Dendritic Drug-drug Conjugates Self-Assembled Hypoxia-Responsive Supramolecular Nanoparticle for Combination Therapy

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Materials

The Chloroambucil (CB, 98%, J&K), Triethylamine (TEA, 99.5%, Aladdin), (7-Azabenzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HATU, 99%, TRC), N, N-Diisopropylethylamine (DIPEA, 99%, Adamas) were used as received. 3-(3-dimethylaminopropyl)-1-ethylcarbodiimide hydrochloride (EDC, 97%, Adamas), and 4-dimethylaminopyridine (DMAP, 99%, Adamas) were used as received. Boc-D2, Azo-CB-COOH, and IR806 were synthesized following the procedure described previously.

Synthesis of Boc-D2-Azo-CB_4

The Azo-CB-COOH (456.3 mg, 1.25 mmol) and (7-Azabenzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HATU) (760 mg, 2 mmol) were dissolved in 10 mL of anhydrous DMF and stirred at 0 °C for 5 h. Following this, DIPEA (348 mg, 2.7 mmol) introduced into the mixture, which was then allowed to warm up to room temperature. Solution of Boc-D2 (105.6 mg, 0.125 mmol) in 5 mL dry DMF was added gradually while stirring continued for an additional forty-eight hours. The reaction was terminated by adding water and subsequently extracted with dichloromethane (DCM). The organic phase was dried using sodium sulfate before removing the solvent under reduced pressure. Finally, the crude product underwent purification through column chromatography on silica gel yielding an orange-red solid Boc-D2-Azo-CB_4 (128.3 mg. 46.2% yield).

Synthesis of IR806-Azo-CB_4

Initially, trifluoroacetic acid (TFA) (1 mL) was added slowly to solution of Boc-D2-

Azo-CB_4 (130 mg, 0.058 mmol) in 1 mL DCM and stirred for 2 h. The solvent along with any unreacted TFA was then evaporated under reduced pressure, resulting in an orange-red solid of D2-Azo-CB_4 after vacuum drying. Next, IR806 (80 mg, 0.1 mmol) and HATU (58 mg, 0.15 mmol) were dissolved in 5 mL dry DMF and stirred for 3 h at 0 ℃. DIPEA (0.5 mL, 0.003 mmol) was introduced into the mixture which was then allowed to warm up to room temperature. A solution of Boc-D2-Azo (217.5 mg, 0.097 mmol) in 2 mL dry DMF was added dropwise into the front solution while stirring continued for another 24 h. The resulting solution was placed into a dialysis tube (MWCO 2000 Da), dialyzed against DMSO for twenty-four hours followed by ultrapure water for an additional twenty-four hours before being freeze-dried to yield IR806-Azo-CB_4 (242.67 mg, 86.3% yield). MALDI-TOF-MS: m/z 2777.1156 (M+)

Fabrication of supramolecular nanoparticle SN@IR806-CB

Generally, IR806-Azo-CB_4 (5 mg) was dissolved in DMF (1 mL), and then aqueous solution (10 mL) of AWBpP6 (1.0 mg/mL) was added into the original solution under vigorous stirring overnight. Then, the mixture solution was transferred into dialysis tube (MWCO 2500 Da) by dialysis against distilled water for 24 h to give the supramolecular nanoparticle SN@IR806-CB.

Detection of $^1O_2$ generation.

The ABDA (0.05 mg/mL) was added into the PBS (20 mL) of SN@IR806-CB with equivalent IR806 concentration of 40 μg/mL at pH 5.0. For the NIR-triggered $^1O_2$ release, the above solution was irradiated with NIR irradiation (808 nm, 1.0 W/cm^2, 5 min). For that ABDA can effectively capture ROS through a rapid reaction with

anthracene moiety, the original UV-Vis absorption of ABDA would gradually reduce in the environment of $^1O_2$.

***In vitro* drug release**

The drug release kinetics of SN@IR806-CB was tested at 37 ± 0.5 °C under continuous agitation. The SN@IR806-CB was transferred to the dialysis bag (MWCO 1000 Da), which was immersed in PBS (pH 5.0 or 7.4) with or without of sodium dithionite (2 mM). At specific time intervals, the aliquots (2 mL) were collected and analyzed by UV-Vis spectrophotometer.

***In vitro* cytotoxicity**

HCT116 cells suspension in DMEM was placed in a 96-well plate at a density of 1 × 10^4 cells/well (200 μL) and incubated for 24 h. Subsequently, the medium in each well was replaced with fresh medium containing SN@IR806-CB with different concentrations under hypoxia or normoxia. For irradiation groups, the cells were irradiated with NIR irradiation (808 nm,1.0 W/cm^2, 5 min), and further incubated for 18 h. Then, the MTT assays were performed to analyze cytotoxicity.

***In vitro* cell internalization and ROS generation**

The HCT116 cells were seeded into a 12-well plate at a density of 1.0 × 10^5 cells/well, and incubated in DMEM for 12 h. Then, the medium was replaced with the fresh DMEM containing SN@IR806-CB with CB concentration of 8 μg/mL for further 4 h incubation under hypoxia or normoxia environment with or without the 808 nm NIR irradiation (1.0 W/cm^2, 5 min). Then, the cells were stained with lysotracker green probe for 20 min, and imaged by confocal laser scanning microscope (CLSM). After

similar treatment, the cells were stained with 2, 7-dichlorodihydrofluorescein diacetate (DCFH-DA) for 20 min, and observed by CLSM.

***In vivo* parmacokinetics analysis**

The experimental protocol was approved by the Nantong University Institutional Animal Care and Use Committee. The pharmacokinetics analysis was performed using SD rats that were randomly divided into two groups (n = 3). The free IR806 and SN@IR806-CB at equivalent IR806 concentrations (5 mg/kg) were injected via the tail vein. The orbital vein blood (0.3 mL) was obtained at a predetermined time, harvested by centrifugation, and frozen at -20 °C. The IR806 level in blood was measured by fluorescence spectroscopy.

***In vivo* antitumor activity**

The HCT116 tumor-bearing mice with a tumor volume (80 mm^3) were spontaneously assigned into five groups (n = 3), and then intravenously injected at 0 day and 6 day with PBS, CB, IR806 + NIR, SN@IR806-CB, or SN@IR806-CB + NIR at CB equivalent dose of 2.5 mg/kg and IR806 dose of 2.2 mg/kg. The tumor volume (V) is calculated according to the following equation: $V = 1/2 \times length \times width^2$. The tumor inhibitory rates (TIR) is calculated by the equation: TIR (%) = 100 × (mean tumor volume of the PBS group - mean tumor volume of others)/(mean tumor volume of the PBS group). At the end of the treatment, all tumors and major organs (heart, liver, spleens, lung, and kidneys) were dissected for histological examination by H&E staining, TUNEL and PCNA assays.

Statistical analysis

The data was expressed as mean ± standard deviations (S.D) using GraphPad Prism software 5.0. The two-tailed analysis of variance and the Student's t-test were used to determine statistical significance. A probability (P) value < 0.01 was indicated to be significant, and P < 0.001 was highly significant.

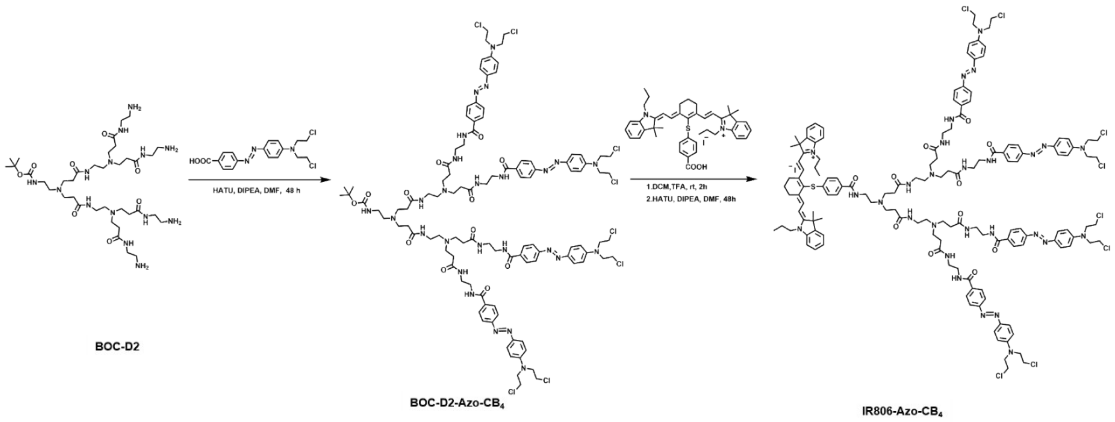

Fig. S1 Synthesis of IR806-Azo-CB_4.

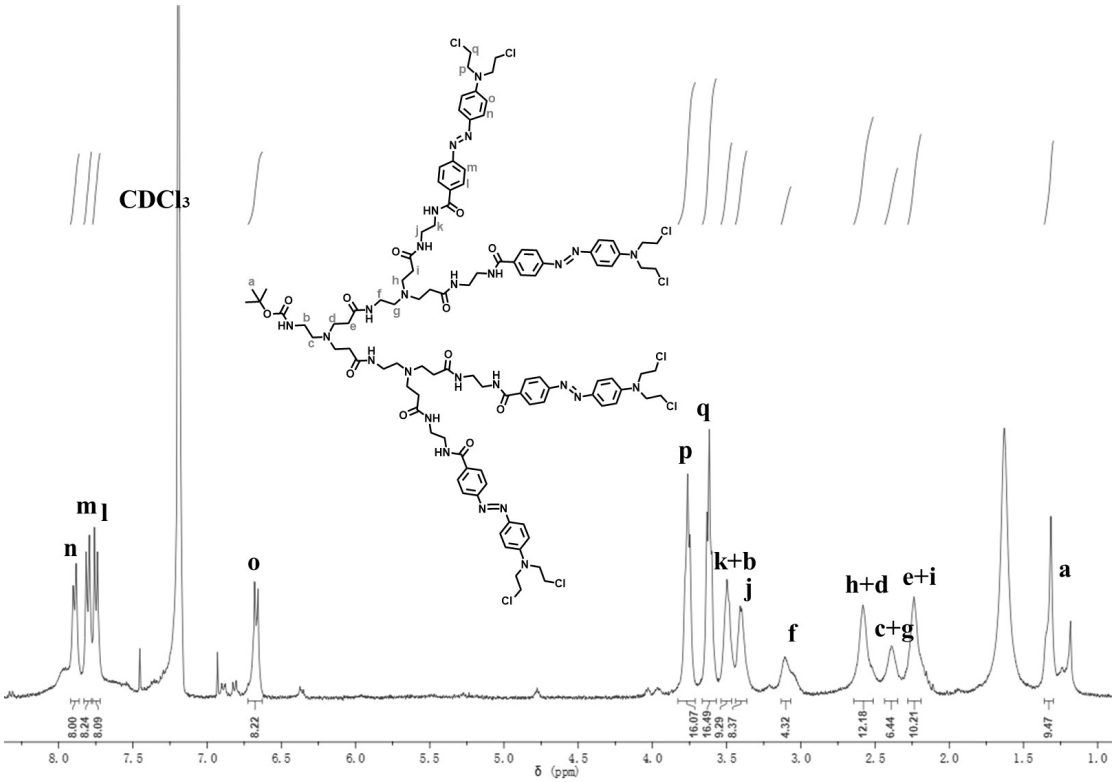

Fig. S2 ^{1}H NMR spectra of Boc-D2-Azo-CB_4 ($CDCl_3$).

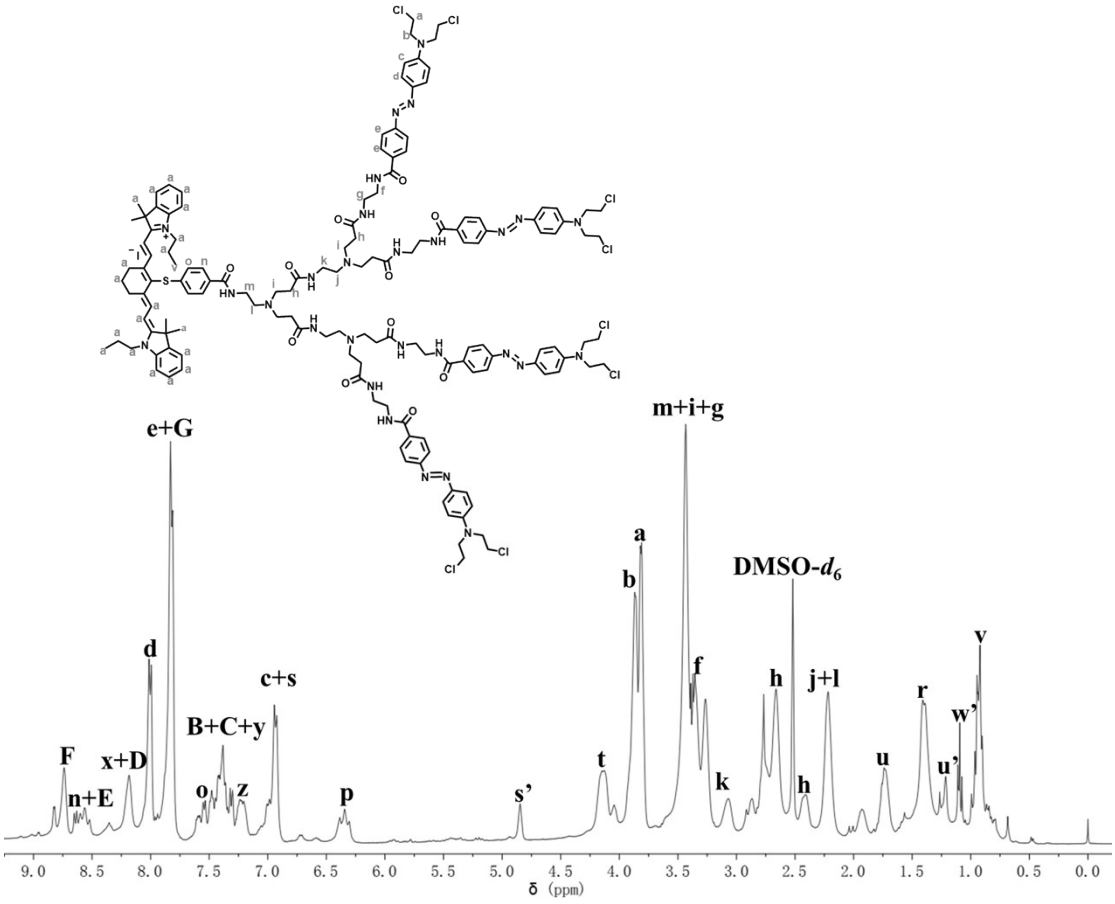

Fig. S3 ^{1}H NMR spectra of IR806-Azo-CB$_4$ (DMSO-d_6).

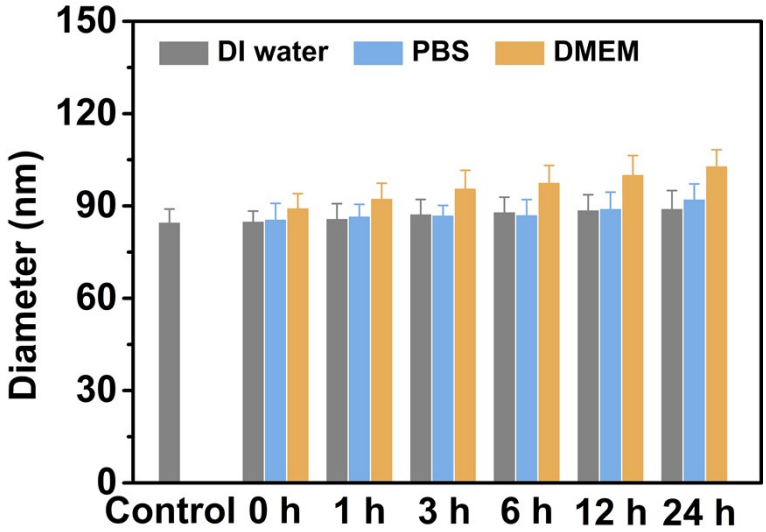

Fig. S4. The dependence of D_h on incubation time in PBS, deionized water (DI water) and dulbecco's modified eagle medium (DMEM) for SN@IR806-CB.

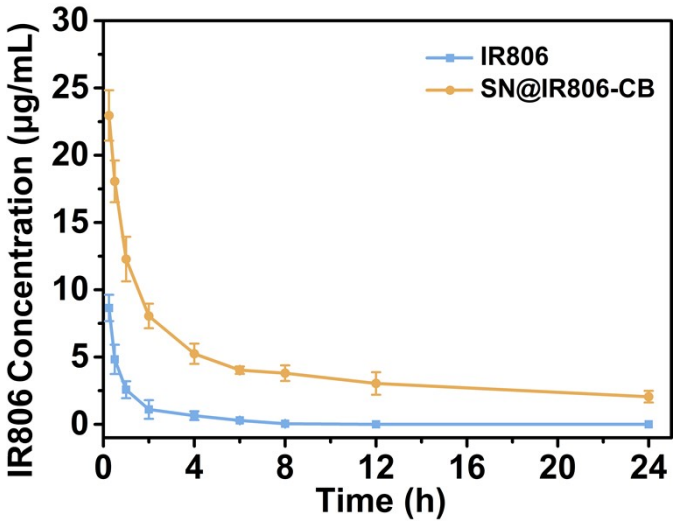

Fig. S5. Representative plasma concentration-time profiles of free IR806 and SN@IR806-CB.

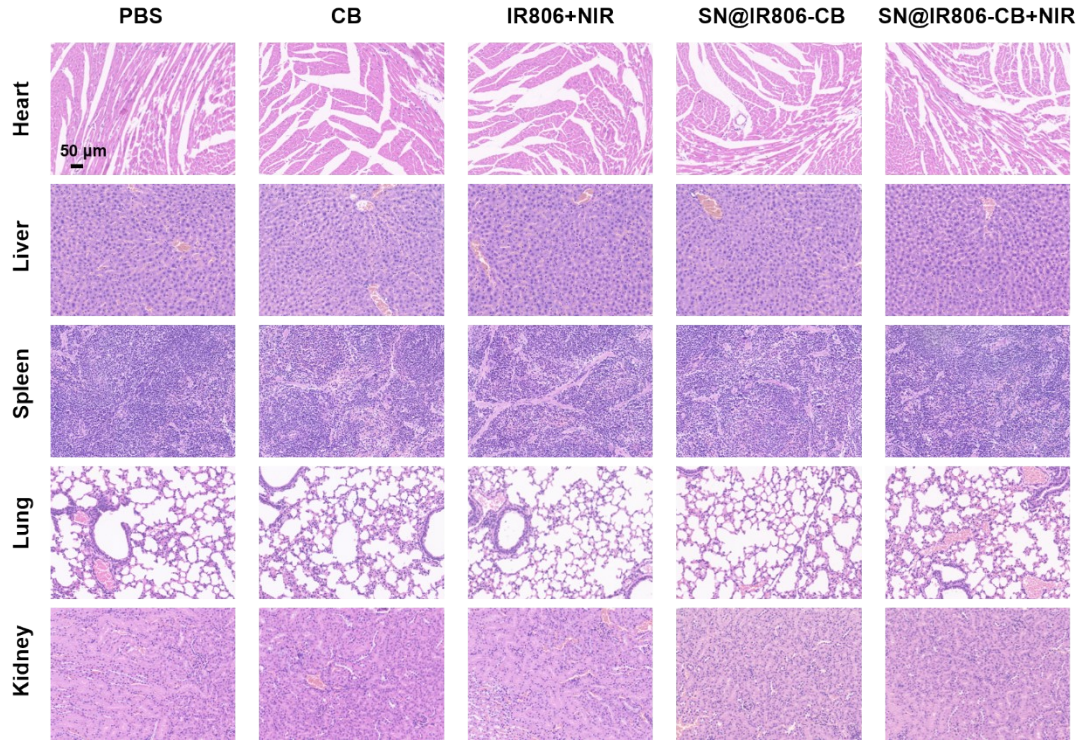

Fig. S6. H&E-stained tissue sections from the major organs (heart, liver, spleen, lung, and kidney) dissected after 21 days treatments (magnification × 400).

Supplementary References

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