Supporting Information Available

An azobenzene-based heteromeric prodrug for hypoxia-activated chemotherapy by regulating subcellular localization

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Exgerimental Section

1.Materials.

N,N-bis(2-hydroxyethyl)aniline, doxorubicin hydrochloride (DOX), 4-aminobenzyl alcohol, 4-nitrophenyl chloroformate, DMAP and triethylamine were obtained from Innochem corp. Methanol and dichloromethane (DCM) were purchased from Shanghai Chemical Co. (China). Phosphorus oxychloride, ethanol, dimethyl sulphoxide (DMSO), $NaNO_2$, Na_2SO_4 were obtained from Aladdin Reagent Co. Ltd. (Shanghai, China). Dulbecco's phosphate buffered saline (PBS), Dulbecco's modified Eagle's medium (DMEM), LysoTracker Green, minimum essential medium (MEM), Roswell Park Memorial Institute-1640 (RPMI-1640) and fetal bovine serum (FBS) were purchased from Invitrogen Corp. Annexin V-FITC, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium-bromide (MTT), PI, MitoTracker Green and Hochest 33342 were purchased from Beyotime Biotechnology (China). All other reagents were of analytical grade and used as received.

2. Cell Cultures.

Murine mammary carcinoma cells (4T1 cells) were cultured in RPMI-1640 medium containing 10% FBS and 1% antibiotics at 37 ^oC. African green monkey kidney cells (COS7 cells), mouse melanoma cells (B16 cells) and mouse fibroblasts were cultured in DMEM medium containing 10% FBS and 1% antibiotics at 37 ^oC, respectively. A humidified atmosphere containing 5% CO_2 was used as normoxic cell culture environment. The hypoxic (1.1% O_2 and 5% CO_2) cell culture environment was adjusted by purging N_2 using ESCO cell culture systems.

3. Synthesis of Compound 1.

Compound **1** was synthesized according to our previous published procedures. Briefly, Phosphorus oxychloride (5 mL) was added into a round bottomed flask and cooled to 0 ^{o}C. And then, N,N-bis(2-hydroxyethyl)aniline (3.9 g) was added slowly. After which, the mixture was heated to reflux at 110 ^{o}C for 1 hour. Subsequently, the mixture was cooled to room temperature and concentrated by rotary evaporating. The residue was dissolved in ethyl acetate (200 mL) and washed thrice with water. The organic layer was dried over anhydrous magnesium. Finally, the residue was purified by silica gel chromatography (methanol/DCM, V/V, 2/98) to obtain **1** as a light yellow viscous liquid.

4. Synthesis of Compound 2.

A solution of sodium nitrite (5.3 g) in water (30 mL) was added into the solution of 4-aminobenzyl alcohol (8.2 g) in water (120 mL) containing concentrated hydrochloric acid (18 mL). After stirring for 20 min at room temperature, the obtained solution was added to the solution of **1** (13.2 g) in ethanol. After 2 h, the solution was diluted with DCM (500 mL) and washed twice with H_2O. The organic layer was collected and dried over Na_2SO_4. The concentrated residue was purified by silica gel chromatography (methanol/DCM, V/V, 1:13) to obtain **2**. The structure of **2** was measured by electrospray ionization mass spectrometry (ESI-MS).

5. Synthesis of Compound 3.

Compound **2** (0.772 g) and DMAP (0.4 g) was dissolved in anhydrous DCM (15 mL) at 0 ^{o}C. And then, a solution of 4-nitrophenyl chloroformate (0.62 g) in

anhydrous DCM (8 mL) was added dropwise and the mixture was stirred for 12 h at room temperature. Finally, the mixture was concentrated by rotary evaporation and the obtained residue of compound **3** was purified by silica gel chromatography (hexane: ethyl acetate, V/V, 3:1).

6. Synthesis of hNDP.

Doxorubicin hydrochloride (0.4 g) and triethylamine (0.29 mL) were dissolved in 6 mL of anhydrous DMF and stirred for 1 h in the shield of light. After which, 3 (0.52 g) in 4 mL of DMF was added and stirred in the dark overnight. Finally, the mixture was precipitated with diethyl ether and collected by centrifugation. The crude product of hNDP was purified by silica gel chromatography (methanol/DCM, V/V, 1:13) and its structure was characterized by ESI-MS.

7. Hypoxia-Regulated Nucleus Targeting of hNDP.

The distribution of hNDP was investigated under hypoxic microenvironment by purging N_2. 4T1 cells, B16 cells and fibroblasts were seeded in Petri dishes and incubated in culture medium for 24 h, respectively. Then, hNDP (20 μM) in 1 mL of culture medium was used to incubate these three types of cells at hypoxic (purged with N_2) or normoxic environment, respectively. After 24 h, the cells were washed by PBS and stained by Hoechst 33342 for 15 min. Finally, the distribution of the fluorescence was observed by CLSM.

Moreover, the distribution of hNDP was also investigated under hypoxic microenvironment in shield of glass cover. 4T1 cells were seeded in Petri dishes and incubated in culture medium for 24 h. Subsequently, the cells were treated with hNDP

(20 μM) in 1 mL of culture medium and covered by coverslip. And the cells without any treatment were used as a control. After incubated in the normoxic environment for 24 h, the cells were washed by PBS and stained by Hoechst 33342. The distribution of the fluorescence was observed and analyzed by CLSM.

Besides, the distribution of hNDP was also investigated by the sequential treatment of normoxic and hypoxic environments using glass cover. Firstly, 4T1 cells were incubated with hNDP (20 μM) for 24 h. Subsequently, the cells, on the one hand, were washed by PBS and stained by Hoechst 33342. The intracellular fluorescence was observed and analyzed by CLSM. On the other hand, the cells treated with hNDP were covered by coverslip and further incubated for 12 h. And then, the cells were stained by Hoechst 33342 and observed by CLSM.

8. The Subcellular Distribution of hNDP under Normoxic and Hypoxic Conditions.

4T1 cells were seeded in Petri dishes and incubated in culture medium for 24 h. Then, the cells were incubated with hNDP (20 μM) in 1 mL of culture medium under the normoxic or hypoxic environment for 24 h. After that, the cells were washed by PBS, stained by LysoTracker Green or MitoTracker Green, and then stained by Hoechst 33342. Finally, the intracellular fluorescence was observed and analyzed by CLSM.

9. The Cellular Uptake and Excretion Behaviors of hNDP.

4T1 cells were seeded in 6-well plate and incubated for 24 h. And then, the cells were co-incubated with hNDP (20 μM) for 24 h. After which, the cells were washed

by PBS and the intracellular fluorescence was analyzed by flow cytometry. For comparison, the cells treated with hNDP were further incubated in fresh culture medium for 12 h and then analyzed by flow cytometry.

10. In Vitro Cytotoxicity

The anti-proliferation ability of hNDP was measured by MTT assay. 4T1 cells and B16 cells were seeded into the 96 well plate (6000 cells/well) and incubated for 24 h, respectively. After which, gradient concentrations of hNDP (20 μM, 13 μM, 8.9 μM, 5.9 μM, 3.9 μM, 2.6 μM, 1.8 μM, 1.2 μM, 0.78 μM, 0.52 μM) in DMEM medium (100 μL) were added into each well. And then, the cells were incubated at 37 ^{o}C for 24 h under normoxic or hypoxic condition, respectively. Subsequently, 20 μL of MTT solution (5 mg/mL) was added into each well. After incubation for 4 h, the culture medium was removed and 150 μL of DMSO was added into each well. The optical density (OD, 570 nm)) of the plate was measured by microplate reader. The relatively cell viability was calculated by the following equation: Cell Viability (%) = $(OD_{sample}/OD_{control})\times100\%$ (OD_{sample} was measured from the cells treated with hNDP, and $OD_{control}$ was obtained from the cells without any treatments).

11. Flow Cytometry Analysis.

The anti-tumor ability of hNDP was also evaluated by flow cytometry. 4T1 cells were seeded in 6-well plates and incubated for 24 h. Afterwards, the cells were incubated with hNDP (20 μM) under normoxic or hypoxic condition by purging N_2. After 24 h, the cells were collected and stained by Annexin V-FITC and PI. Subsequently, the cell status and the corresponding real time fluorescence images

were measured by flow cytometry.

12. Western Blotting Analysis.

4T1 cells were seeded in 6-well plates and incubated for 24 h prior to experiments. And then, the cells were treated with hNDP (20 μM) and then cultured for 24 h under normoxic or hypoxic condition. After which, the cells was collected and washed by PBS. Total proteins were extracted from 4T1 cells by RIPA lysis buffer supplemented with protease and phosphatase inhibitors. Protein lysates (30 μg) were electrophoresed on 10% SDS-PAGE gels and electroblotted onto polyvinylidene fluoride membranes (Immobilon, Millipore). Membranes were blocked with 5% milk in tris-buffered saline with tween(TBST) for 1 hour and incubated with the following primary antibodies at 4 ℃ overnight: anti-Bad (Cell Signaling Technology, #9292), anti-Bax (Cell Signaling Technology, #2772), anti-Bcl-2 (Cell Signaling Technology, #3498), anti-Cleaved Caspase-3 (Cell Signaling Technology, #9664) and anti- alpha Tubulin (Abcam, ab52866). The membranes were then washed with TBST and incubated with the following secondary antibodies for 2 hours at room temperature: goat anti-rabbit immunoglobulin G (IgG)-horseradish peroxidase (HRP) (Santa Cruz, sc-2030). Membranes were eventually developed by SuperSignal West Pico Chemiluminescent Substrate kit (Pierce Biotechnology) and scanned for relative protein level analysis. The intensity of each protein band was determined by densitometry and divided by alpha Tubulin gray value for normalization.

13. Anti-Tumor Effects

To construct a tumor-bearing animal model, 4T1 cells were subcutaneously

injected into the right hind limbs of the Balb/c mice. The mice were randomized divided into three groups when the tumor volume reached about 100 mm^3. After which, the mice were subcutaneously injected with PBS, DOX (5 mg/kg per mouse) or equiequivalent of hNDP (7.95 mg/kg per mouse), respectively. The tumor volume and the body weight of the mice were recorded every two days. After treatment for 12 days, the mice were sacrificed and the main organs and the tumor tissues were collected for H&E analysis. The tumor volume was calculated by the equation of $V = W^2 \times L/2$, where W and L represented the minor and major length, respectively.

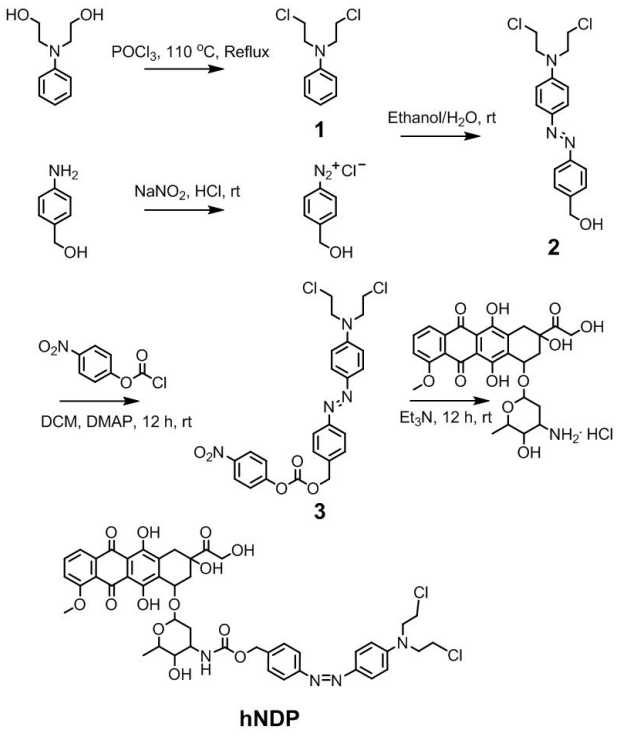

Fig. S1 Synthetic procedures of hNDP.

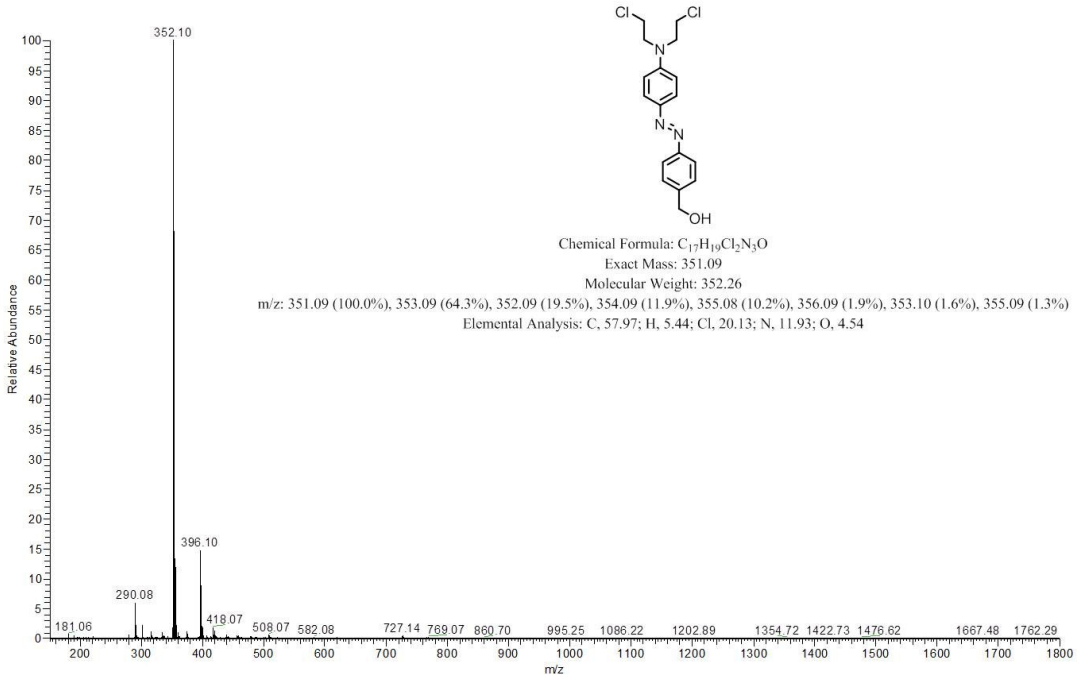

Fig. S2 ESI-MS of compound 2.

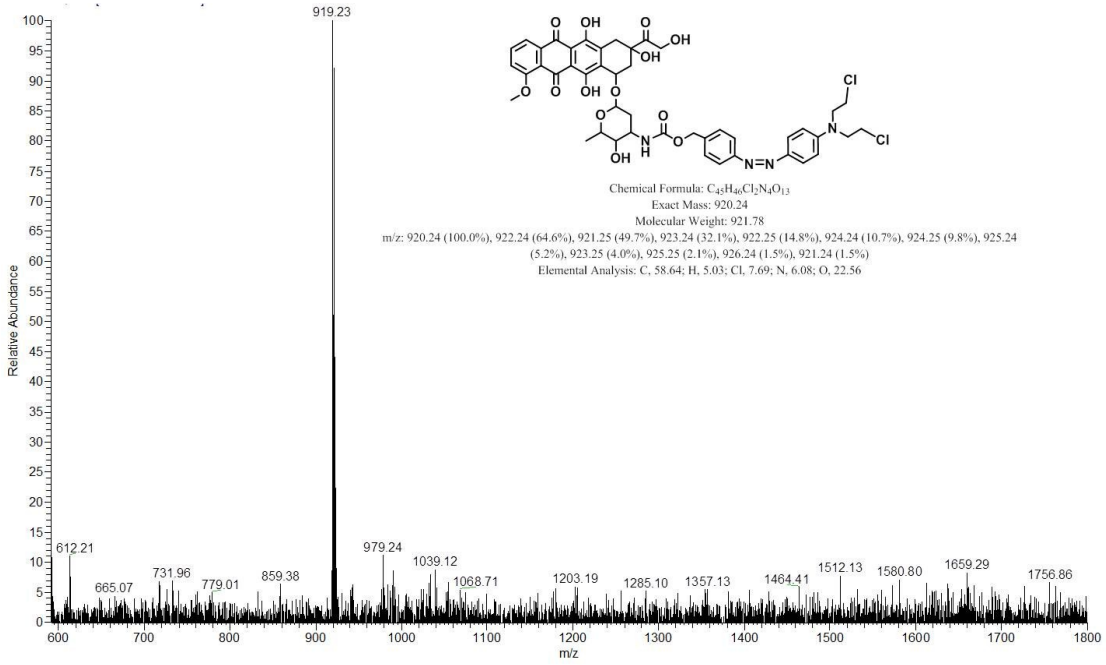

Fig. S3 The chemical structure and ESI-MS of hNDP.

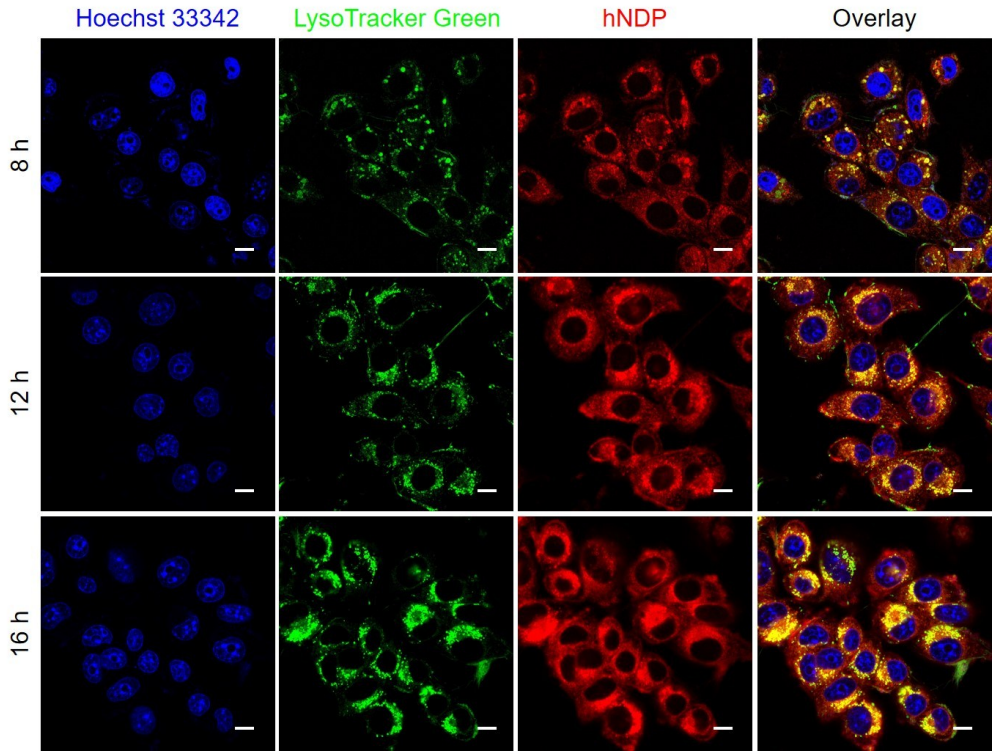

Fig. S4 CLSM images of 4T1 cells after treatment with hNDP for 8 h, 12 h, 16 h and stained by Hoechst 33342 and LysoTracker Green. Scale bar: 30 μm.

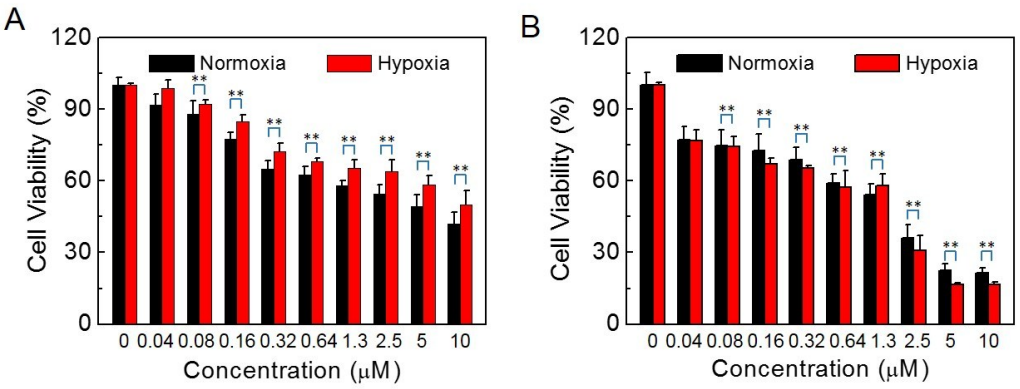

Fig. S5 Cell viabililties of A) 4T1 cells and B) B16 cells after treatment with gradient concentrations of DOX under normoxia or hypoxia conditions. *p < 0.05 and **p < 0.01 were determined by a Student's t-test when the cells incubated under normoxia was compared with those incubated under hypoxia.

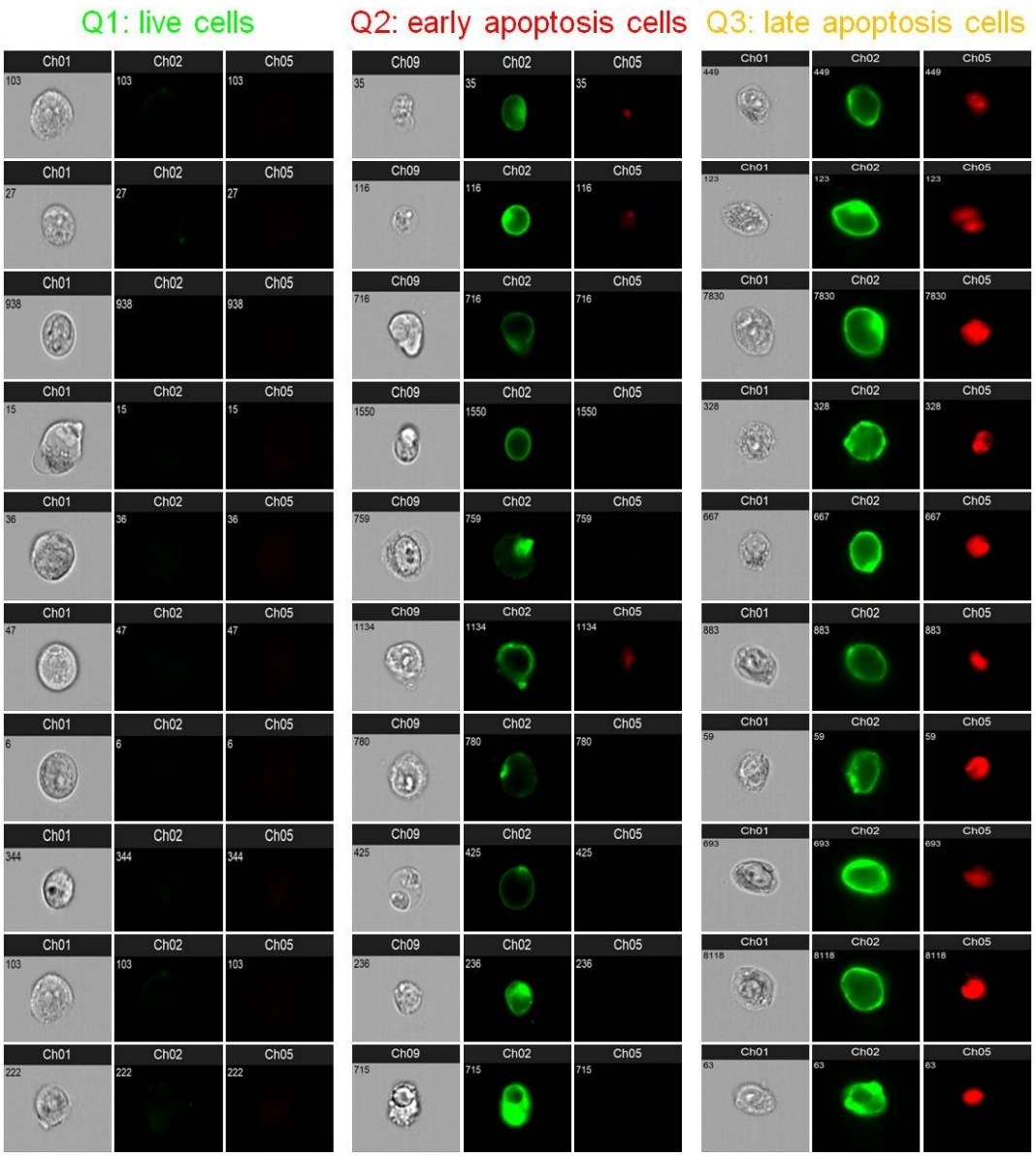

Fig. S6 Real time fluorescence images of 4T1 cells after treatment with hNDP (20 μM) under normoxic condition.

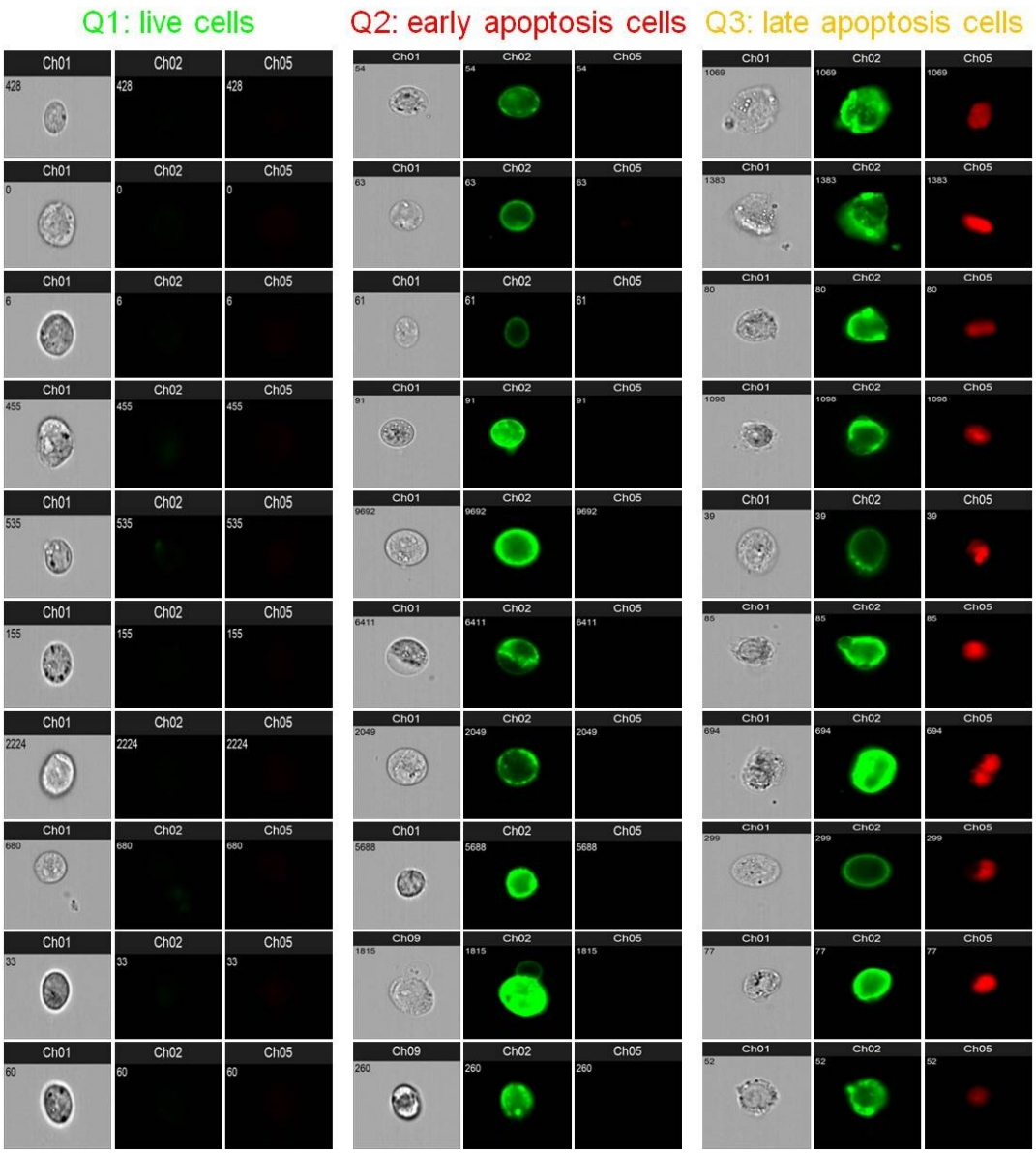

Fig. S7 Real time fluorescence images of 4T1 cells after treatment with hNDP (20 μM) under hypoxic condition.

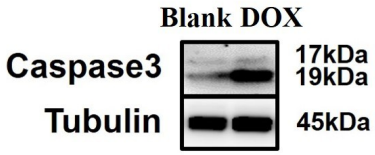

Fig. S8 Western blot analysis of 4T1 cells after treatment with DOX for 24 h under normoxia condition using 4T1 cells without any treatment as a blank control.

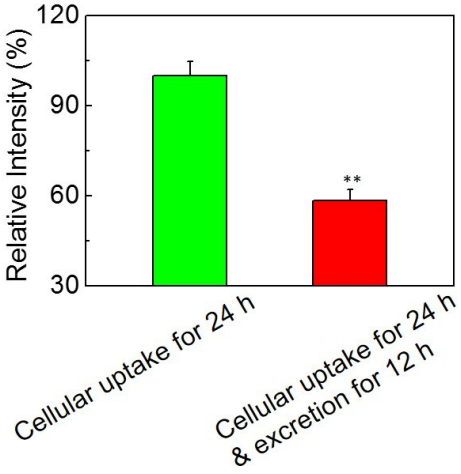

Fig. S9 The relative fluorescence intensity of 4T1 cells after treatment with hNDP (20 μM) for 24 h and then incubated in fresh culture medium for another 12 h under normoxic condition. **$p < 0.01$ was determined by a Student's t-test.

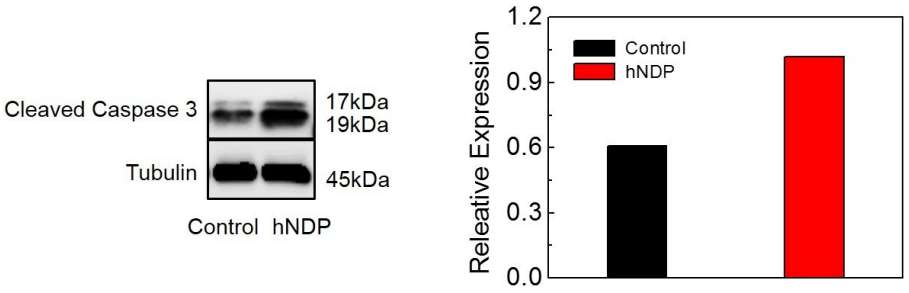

Fig. S10 Western blot analysis of 4T1 cells after treatment with hNDP (20 μM) for 24 h under hypoxia conditions using 4T1 cells without any treatment as a blank control.

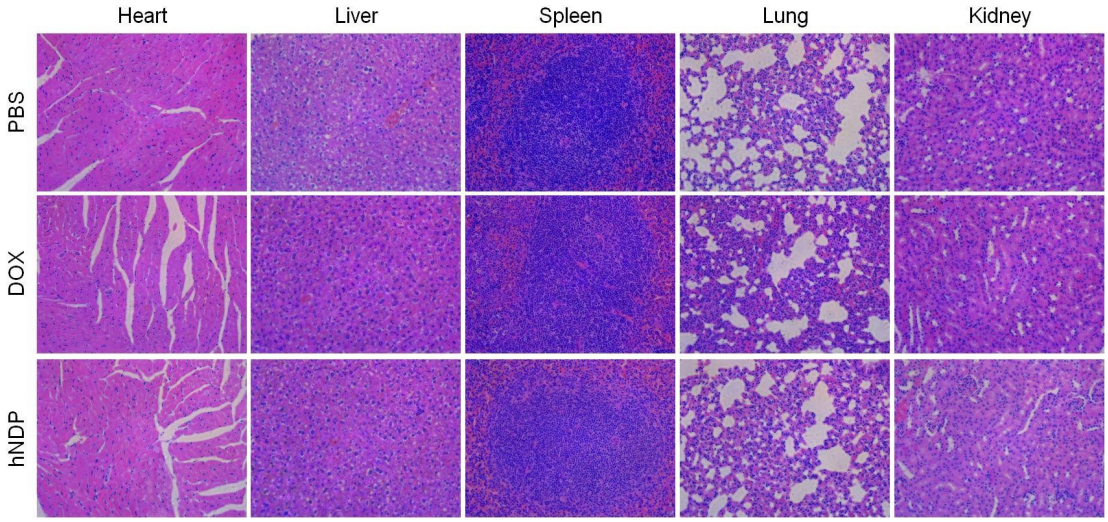

Fig. S11 H&E staining images of the heart, liver, spleen, lung and kidney in different groups after treatment with PBS, DOX or hND P at the 12th day.