Supplementary information

A dual-sensitive nanoparticle-mediated deepening synergistic therapy strategy involving DNA damage and ICD stimuli to treat triple-negative breast cancer

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1. Experimental Section

1.1. Sensitive response of HDP

1.1.1. H_2O_2 sensitive response

Briefly, HDP was incubated in DMSO-H_2O (1:1, v/v) containing 1 mM H_2O_2 and 1 mM GSH for 2 h. The surplus of GSH was used to detect the H_2O_2 sensitive response of HDP by HPLC (LC-20A, Shimadzu, Japan). The main HPLC conditions were set as following: mobile phase (acetonitrile/water 1/9, v/v), chromatographic column (Hypersil BDS C18 5 μm, 4.6 mm×250 mm) and ultraviolet detector (210 nm).

1.1.2. HAase sensitive response

HDP was incubated in PBS (pH=7.4) containing 200 U/mL HAase for 6 h at 37℃. DOX was used to detect the HAase sensitive response of HDP by HPLC. The main HPLC conditions were set as following: mobile phase (acetonitrile/0.05% trifluoroacetic acid water solution 55/45, v/v), chromatographic column (Hypersil BDS C18 5 μm, 4.6 mm×250 mm) and ultraviolet detector (496 nm).

1.2. Stability study of Ce6/HDP NPs

In order to future application, the macroscopic morphology of Ce6/HDP NPs was evaluated by photographs at preparation process. The solution stability of Ce6/HDP NPs was also evaluated with particle size and polydispersity index (PDI). For the long-term storage, the solution stability of Ce6/HDP NPs in 4℃ was evaluated. For physiological conditions, Ce6/HDP NPs were incubated with PBS (pH7.4) at 37℃ and cell culture medium at 37℃, respectively. At planned time points, the incubated buffer was evaluated by DLS.

1.3. Hemolysis investigation

Red blood cell (RBC) suspension (2%, v/v) was obtained from the blood gathered from BALB/c mice and subsequent extraction with normal saline (NS). A series of Ce6/HDP NPs solutions (0.25-1 mg/mL) were singly added in the RBC suspension with the same volume. DW or NS which replaced ahead NPs serve as positive control or negative control. These mixed samples were incubated in water bath (37℃, 1 h) and centrifugated (at 3000 rpm, 10 min). Acquired supernatant was measured with UV-visible spectrophotometer (541 nm). The hemolysis rate was calculated according to

the following equation:

Hemolysis ratio (%) = (A^{sample} - $A^{negative}$) / ($A^{positive}$ - $A^{negative}$) × 100

"A^{sample}", "$A^{negative}$", and "$A^{positive}$" represent the absorbance of Ce6/HDP NPs group, negative control group and positive control group accordingly.

1.4. Singlet oxygen generation in vitro solution

To evaluate the photochemical activity of Ce6/HDP NPs, 1,3-diphenylisobenzofuran (DPBF) was applied to monitor the singlet oxygen (a sort of ROS) generation through the decrease of DPBF. Fresh DPBF solution (dissolved in DMF) was added in Ce6 or Ce6/HDP NPs aqueous solution (the result concentrations of DPBF and Ce6 were 20 μg/mL and 2 μg/mL). The mixture was irradiation (660 nm, 100 mW/cm^2) for 2 min in total. The absorbance of DPBF (426 nm) in mixture was recorded at different time points with UV-visible spectrophotometer.

1.5. Cellular uptake

The 4T1 cells (3×10^5 per well) were seeded in 6-well plates and incubated for 24 h. Then, cells were cultured with Ce6 (2 μg/mL) or Ce6/HDP NPs (2 μg/mL of Ce6 equivalent) for 1 or 4 h. For qualitative research, cells were fixated with 4% paraformaldehyde and stained with DAPI before CLSM observation (Dragonfly 200, Andor, England). For quantitative research, the cellular uptake efficiency was measured by flow cytometry (FCM, Accuri C6 Plus, BD, USA). To investigate the active targeting capability of Ce6/HDP NPs, 4T1 cells were pretreated with HA for 4 h before culturing with NPs. Other operations were conducted as described above.

1.6. Cell viability assay

Briefly, the 4T1 cells (1×10^4 per well) were seeded in 96-well plates and incubated for 24 h. The culture medium was replaced with DOX, Ce6, DOX+Ce6, HDP or Ce6/HDP at different concentrations. After 12 h incubation and PBS washing, cells were irradiated with or without laser (660 nm, 100 mW/cm^2, 1 min) and cultured for another 12 h. The cell viability was evaluated by CCK-8 according to manufacturer's protocol. The IC_{50} values of all groups were calculated by GraphPad Prism 8.0. The combination index (CI) of HDP and Ce6 with laser irradiation was evaluated by CompuSyn 1.0, as well as for the CI of DOX and Ce6 with laser irradiation.

1.7. Apoptosis assay

The 4T1 cells (1×10^5 per well) were seeded in 12-well plates and incubated for 24 h. Then, cells were incubated with fresh medium, DOX (1 μg/mL), HDP (1 μg/mL of DOX equivalent), Ce6 (1.3 μg/mL), DOX+Ce6 (1 μg/mL of DOX equivalent, 1.3 μg/mL of Ce6 equivalent) or Ce6/HDP (1 μg/mL of DOX equivalent, 1.3 μg/mL of Ce6 equivalent) for 12 h. Cells were conducted laser irradiation (660 nm, 100 mW/cm^2, 1 min), or not after PBS washing and incubated with fresh medium for 4 h. Finally, collected cells were stained with annexin V-FITC/PI for FCM analysis.

1.8. In vivo pharmacokinetics and fluorescence imaging study

In order to evaluate the pharmacokinetic of Ce6/HDP NPs in vivo, DOX (4 mg/kg), Ce6 (5 mg/kg) or Ce6/HDP (4 mg/kg of DOX equivalent, 5 mg/kg of Ce6 equivalent) was injected into healthy female BALB/c mice (n=3) independently. After blood collection and plasma separation, 90 μL of acetonitrile was added into 10 μL of plasma, and then the mixture was vortexed and centrifugated (at 14000 rpm, 5 min). The fluorescence intensities of Ce6 and DOX (Ce6, excitation at 400 nm and emission at 660 nm; DOX, excitation at 480 nm and emission at 590 nm) were measured via multimode plate reader (EnSight, PerkinElmer, USA). The pharmacokinetic parameters of Ce6 and DOX were analyzed by DAS 2.0 in non-compartment model.

In order to evaluate the in vivo distribution of Ce6/HDP NPs, 4T1 tumor-bearing mice were randomly assigned to two groups (n=15). Ce6 (5 mg/kg) or Ce6/HDP NPs (5 mg/kg of Ce6 equivalent) was intravenously injected into each group. For in vivo fluorescence imaging, three mice of each group were continuously anesthetized and conducted fluorescence imaging on IVIS spectrum imaging system (PerkinElmer, USA) at 2 h, 6 h, 12 h, 24 h after administration. For ex vivo fluorescence imaging, every three mice of each group were euthanized at previous time points. Major organs (hearts, livers, spleens, lungs, kidneys) and tumors were collected and conducted for fluorescence imaging. Data were analyzed via Living Image 4.5 (PerkinElmer, USA).

1.9. In vivo singlet oxygen detection

4T1 tumor-bearing mice were randomly assigned to 7 groups (n=3) and intravenously injected with NS, DOX (4 mg/kg), HDP (4 mg/kg of DOX equivalent),

Ce6 (5 mg/kg), DOX+Ce6 (4 mg/kg of DOX equivalent, 5 mg/kg of Ce6 equivalent) or Ce6/HDP (4 mg/kg of DOX equivalent, 5 mg/kg of Ce6 equivalent), respectively. All mice were intraperitoneal injected with SOSG at 5.5 h post-intravenous injection. After another 0.5 h, tumor location of mice was received laser irradiation (660 nm, 200 mW/cm^2, 10 min), or not. At last, tumors of mice were separated for CLSM observation after euthanasia.

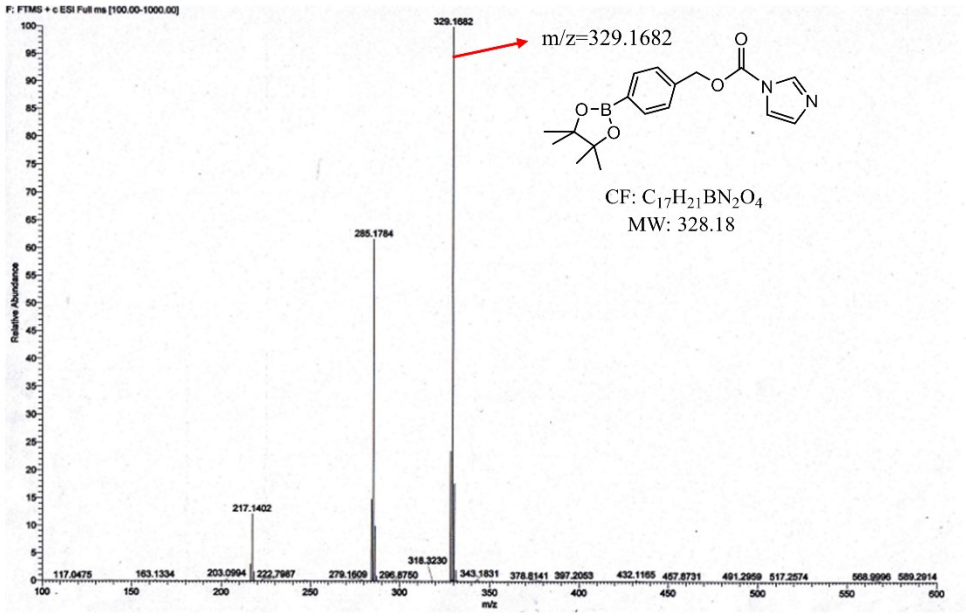

Figure S1. The HRMS result of PBAP-CDI, $[M+H]^+$, at m/z = 329.1682.

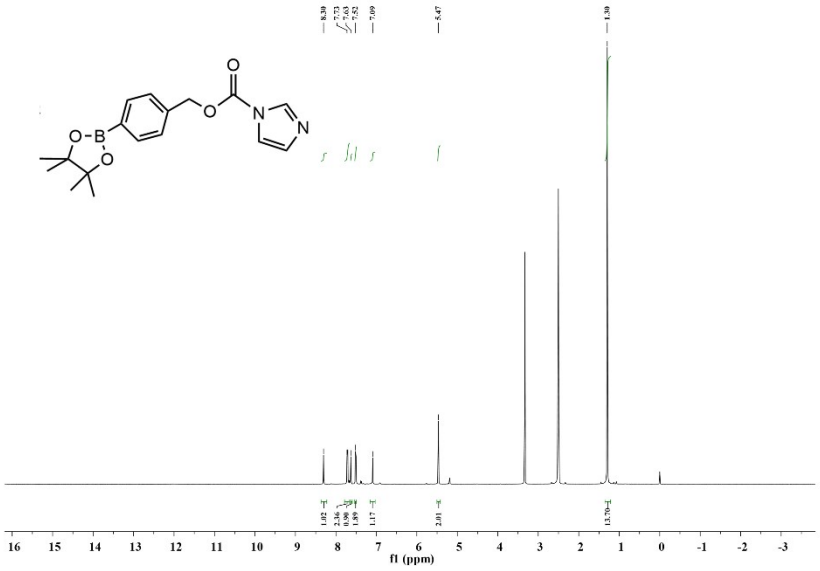

Figure S2. The ^1H NMR spectra of PBAP-CDI.

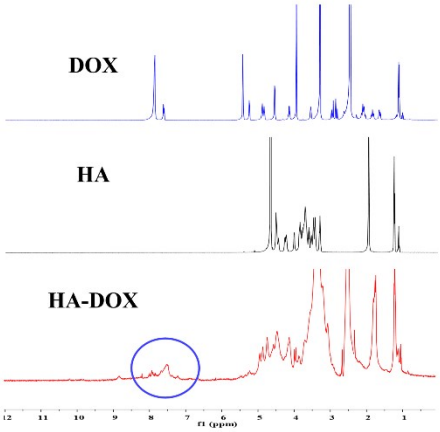

Figure S3. The ^{1}H NMR spectra of DOX, HA and HA-DOX.

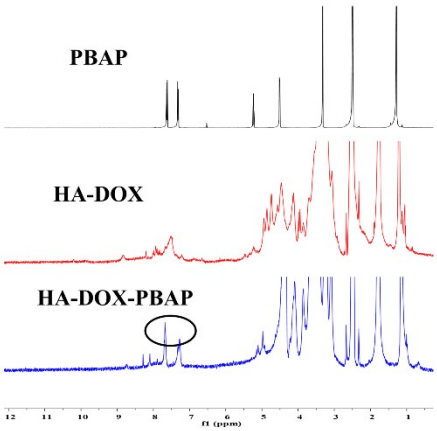

Figure S4. The ^{1}H NMR spectra of PBAP, HA-DOX and HA-DOX-PBAP (HDP).

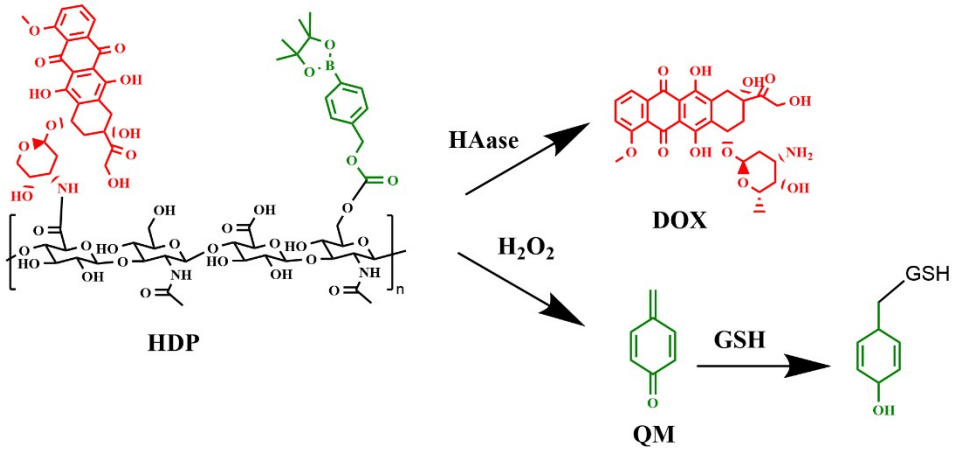

Figure S5. The dual-sensitive disassembly pathways of HDP.

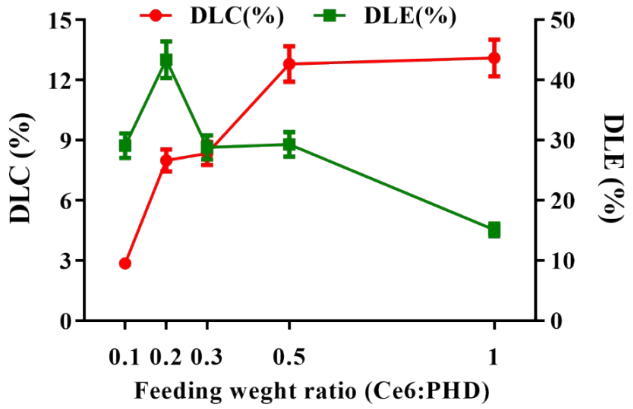

Figure S6. Drug loading content (DLC) and drug loading efficiency (DLE) of Ce6-loaded HDP NPs with different feeding weight ratios of Ce6 to HDP.

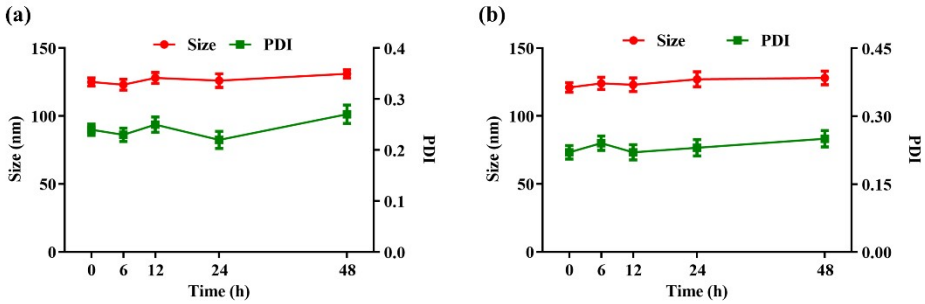

Figure S7. Stability of Ce6/HDP NPs in PBS at 37℃ for 48 h (a) and in cell culture medium at 37℃ for 48 h (b) (n = 3, mean ± SD).

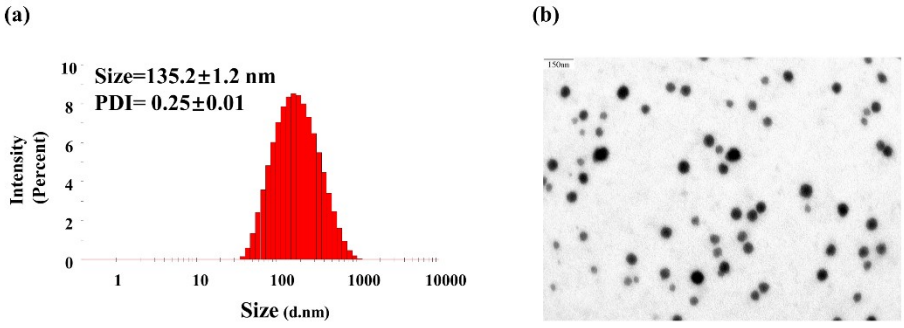

Figure S8. Particle size distributions and TEM images of Ce6/HDP NPs after freeze drying and dispersing in PBS (n = 3, mean ± SD).

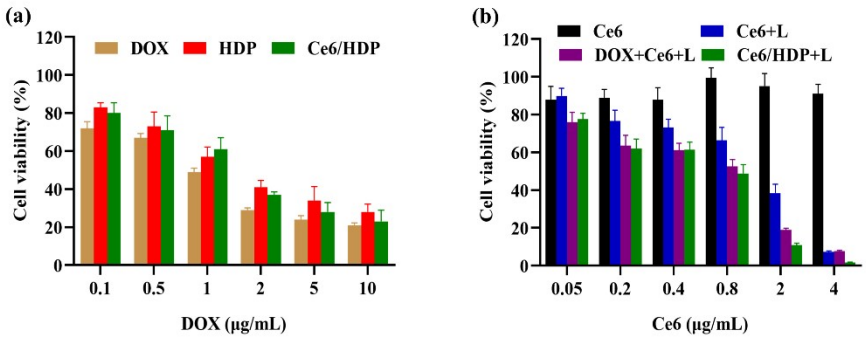

Figure S9. Cell viability of DOX related formulations(a) and Ce6 related formulations at different concentrations (b) (n = 3, mean ± SD).

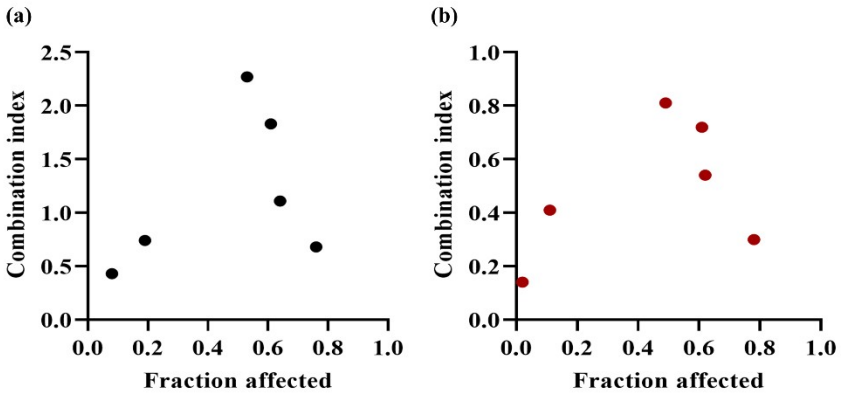

Figure S10. (a) The combination index (CI) of DOX and Ce6 with laser irradiation in DOX+Ce6+L group. (b) The CI of HDP and Ce6 with laser irradiation in Ce6/HDP+L group.

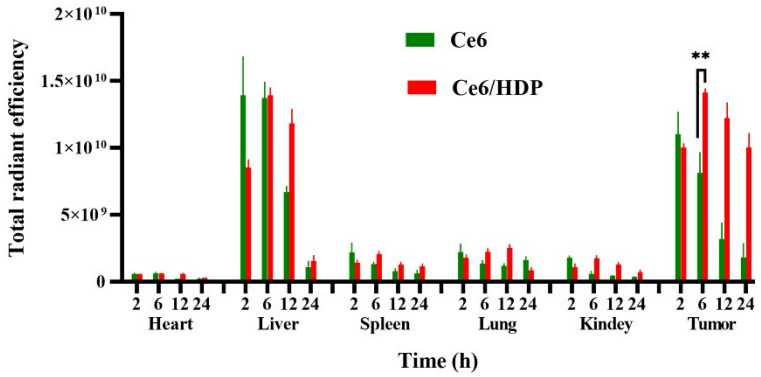

Figure S11. Semiquantitative MFI of Ce6 and Ce6/HDP NPs in major organs and

tumors at different time points after intravenous injection (n = 3, mean ± SD).

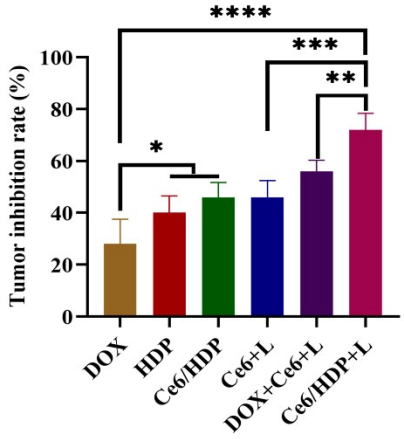

Figure S12. Inhibition rates of 4T1 tumors in different groups (n = 5, mean ± SD).

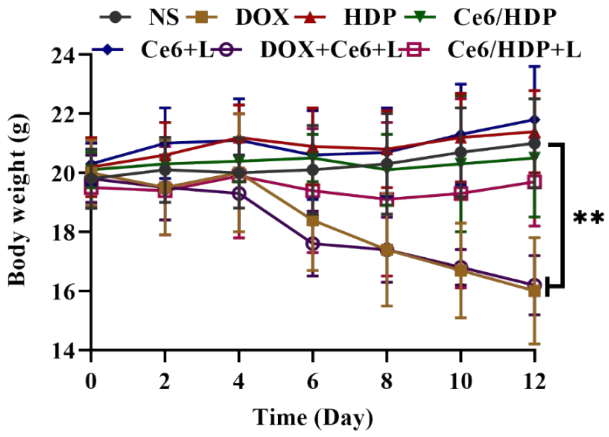

Figure S13 Body weight curves of 4T1 tumor-bearing mice after different treatments (n = 5, mean ± SD). $p < 0.01$ (**).

Polymers	GPC		PDI
	M_w	M_n	M_w/M_n
HA	10910	6905	1.58
HA–DOX	12437	7066	1.76
HDP	14704	7389	1.99

Table S1. GPC-determined molecular data of polymers

(a)

Parameter	DOX	Ce6/HDP
$AUC_{(0-t)}$ (μg/L*h)	1221.2±318.9	3370.5±334.8
$t_{1/2}$ (h)	2.8±0.5	9.9±3.9
Cl (L/h/kg)	3.9±0.9	1.4±0.2
$MRT_{(0-t)}$ (h)	1.6±0.3	3.6±1.1

(b)

Parameter	Ce6	Ce6/HDP
$AUC_{(0-t)}$ (μg/L*h)	3601.4±335.7	5863.5±286.2
$t_{1/2}$ (h)	2.0±0.3	3.4±0.6
Cl (L/h/kg)	1.4±0.1	0.8±0.1
$MRT_{(0-t)}$ (h)	1.2±0.1	2.2±0.4

Table S2. Pharmacokinetic parameter of BALB/c female mice after intravenous administration of DOX and Ce6/HDP NPs at an equivalent DOX dose of 4 mg/kg (a), Ce6 and Ce6/HDP NPs at an equivalent Ce6 dose of 5 mg/kg (b) (n = 3, mean ± SD).

Group	RBC (10^{12}/L)	WBC (10^9/L)	PLT (10^9/L)	HGB (g/L)	RDW (%)	MCV (fL)
NS	10.3±0.5	6.5±0.2	857±32	164.2±8.2	14.2±0.8	50.3±1.2
DOX	10.6±0.2	4.3±0.7**	765±27*	159.0±9.0	13.9±0.5	48.7±0.8
HDP	9.8±0.7	6.3±0.4	846±38	157.4±5.3	13.6±0.5	51.2±1.5
Ce6/HDP	10.1±0.2	6.2±0.3	850±17	162.7±3.2	14.4±0.2	50.7±1.8
Ce6+L	9.9±0.4	6.6±0.6	871±13	165.1±7.1	14.7±0.4	49.6±1.1
DOX+Ce6+L	10.4±0.7	4.7±0.5**	747±45*	161.4±8.7	15.0±0.5	48.5±1.5
Ce6/HDP+L	10.2±0.6	6.1±0.5	870±22	157.3±6.1	14.8±0.3	49.4±1.3

Table S3. Hematological parameters of the blood collected from healthy mice with different treatment (n = 3, mean ± SD).