Supporting Information

A GPX4-targeted photosensitizer to reverse hypoxia-induced inhibition of ferroptosis for non-small cell lung cancer therapy

Qiao Hu, Wanjie Zhu, Jianjun Du,*[ab] Haoying Ge, Jiazhu Zheng, Saran Long,[ab] Jiangli Fan [ab] and Xiaojun Peng [a]

[a] State Key Laboratory of Fine Chemicals, Frontiers Science Center for Smart Materials Oriented Chemical Engineering, School of Chemical Engineering, Dalian University of Technology, Dalian 116024, China.

[b] Ningbo Institute of Dalian University of Technology, 26 Yucai Road, Jiangbei District, Ningbo 315016, P. R. China.

*Correspondence author. E-mail: dujj@dlut.edu.cn

Experimental section

1. Materials and instruments

The general chemical reagents used in the report were purchased from Energy Chemical Co., and J&K Scientific Ltd., and all of the solvents were of analytic grade. DMEM, PBS, purchased from KeyGEN BioTECH Ltd. All reactions were monitored by layer chromatography (TLC) using 0.25 mm silica gel plates with UV indicator (GF-254).

^{1}H NMR and ^{13}C NMR spectra were detected by Vaian DLG 400 spectrometer and Bruker Avance III 400 spectrometer. Mass spectrometric (MS) data were carried out using LTQ Orbit rap XL instruments and TOF LC/MS instruments. Absorption spectra were measured on a PerkinElmer Lambda 35 UV/vis spectrophotometer (PerkinElmer). Fluorescence spectra were obtained with a VAEIAN CARY Eclipse fluorescence spectrophotometer (Serial No. FL0812: M018). Confocal laser scanning microscope (CLSM) images were performed on Olympus FV3000 confocal laser. The mice fluorescence pictures were obtained from NightOWL II LB983 living imaging system.

2. Synthesis of ENBS-ML210

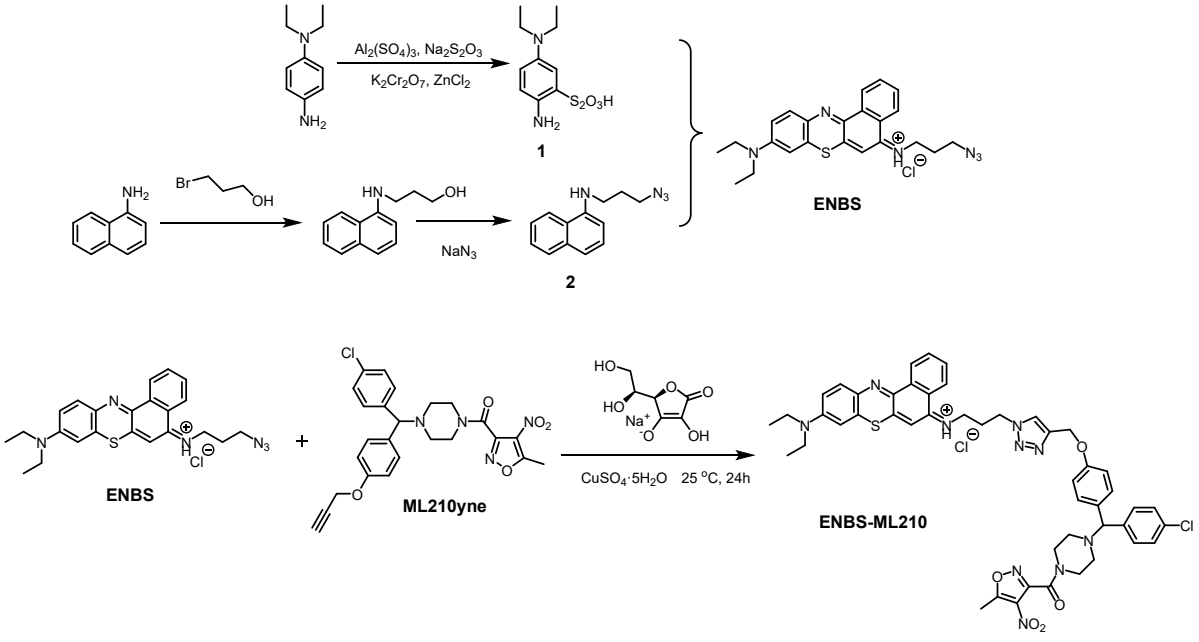

Fig. S1 Synthetic route of **ENBS-ML210**

Synthesis of **ENBS:** Compound **1** and **2** were synthesized according to reference.[1, 2] Add compound **1**(300 mg, 1.33 mmol) and compound **2** (267 mg, 0.966 mmol) into a single ended round bottom flask and dissolve them with 6 mL of dimethyl sulfoxide. Add potassium dichromate (312 mg, 1.06 mmol) and stir for 20 minutes at room temperature. Then transfer the reaction solution to a single round bottom flask filled with 60 mL methanol, add slowly 6 mL hydrochloric acid solution with a concentration of 2 mol/L, and stir for 40 min. After the reaction, extract the reaction solution with water and ethyl acetate, take the organic phase, dry it with anhydrous sodium sulfate, filter it, take the filtrate, spin it under reduced pressure, and separate the product (eluent: CH_2Cl_2/MeOH) by column chromatography to obtain 400 mg of blue solid **ENBS** with a yield of 88.7%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.58 (s, 1H), 8.84 (q, *J* = 5.8, 4.7 Hz, 2H), 7.88 (d, *J* = 9.4 Hz, 1H), 7.75 – 7.68 (m, 2H), 7.13 – 7.09 (m, 1H), 6.92 (s, 1H), 6.81 (d, *J* = 2.7 Hz, 1H), 3.82 (d, *J* = 6.5 Hz, 2H), 3.61 (q, *J* = 7.2 Hz, 4H), 3.54 (t, *J* = 6.3 Hz, 2H), 2.18 (p, *J* = 6.6 Hz, 2H), 1.35 (t, *J* = 7.1 Hz, 7H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 154.09, 150.65, 140.00, 137.09, 134.86, 133.25, 132.34, 131.19, 130.73, 130.21, 124.88, 124.66, 124.60, 116.49, 104.53, 102.05, 77.27, 53.46, 49.07, 45.81, 40.99, 28.14, 18.44, 12.81, 1.02. HRMS: m/z calculated for ($[C_{23}H_{25}N_6S^+]$): 417.1856; found:417.1843.

Synthesis of **ENBS-ML210:** ML210yne was synthesized according to reference.[3] Add the **ENBS** (100 mg, 0.230 mmol), (136 mg, 0.230 mmol), sodium ascorbate (54 mg, 0.275 mmol) and copper sulfate pentahydrate (38 mg, 0.151 mmol) into two round bottom flasks. Dissolve 14 mL of mixed solvent ($CHCl_3$/MeOH/H_2O = 12/1/1), and react at room temperature for 24 h under nitrogen protection and dark condition. At the end of the reaction, the reaction solution solvent is decompressed and spun, and the product was separated by column chromatography (eluent: CH_2Cl_2/MeOH) to obtain about 20 mg of blue solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.82 (s, 1H), 9.00 (s, 1H), 8.52 (d, *J* = 8.2 Hz, 1H), 8.29 (s, 1H), 8.09 – 7.79 (m, 3H), 7.54 – 7.39 (m, 5H), 7.32 (dd, *J* = 15.6, 8.2 Hz, 5H), 6.96 (d, *J* = 8.3 Hz, 2H), 5.07 (s, 2H), 4.58 (t, *J* = 6.8 Hz, 2H), 4.38 (s, 1H), 3.76 – 3.64 (m, 8H), 2.81 (s, 3H), 2.37 (q, *J* = 7.6, 6.9 Hz, 4H), 2.20

(t, J = 5.0 Hz, 2H), 1.24 (d, J = 3.9 Hz, 8H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 173.88, 157.66, 156.57, 153.52, 153.40, 151.49, 143.27, 142.14, 140.23, 137.52, 134.37, 134.04, 133.72, 132.92, 131.83, 131.71, 129.96, 129.75, 129.26, 129.00, 128.71, 125.18, 124.66, 123.87, 118.23, 115.27, 105.95, 103.33, 73.15, 61.62, 56.49, 55.38, 51.83, 51.07, 47.72, 46.58, 45.68, 29.49, 22.56, 19.02, 14.02, 13.14. HRMS: m/z calculated for ([$C_{48}H_{48}ClN_{10}O_5S^+$]): 911.3213; found: 911.3209.

3. DFT theoretical calculation

All the quantum calculation were carried out with the Gaussian 09. The geometry optimizations of the ENBS and ENBS-ML210 for ground state were performed using density functional theory (DFT) in combined with B3LYP/6-31g(d) basis set in MeOH.

4. Cell imaging

In this experiment, non-small cell lung cancer cells (H1299) were used for biomedical research in vitro and evaluation of antitumor effect in vivo. The cells were cultured in 1640 medium containing 10% serum and 1% penicillin/streptomycin at 37 °C and 5% CO_2 to the logarithmic growth phase, and then digested by trypsin to prepare l x 10^5 cells/mL cell suspension for subsequent different experimental programs.

4.1 Intracellular ROS detection

Take 100 μL H1299 cell suspension (1x10^5 cells/mL) and place it in a confocal dish containing 2 mL of fresh culture medium. Incubate it at 37 °C and 5% CO_2 for 24 h to the logarithmic growth phase, and then add 1 μL **ENBS-ML210** for incubation for 2 h. After washing by PBS, the cells were labeled by DCFH-DA (2 μM), DHE (10 μM), SOSG (1 μM) and HPF (10 μM) for 0.5 h, respectively. Then each group was given 660 nm (20 mW/cm^2) light for 15 min. Subsequently, detection of intracellular fluorescence signals using confocal microscopy.

4.2 Cell apoptosis analysis

After incubation for 24 h, H1299 cells in six-well plates were added with **ENBS** (1 μM), **ENBS-ML210** (1 μM) for 2 h. Then, the cells were digested and collected by centrifugation at 1500 rpm for 5 min after LED light irradiation (660 nm, 20 mW/cm^2) for 10 min. After Annexin V-FITC and PI staining for 20 min, the cells were finally evaluated for apoptosis detection by flow cytometry.

4.3 Live/dead cell staining imaging

H1299 cells were cultured in a confocal dish (containing 2 mL medium) until they are suitable for imaging. **ENBS** (1 μM), **ENBS-ML210** (1 μM) were respectively incubated for 2 h. After that, the above cells were stained with Calcein AM and PI for 0.5 h after the irradiation with LED light (660 nm, 20 mW/cm^2) for 10 min, and the fluorescence intensity and position of each group were observed by CLSM.

4.4 Detection of lipid peroxidation

BODIPY (581/591)-C11 was employed as the probe to assess the LPO. Above all, **ENBS** (1 μM), **ENBS-ML210** (1 μM) were respectively incubated with H1299 cells for 2 h. After the irradiation with LED light (660 nm, 20 mW/cm^2) for 10 min, the cells were further stained by C11-BODIPY (581/591) (5 μM) for 30 min, H1299 cells were washed with PBS for two times, followed by observation of in intracellular fluorescence by CLSM.

4.5 MTT assay

Mitochondrial dehydrogenases in living cells can reduce MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to formazan crystals. H1299 cells were prepared into a single-cell suspension with a concentration of 5 ×10^4 cells/mL, and 100 μL per well was seeded into a 96-well plate, and the surrounding 32 wells were filled with 100 μL PBS buffer, 37 °C, 24 h incubation. After that, in the experimental group, **ENBS-ML210** and **ENBS** with different concentrations (0-1 μM) were added respectively, 6 duplicate wells were set per group, and were incubated for 2 h, then the cells were irradiated with LED light (660 nm, 20 mW/cm^2) for 15 min, followed by another 24 h incubation. In addition, in the control group, the cell treatment method was the same as that of the experimental group without light. Replace the original culture medium with 100 μL of medium solution containing 0.5 mg/mL MTT. After the cells were incubated for 4 h, the culture medium was carefully removed, the supernatant was aspirated, and 100 μL of DMSO was added to each well and shaken on a shaker for 10 min to dissolve the blue formazan. Finally, absorbance (OD value) was measured with a UV-Vis microplate reader at wavelengths of 490 nm and 570 nm (630 nm).

5. Western blot

To analyze the expression of GPX4 protein, **ENBS-ML210** (1 μM) was incubated with H1299 cells for 2 h, then through different treatment (light or without light). The cells were lysed with RIPA lysis buffer on ice, the total protein was obtained by collecting the supernatant after centrifugation at 12000 rpm for 5 min. Afterward, the proteins were isolated by electrophoresis in SDS-PAGE and transferred to PVDF membrane. After sealed with western sealing fluid, the PVDF membrane was incubated with anti-GPX4 at 4 °C overnight, followed by treatment with HRP-marked secondary antibodies for 2h. Ultimately, the GPX4 expression was detected via chemiluminescence.

6. *In vivo* fluorescence imaging

All animal experiments involved in this study were conducted in accordance with the "Guidelines for the Care and Use of Laboratory Animals" published by the National Institutes of Health (8th edition, 2011) and approved by the Animal Ethics Review Committee of Dalian University of Technology. 5-6 weeks Balb/c female nude mice were obtained from Liaoning Chang Sheng Biotechnology Co., Ltd. The preparation concentration is 1×10^7 cells/mL of NCI-H1299 cells suspension in PBS, 100 μL was injected subcutaneously into the right thigh of mice with a 1 mL syringe. After the tumor volume reached about 100 mm^3 (about 20 days), it was used for *in vivo* tumor-targeted fluorescence imaging. The above tumor-bearing mice were taken and injected with 100 μL **ENBS-ML210** (1 μM) from the tail vein, and then the distribution of **ENBS-ML210** at different time points (0.5, 1.5, 3, 5, 6, 8, 12 and 24 h) was observed with a small animal imager. After imaging, the test mice were euthanized, tumor and organs such as heart, liver, spleen, lung and kidney were dissected and collected, and corresponding fluorescent images were collected.

7. *In vivo* antitumor study

The H1299 tumor-bearing mouse were divided into 5 groups and treated differently (Control, **ENBS**, **ENBS**+Light, **ENBS-ML210**, **ENBS-ML210**+Light). Wherein, light group with 660 nm LED (100 mW/cm^2, 20 min). During the treatment, the tumor volume and mice weight were measured every 2 days and the mice were sacrificed at

the 14th day. The main organs and tumor tissues were harvested to carry out H&E and Tunnel staining.

8. Supporting figures

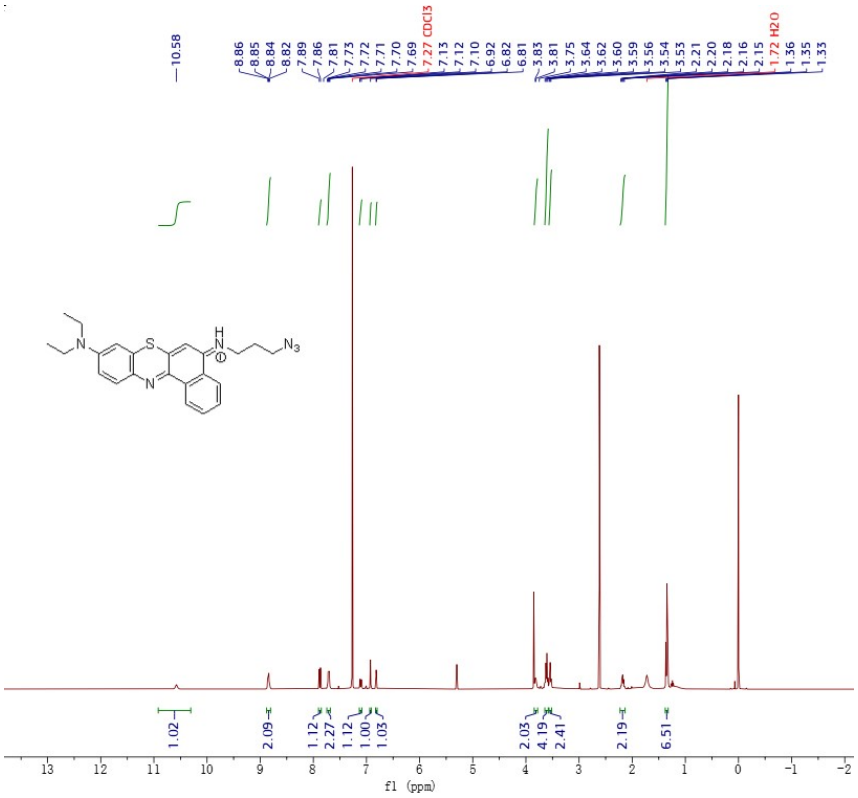

Fig. S2 ^{1}H NMR spectrum of **ENBS** in $CDCl_3$ (400 MHz).

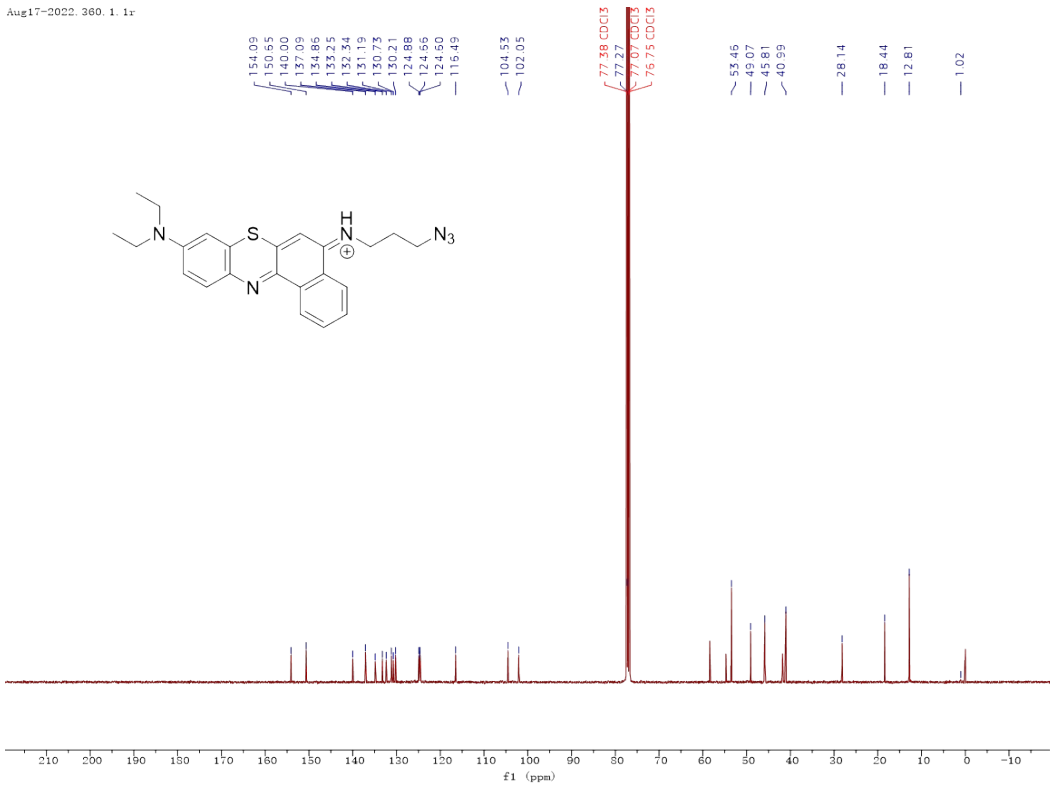

Fig. S3 ^{13}C NMR spectrum of **ENBS** in $CDCl_3$ (101 MHz).

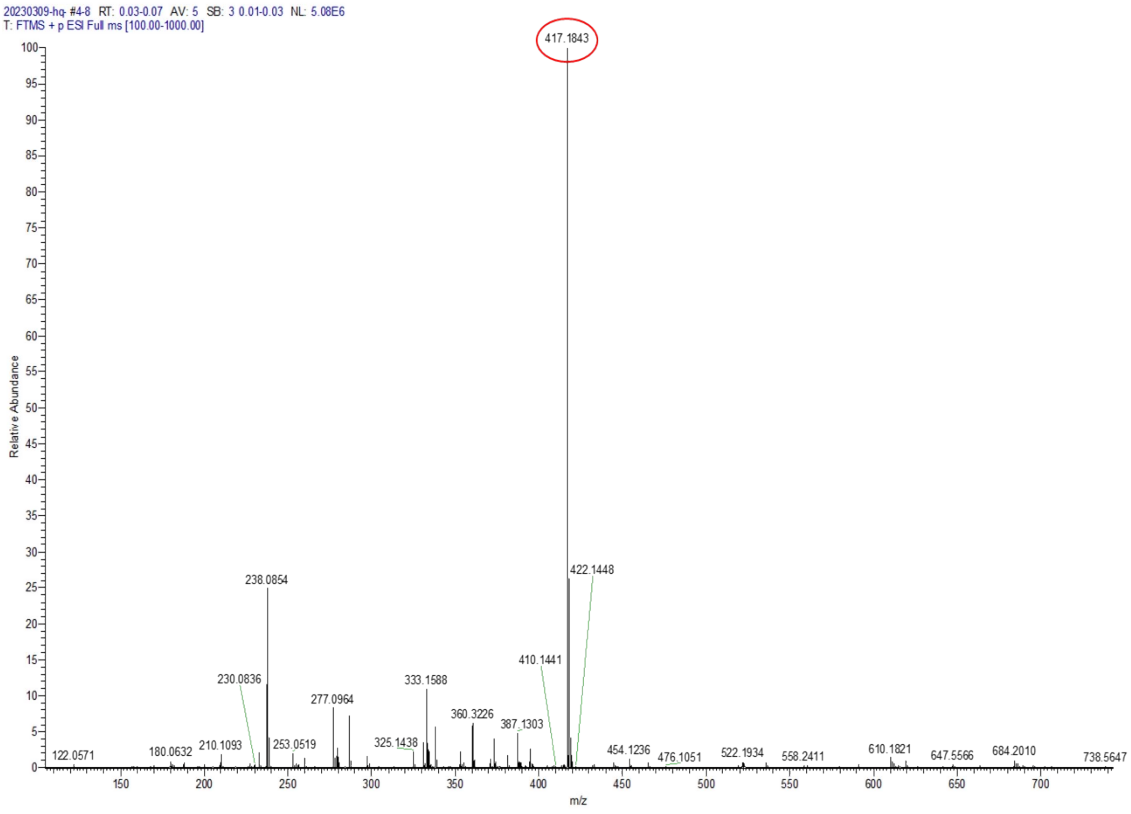

Fig. S4 HRMS (ESI) spectrum of **ENBS**.

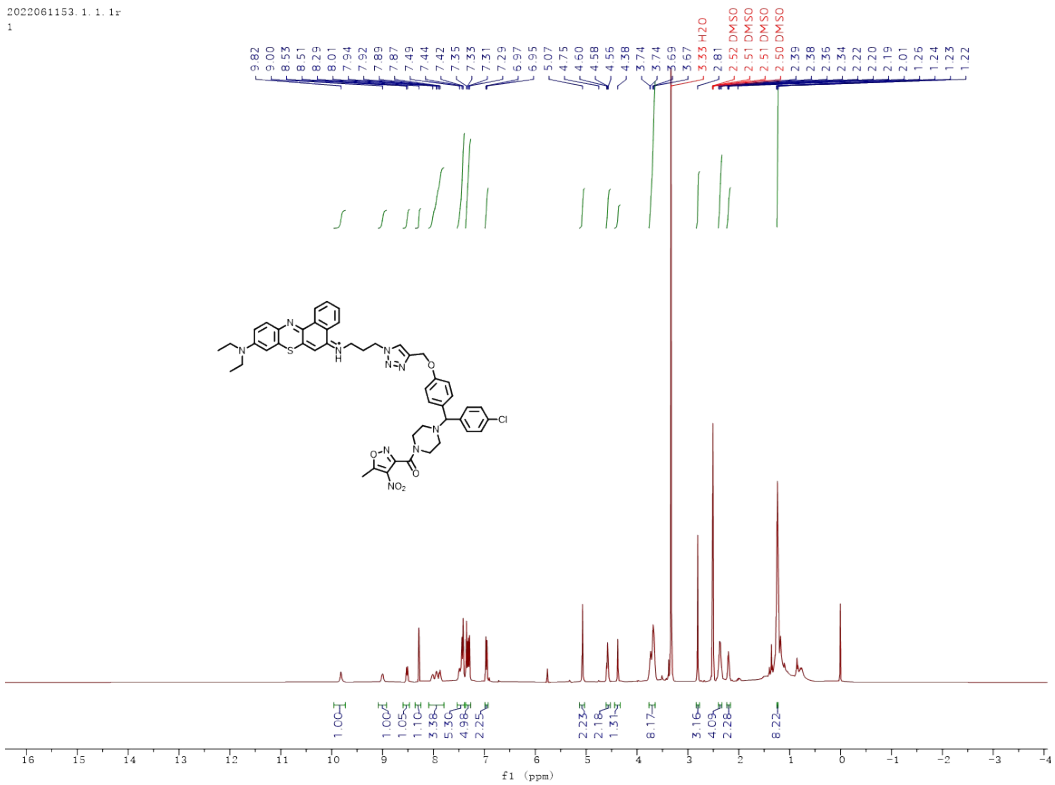

Fig. S5 ^{1}H NMR spectrum of **ENBS-ML210** in DMSO-d_6 (400 MHz).

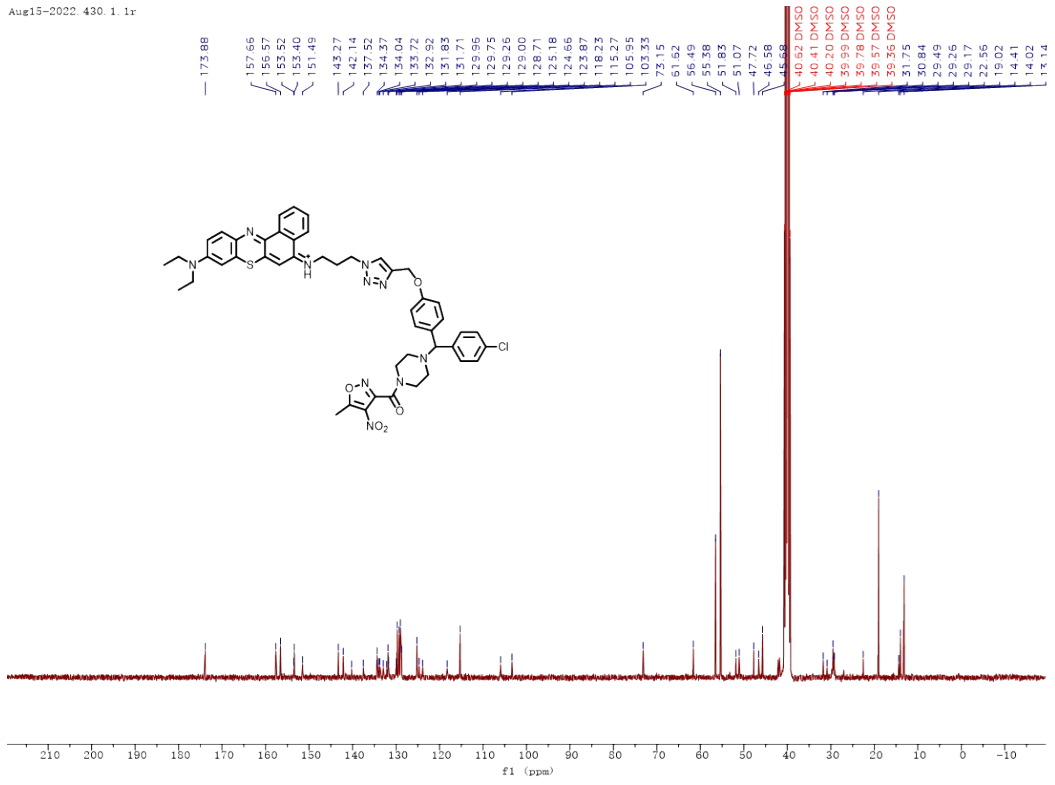

Fig. S6 ^{13}C NMR spectrum of **ENBS-ML210** in DMSO- d_6 (101 MHz).

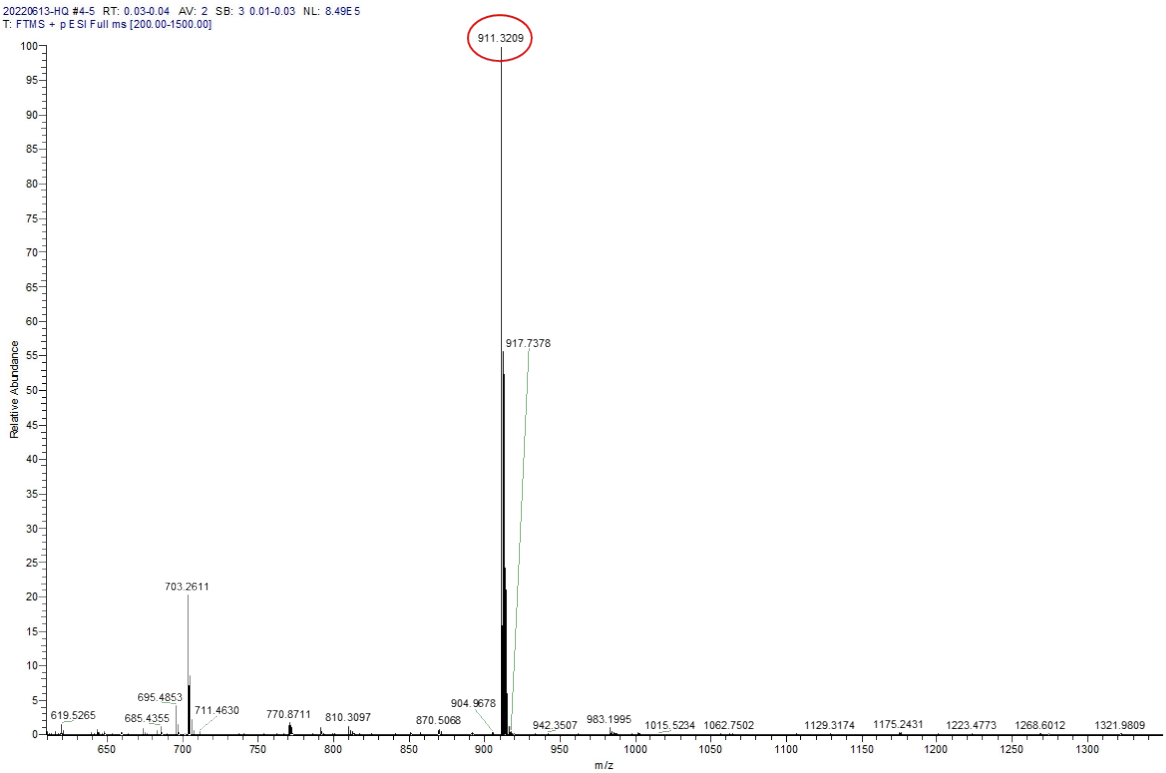

Fig. S7 HRMS (ESI) spectrum of **ENBS-ML210**

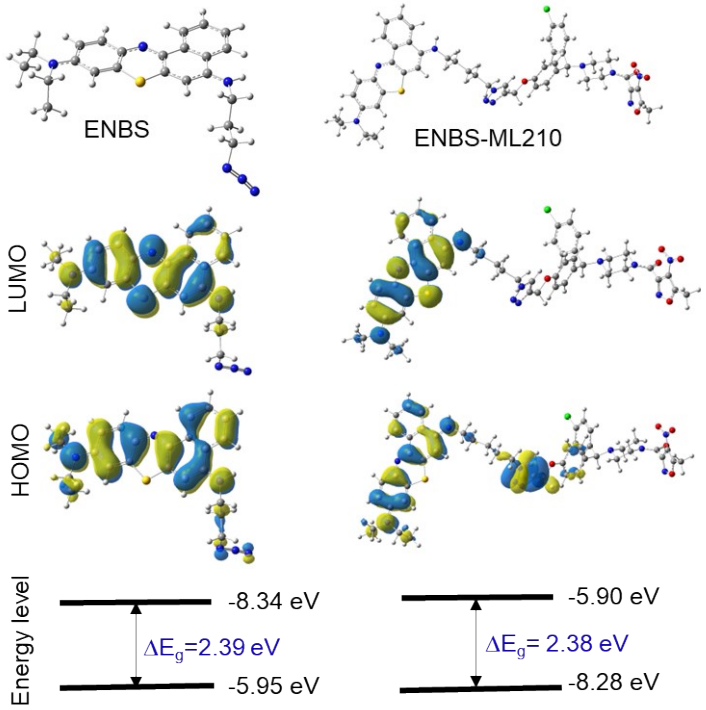

Fig. S8 DFT theoretical simulation of **ENBS** and **ENBS-ML210**.

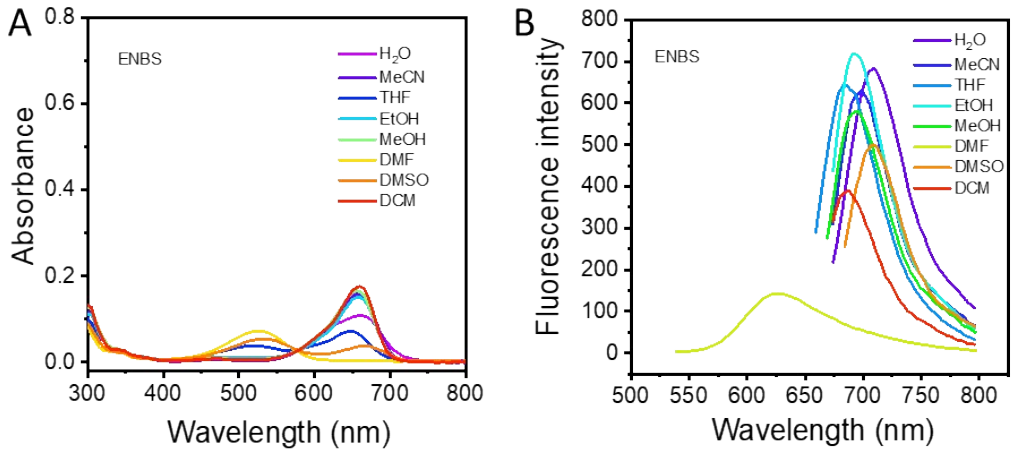

Fig. S9 (A) UV-vis absorption and (B) fluorescence spectra of **ENBS** in different solvents.

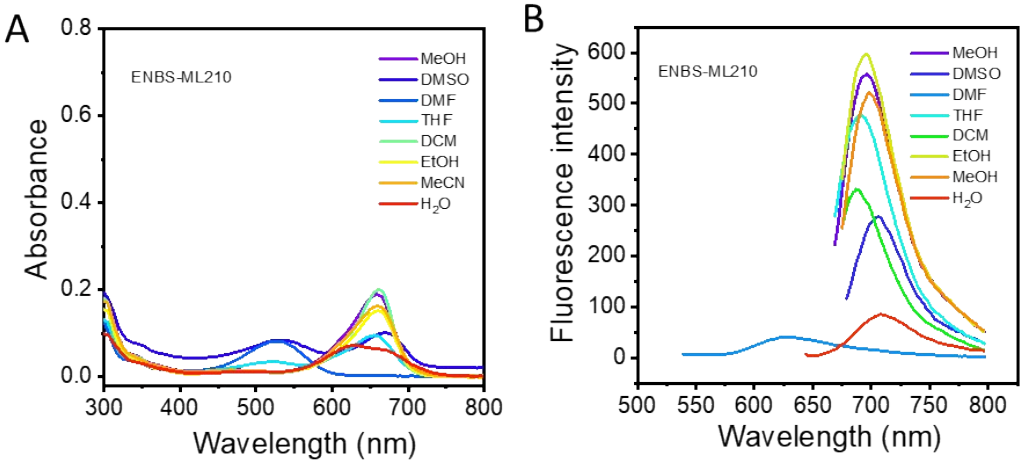

Fig. S10 (A) UV-vis absorption and (B) fluorescence spectra of **ENBS-ML210** in different solvents.

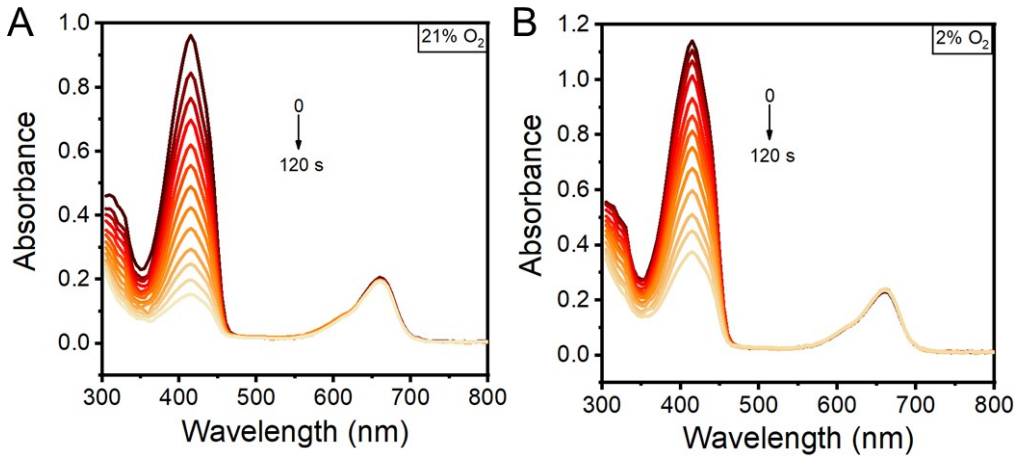

Fig. S11 Photodegradation curves of DPBF with **ENBS-ML210** under (A) 21% O_2 and (B) 2% O_2 conditions.

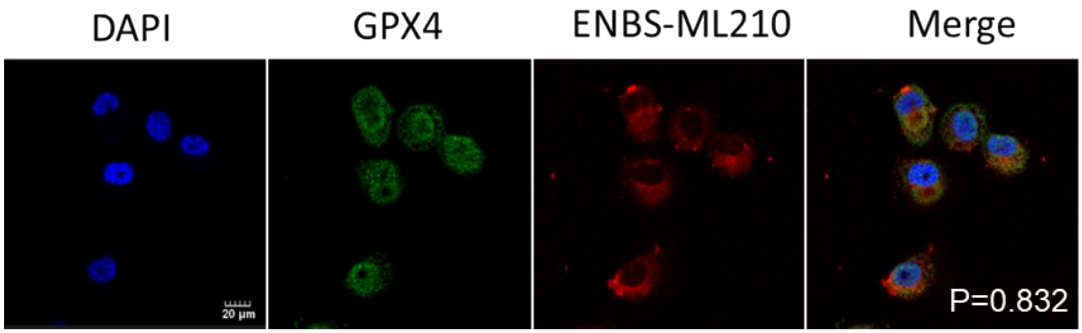

Fig. S12 Immunofluorescence colocalization imaging in H1299 cells. DAPI (blue): λex = 405 nm, λem = 440 nm–480 nm; GPX4 (Alexa fluor 488-labeled secondary antibody, green): λex = 488 nm, λem = 500 nm–550 nm; **ENBS-ML210** (red): λex = 640 nm, λem = 600 nm–700 nm (scale bar = 20 μm).

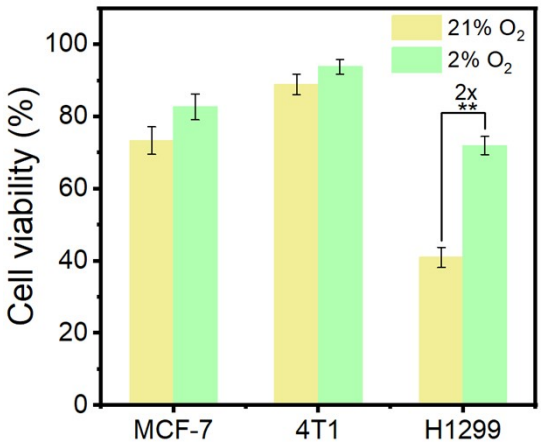

Fig. S13 Comparison of cytotoxicity of 10 μM ML210 in MCF-7, 4T1, and H1299 cells at 21% and 2% O_2 conditions.

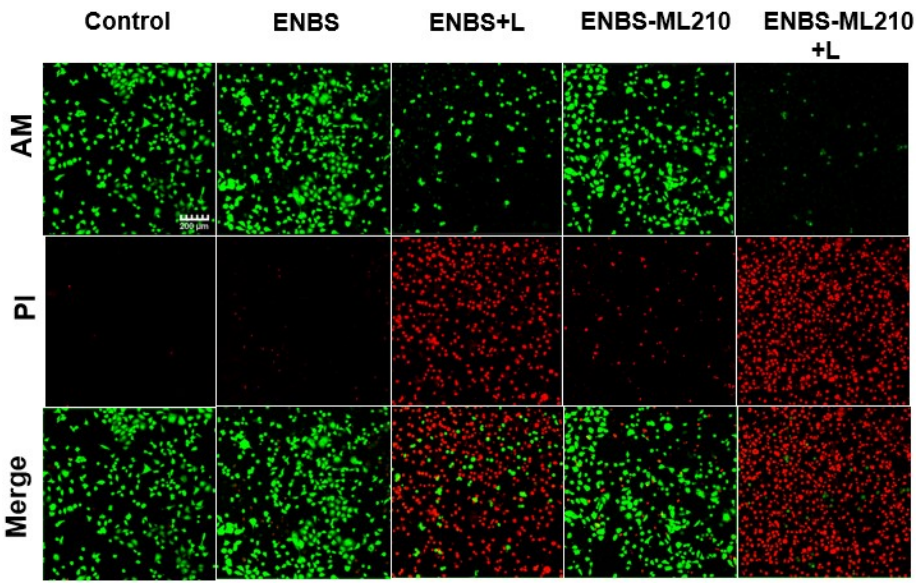

Fig. S14 Live/Dead cell co-staining fluorescence imaging.

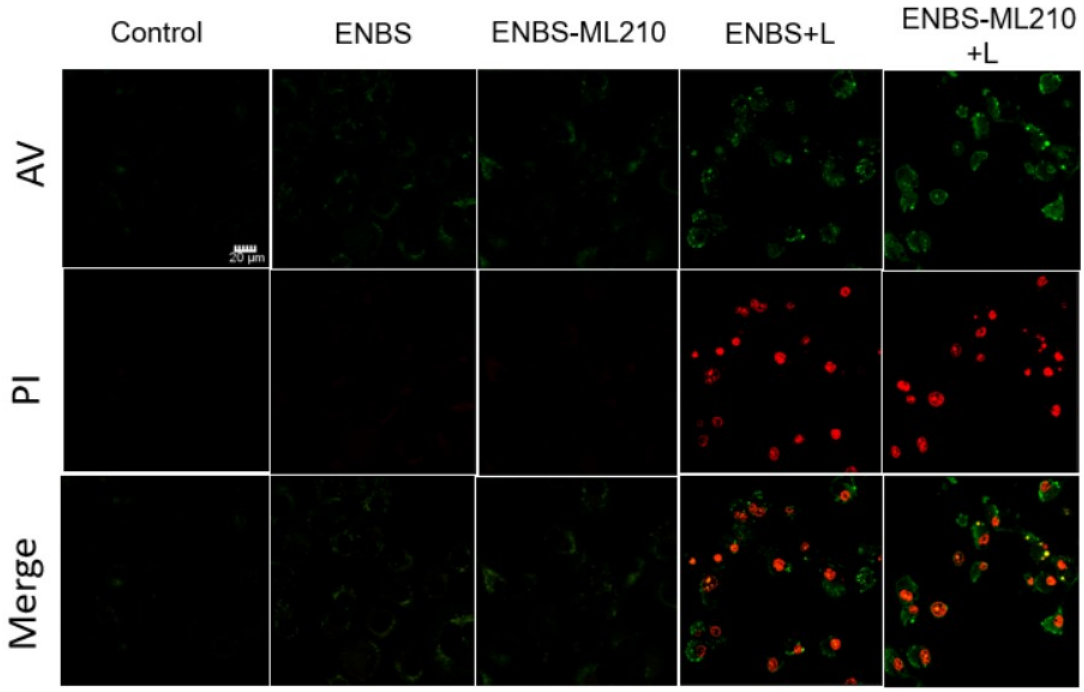

Fig. S15 FITC-Annexin V/PI co-staining for cell apoptosis (scale bar = 20 μm).

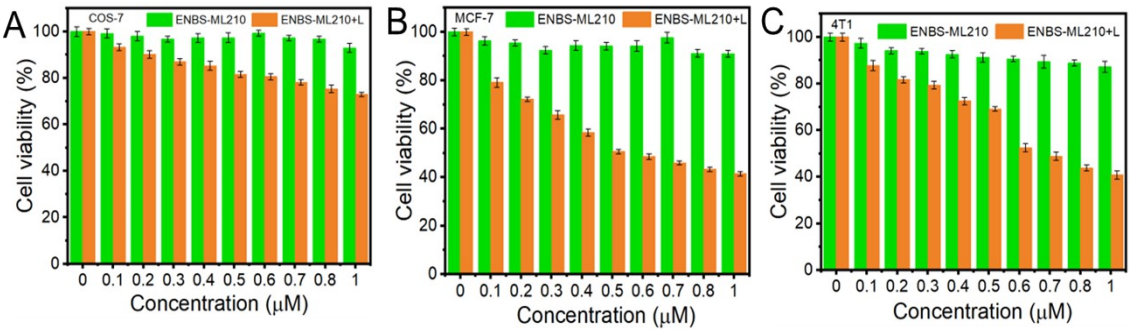

Fig. S16 Cytotoxicity of **ENBS-ML210** in (a) COS-7, (b) MCF-7 and (c) 4T1 cells.

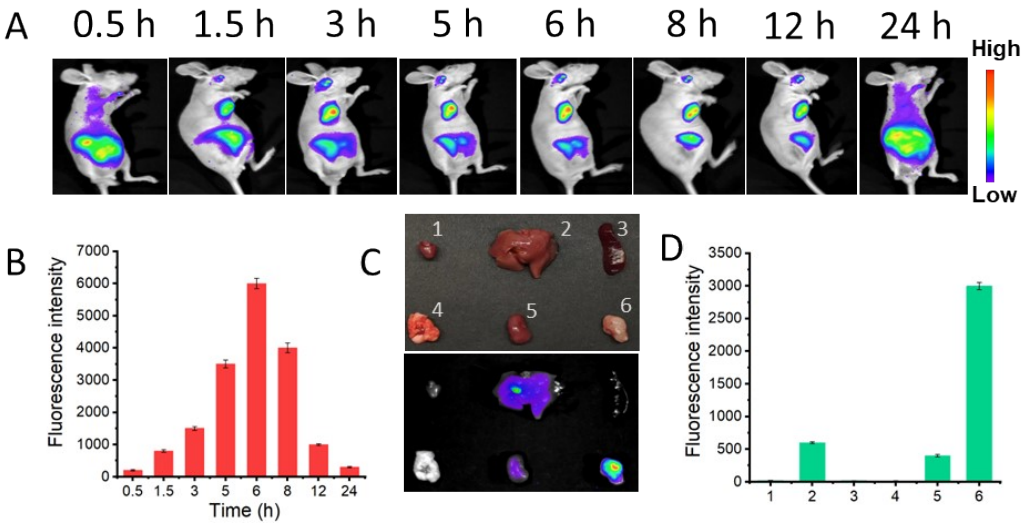

Fig. S17 *In vivo* and *ex vivo* fluorescence imaging of **ENBS-ML210** in H1299-bearing mice. (a) Fluorescence imaging of tumor-bearing mice by tail vein injection of **ENBS-ML210** (λex: 640 ± 20 nm, λem: 700 ± 20 nm). (b) Quantitative analysis of mean fluorescence intensity at the tumor site according to optical imaging at different time. (c) *In vitro* images of organs and tumor. (d) Quantitative analysis of mean fluorescence intensity of the organs at 24 h. (inset:(1) heart, (2) liver, (3) spleen, (4) lung, (5) kidney, and (6) tumor).

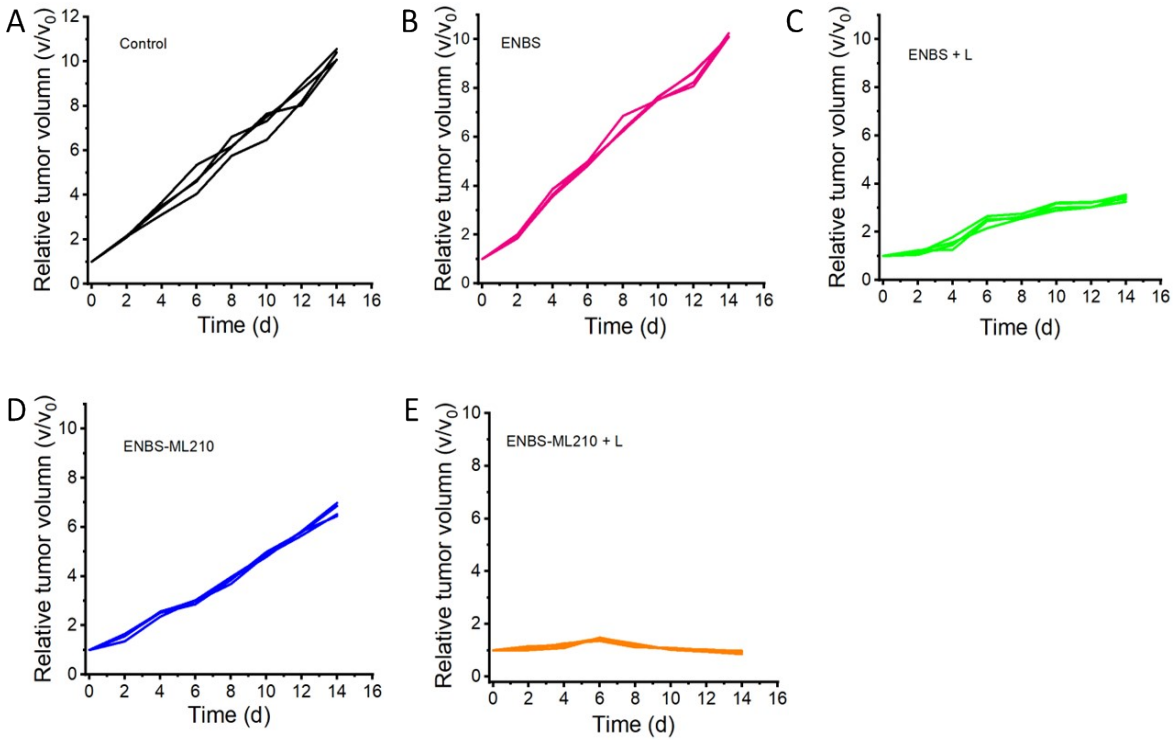

Fig. S18 Tumor growth curve of mice in each group (A) control, (B) **ENBS**, (C) **ENBS**+L, (D) **ENBS-ML210**, and (E) **ENBS-ML210**+L.

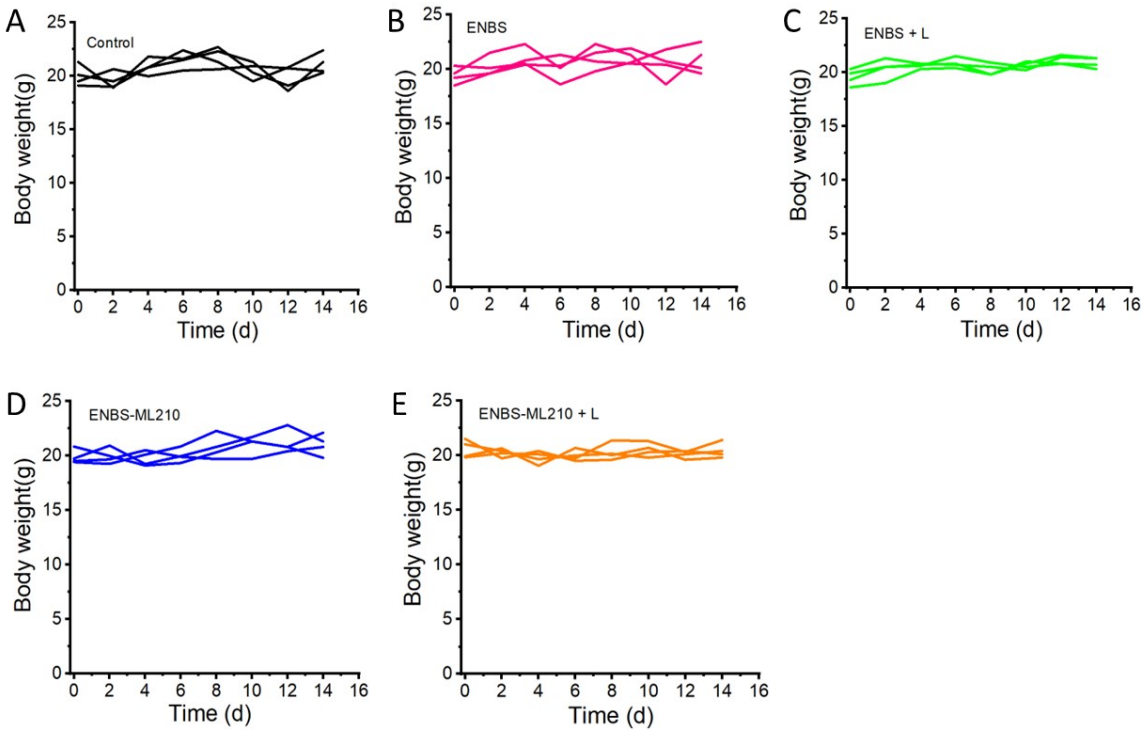

Fig. S19 Body weight growth curve of mice in each group (A) control, (B) **ENBS**, (C) **ENBS**+L, (D) **ENBS-ML210**, and (E) **ENBS-ML210**+L.

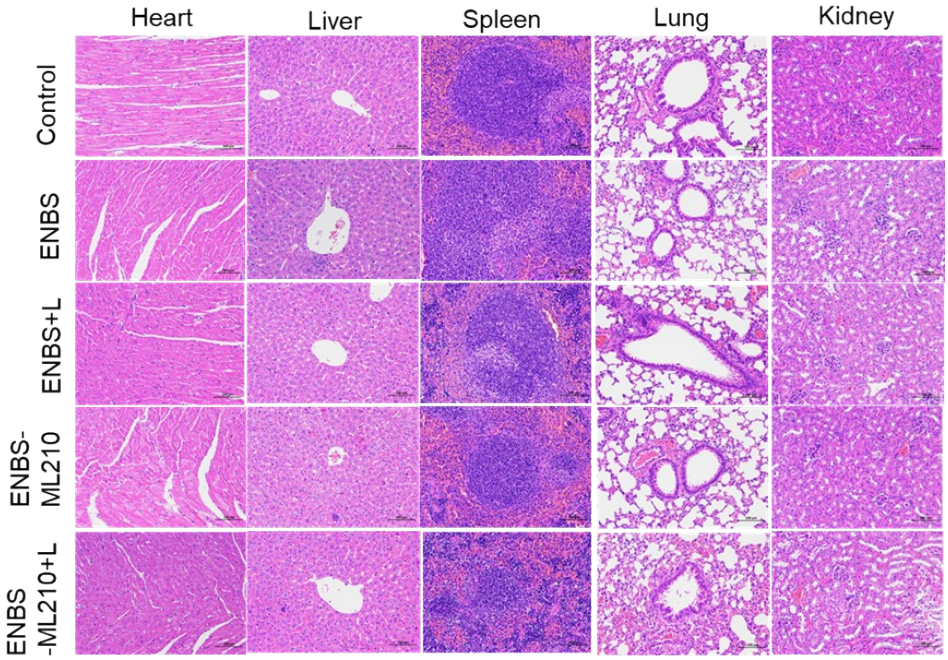

Fig. S20 H&E staining of the major organs dissected from mice at 14 days after various treatments. Scale bar: 100 μm.

References:

1. S. Verma, U. W. Sallum, H. Athar, L. Rosenblum, J. W. Foley and T. Hasan, *Photochem. Photobiol.*, 2009, **85**, 111-118.
2. J. Fan, S. Guo, S. Wang, Y. Kang, Q. Yao, J. Wang, X. Gao, H. Wang, J. Du and X. Peng, *Chem. Commun.*, 2017, **53**, 4857-4860.
3. J. K. Eaton, L. Furst, R. A. Ruberto, D. Moosmayer, A. Hilpmann, M. J. Ryan, K. Zimmermann, L. L. Cai, M. Niehues, V. Badock, A. Kramm, S. Chen, R. C. Hillig, P. A. Clemons, S. Gradl, C. Montagnon, K. E. Lazarski, S. Christian, B. Bajrami, R. Neuhaus, A. L. Eheim, V. S. Viswanathan and S. L. Schreiber, *Nat. Chem. Biol.*, 2020, **16**, 497-506.