (a) Fluorescence images of tumor sites. (b) The changes of tumor in all the mice during the process of different treatments (Inserted graph: tumor from mice in each group). (c) The mean weight of tumor in each group of mice after received different treatments (n = 5, mean ± SD). (d) TUNEL staining images for tumor slices collected from mice in each group after different treatments. Scale bar: 20 μm

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