Supporting Information

Activatable fluorogenic probe for accurate imaging ulcerative colitis hypoxia *in vivo*

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1. Material , instruments and general experimental procedure

1.1 Material and instruments

All reagents and solvents were purchased commercially and used without further purification. Nile Blue was purchased from Beijing Mreda Technology Co., Ltd (China). Triphosgene, 4-nitrobenzyl alcohol and triethylamine were purchased from Aladdin Company (Shanghai, China). Column chromatography was performed using silica gel (200-300 mesh, Qingdao Ocean Chemicals, Qingdao, China). *Escherichia coli* nitroreductase and nicotinamide adenine dinucleotide phosphate (NADH) were purchased from Sigma-Aldrich (USA). The ultrapure water (18 Ω) used in all experiments was obtained from the Milli-Q system (Millipore, USA).

Spectroscopic data was measured on a FS5 Spectrofluorometer (Edinburgh Instruments Ltd., UK) or SpectraMax M4 by Molecular Device. High-performance liquid chromatography (HPLC) chromatograms were performed on Agilent Infinity 1260 with a C18 column (100 Å, 30 × 4.6 mm). The test conditions are as follows: mobile phase: acetonitrile/H_2O (0.1% CF_3COOH) = 5/95 (*v*/*v*) to 95/5 (*v*/*v*); flow rate: 1 mL·min^{-1}; detection wavelength: 600 nm. Photostability data was measured on a FS5 Spectrofluorometer with an intermittent excitation of xenon lamp (150 W, once per 2 min, 90 times). High-resolution electrospray ionization mass spectrometry (HRMS-ESI) was acquired on an LTQ Orbitrap XL mass spectrometer (Thermo Scientific, USA). ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker Advance spectrometer with TMS as an internal standard. Fluorescence images of the cells were carried out on a Nikon Ti-e confocal laser scanning microscope. The *in vivo* fluorescence images were acquired with the IVIS spectrum system (PerkinElmer, USA). The fluorescence intensity of the region of interest was analyzed by Living Image 4.5 Software.

1.2 Synthesis of probe NB-NO_2

Nile Blue (**NB**) (0.18 g, 0.25 mmol) was dissolved in HPLC grade acetonitrile (10 mL), then triethylamine (34 μL, 0.25 mmol) and triphosgene (0.15 g, 0.50 mmol) dissolved in HPLC grade DCM (10 mL) were slowly added. The reaction mixture was stirred in an ice bath for 0.5 h, and then refluxed at 90°C for 12 h under N_2 protection. After that, 4-nitrobenzyl alcohol (0.075 g, 0.50 mmol) was added to the reaction system. After reacting for 12 h, the mixture was cooled to room temperature and evaporated. The crude residue was then purified by flash column chromatography using an eluting solvent (DCM: CH_3OH=50:1 to 20:1) to afford the desired purple solid **NB-NO_2** (0.04 g, 16%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.65 (d, *J* = 7.8 Hz, 1H), 8.51 (d, *J* = 7.8 Hz, 1H), 8.26 (d, *J* = 8.7 Hz, 2H), 7.71 (d, *J* = 7.1 Hz, 1H), 7.66 (d, *J* = 8.7 Hz, 2H), 7.61 (dd, *J* = 13.4, 8.5 Hz, 2H), 6.71 (s, 2H), 6.42 (d, *J* = 2.4 Hz, 1H), 5.42 (s, 2H), 3.48 (q, *J* = 7.0 Hz, 4H), 1.26 (t, *J* = 6.4 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 163.19, 150.83, 149.68, 148.09, 147.49, 146.61, 143.80, 139.53, 131.60, 130.99, 130.87, 129.66, 128.42, 126.77, 125.40, 123.72, 123.60, 123.51, 110.09, 99.95, 95.98, 66.11, 44.99, 12.43. HR-MS *m/z* ($C_{28}H_{25}N_4O_5^+$) calculated $[M]^+$: 497.1819, found: 497.1820.

1.3 The general procedure for in vitro spectra measurement

All spectroscopic were measured in PBS buffer (10 mM, pH =7.4) containing 500 μM NADH at 37°C. A stock solution of **NB-NO_2** (1 mM) was prepared in HPLC grade DMSO and stored at 4°C. Freshly diluted **NB-NO_2** (10 μM) in PBS buffer in a 10 mm path length quartz cuvette was utilized to perform the *in vitro* measurement. Other analytes were prepared by dissolving the relevant analytes with the same PBS buffer or distilled water to the final concentrations, including NaCl, $MgCl_2$, $CaCl_2$, NaHS, Cys, GSH, H_2O_2, Vitamin C (1 mM respectively), Leucine aminopeptidase (LAP), Alkaline

phosphatase (ALP), β-Galactosidase (β-Gal) (10 U/mL in distilled water respectively) and *Escherichia coli* Nitroreductase (NTR) (10 μg/mL in distilled water). λ_{ex} = 590 nm, λ_{em} =670 nm.

Cell culture, cytotoxicity assay and cell imaging

Human lung carcinoma (A549) cell line was purchased from the Cell Resource Centre, Peking Union Medical College. The cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 culture medium containing 10% (*v*/*v*) FBS (Gibco, Thermo Fisher Scientific Company, USA) and 1% (*v*/*v*) penicillin-streptomycin (Gibco, Thermo Fisher Scientific Company, USA) at 37°C in a 5% CO_2 humidified atmosphere.

The cytotoxicity of **NB-NO_2** to A549 cells was measured by using the standard Cell Counting Kit-8 system (CCK-8) assays. A549 cells were seeded in a 96-well plate at the same density of 1.0×10^4 cells/well and cultured for 24 h. Then, various concentrations of **NB-NO_2** (0-50 μM) were added to 96-well plate and incubated for another 24 h. After that, the medium was removed, and the cells were washed 3 times with fresh PBS. The cells were then treated with 100 μL of CCK-8 solution and incubated for 1 h. The absorption at 450 nm was measured and the cytotoxicity was reported as mean±standard.

The anaerobic and microaerobic packs from Mitsubishi Gas Chemical Company, Inc. (Japan) were used for confocal imaging tests in A549 cells under different oxygen conditions. Cells (normal oxygen state) were cultured in the constant temperature incubator containing 5% CO_2 and 21% oxygen. The hypoxic or microaerobic cells were cultivated overnight in a constant temperature incubator under an anaerobic or microaerobic atmosphere to simulate different degrees of hypoxic conditions.

For cell fluorescence imaging, A549 cells were plated in a glass-bottom confocal

dish for 24 h before the experiment. Cell fluorescence imaging of **NB-NO_2** in A549 cells were divided into 3 groups: normoxic, microaerobic and hypoxic. After the cells were incubated with **NB-NO_2** probe for 1 h, the three groups of cells were transferred to different hypoxic conditions and incubated for another 12 h. The cells of the normoxic group were cultured under normal conditions (37℃, 5% CO_2 and 21% O_2), while the microaerobic/hypoxic cells were cultivated at 37℃ in anaerobic and microaerobic packs to simulate the microaerobic and hypoxic condition. Fluorescence images of the cells were captured by a Nikon Ti-e microscope (λ_{ex} = 635 nm, λ_{em} = 663-738 nm).

1.4 In vivo fluorescence imaging of A549-bearing xenograft model

All animal procedures were performed in accordance with the Ethics Committee of Beijing University of Technology, China. Female BALB/c mice (body weight, around 18-20 g) were purchased from Beijing HFK Bioscience Co., Ltd., and maintained under standard conditions. The A549-bearing xenograft model was obtained by subcutaneously injecting A549 cells into the lower limbs of mice for 2 weeks. When the tumor grew to an appropriate size, the mouse was used for *in vivo* fluorescence imaging. The mice were anesthetized by 2 % isoflurane delivered via a nose cone during the experiments. After mice were injected intratumorally with the probe (100 μM, 50 μL), they were placed in the IVIS system for imaging at different time points (0, 30, 60, 90, 120, 180, 240 min). Data was analyzed with the Living Image 4.5 Software and ROIs of equal area in the regions of interest were obtained for further analysis. The excitation wavelength of the IVIS system is 640 nm, and the emission wavelength is 680-700 nm in the meanwhile.

1.5 In vivo fluorescence imaging of DSS-induced ulcerative colitis model

For *in vivo* imaging of (dextran sulfate sodium) DSS-induced ulcerative colitis,

female BALB/c mice were divided into 2 groups (n = 3). For the DSS group, the mice were fed 3.5% DSS solution for 7 days. The mice in the control group were kept drinking distilled water for 7 days. On day 7, all mice were injected intravenously with **NB-NO_2** (200 μM, 50 μL). Subsequently, the mice were optically imaged by the IVIS imaging system at designated time point (0, 0.5, 1, 1.5, 2 and 3 h). The mice were sacrificed at 3 h after probe administration, and the major organs (heart, liver, spleen, lung, kidney and colon) were excised for *ex vivo* imaging. The excitation wavelength of the IVIS system is 640 nm, and the emission wavelength is 680-700 nm in the meanwhile.

1.6 Histology

After *in vivo* fluorescence imaging, colons of 2 groups mice were collected, washed with normal saline and fixed in 10% formaldehyde solution for paraffin embedding. Then the embedded colons were sectioned and the tissue slices were stained with hematoxylin and eosin (H&E) for histological examination.

1.7 Determination of the fluorescence quantum yield

Quantum yields for all the fluorescent compounds were measured by dividing the integrated emission area of their fluorescent spectrum against the area of Rhodamine B in Ethanol excited at 540 nm ($\Phi_{\text{Rhodamine B}}$ = 0.71). Quantum yields where then calculated using equation (1), where F represents the integrated emission area of fluorescent spectrum, and Abs represents absorbance at excitation wavelength selected for standards and samples. Emission was integrated from 565 nm to 720 nm.

$$\Phi_{flu}^{sample} = \Phi_{flu}^{reference}\left(\frac{F^{sample}}{F^{reference}}\right)\left(\frac{\eta^{sample}}{\eta^{reference}}\right)\left(\frac{Abs^{reference}}{Abs^{sample}}\right) \quad (1)$$

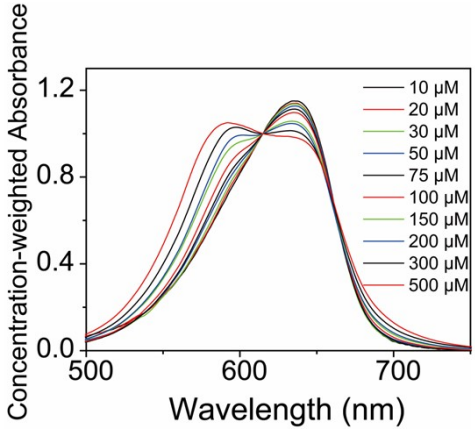

Figure S1 H-type aggregation and concentration-weighted absorbance spectra of **NB** measured at different concentrations between 10-500 μM in H_2O.

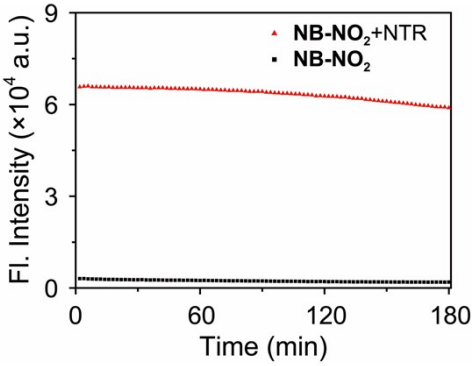

Figure S2 Time dependent fluorescence change of **NB-NO$_2$** (10 μM) before and after adding NTR, fluorescence intensity of **NB-NO$_2$** and corresponding product were monitored at 680 nm.

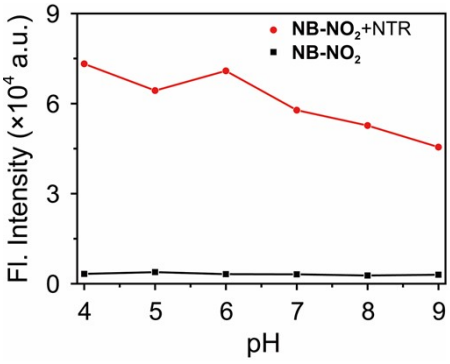

Figure S3 pH-dependent fluorescence intensity changes of **NB-NO$_2$** in the absence and presence of NTR.

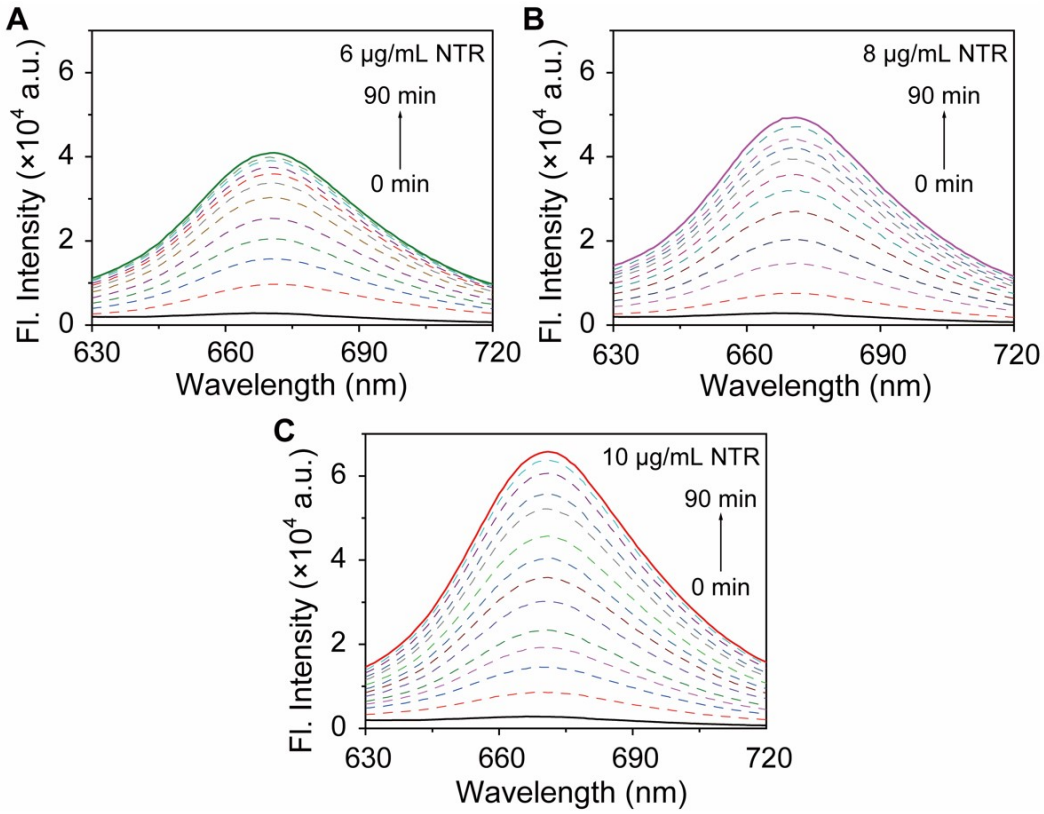

Figure S4 The fluorescence changes of **NB-NO$_2$** (10 μM) to different concentrations of NTR (6, 8, 10 μg/mL).

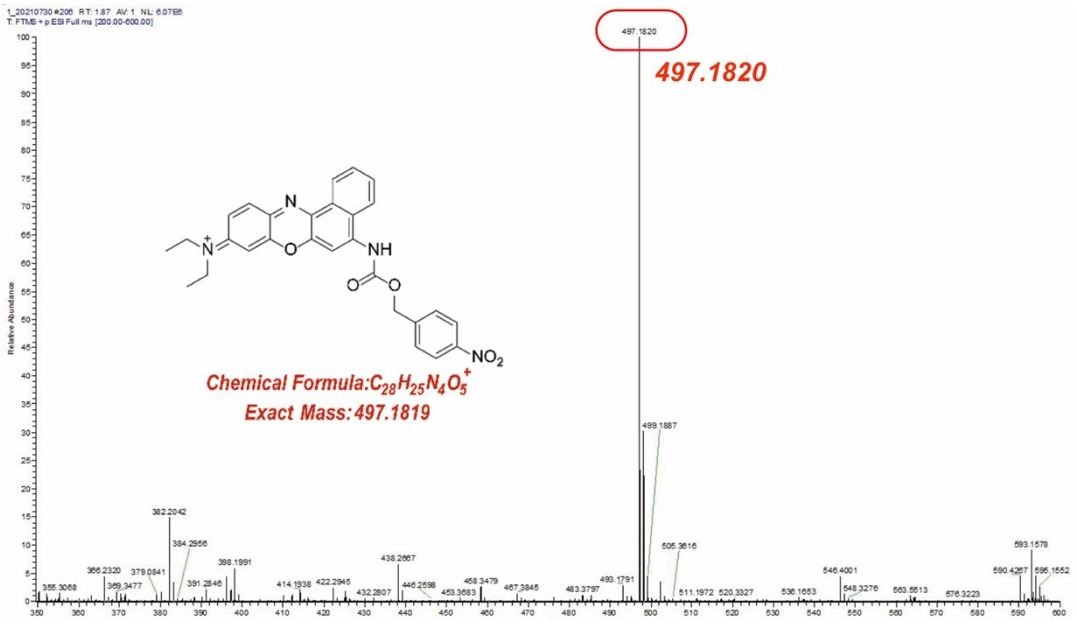

Figure S5 HRMS spectrum of **NB-NO$_2$.**

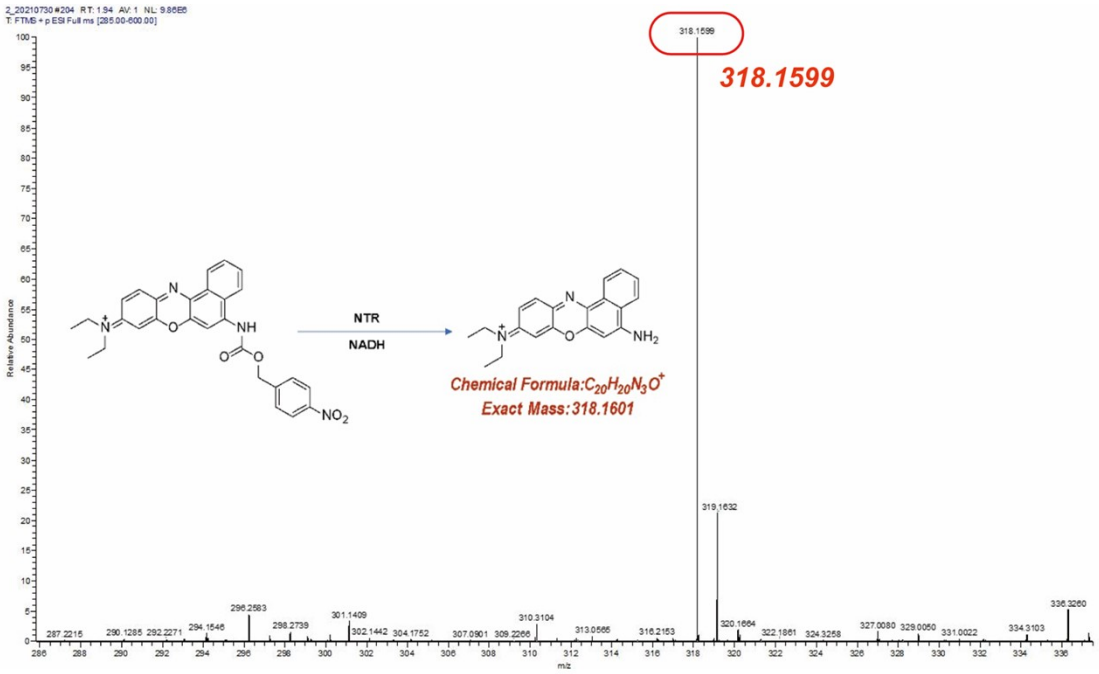

Figure S6 HRMS spectrum of the reaction solution of **NB-NO_2** with NTR.

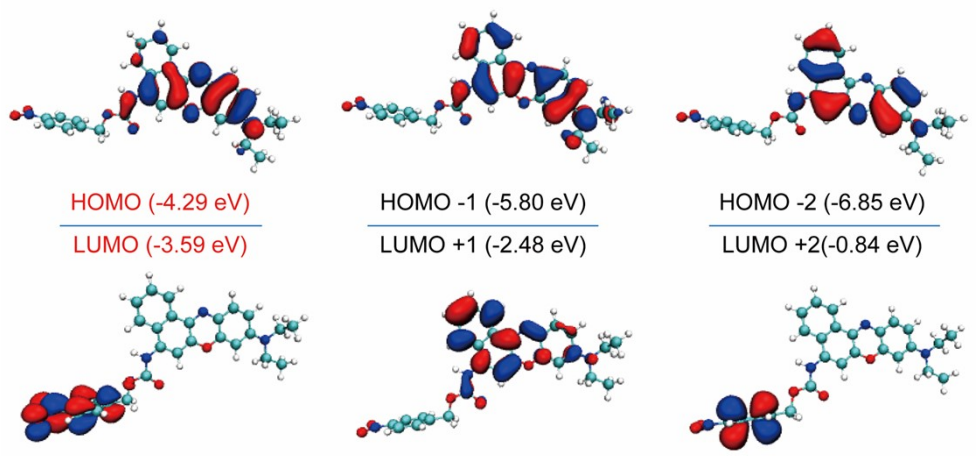

Figure S7 The frontier molecular orbitals (MOs) of **NB-NO_2**.

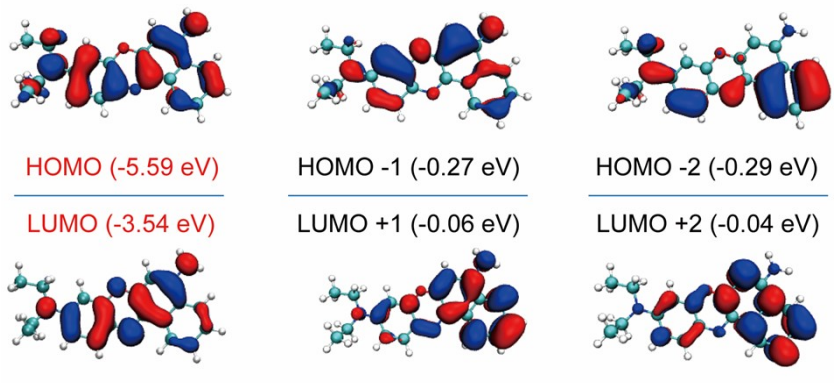

Figure S8 The frontier molecular orbitals (MOs) of **NB**.

	TDDFT//PBE/6-31G(d)			
	Energy[a]	***f***[b]	**Composition**[c]	**CI**[d]
NB	2.0503 eV (604.72 nm)	1.2097	H→L	0.70642
	2.9473 eV (420.67 nm)	0.0039	H-1→L	0.68722
	3.5406 eV (350.18 nm)	0.0002	H-2→L	0.65736
NB-NO_2	0.6966 eV (1779.86 nm)	0.0000	H→L	0.99905
	1.8097 eV (685.10 nm)	0.0024	H→L+1	0.91977
	2.2172 eV (559.20 nm)	0.6578	H-2→L	0.92382

Table S1 Selected parameters for the vertical excitation (UV-vis absorptions) of the compounds. Electronic excitation energies (eV) and oscillator strengths (f), configurations of the low-lying excited status of the **NB** and **NB-NO_2**. Calculated by TDDFT//PBE/6-31G(d), based on the optimized ground state geometries.

[a] The selected excited states were measured. The number in parentheses are the excitation energy in wavelength.

[b] Oscillator strength.

[c] H means the HOMO and L means the LUMO.

[d] Coefficient of the wavefunction for each excitation.

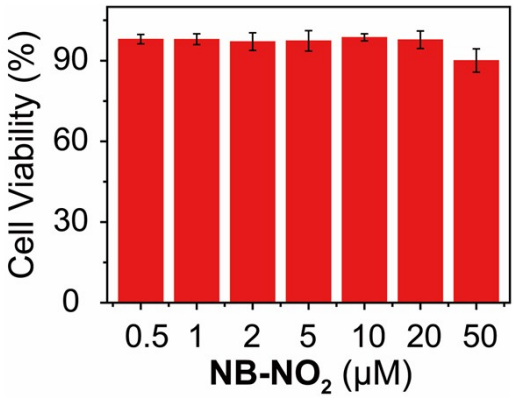

Figure S9 Cytotoxicity of **NB-NO_2** for A549 cells. Cells were incubated with the probe at corresponding concentrations for 24 h. Cell viability was measured by MTT assay and the results are reported as percentage relative to untreated cells (mean ± SD).

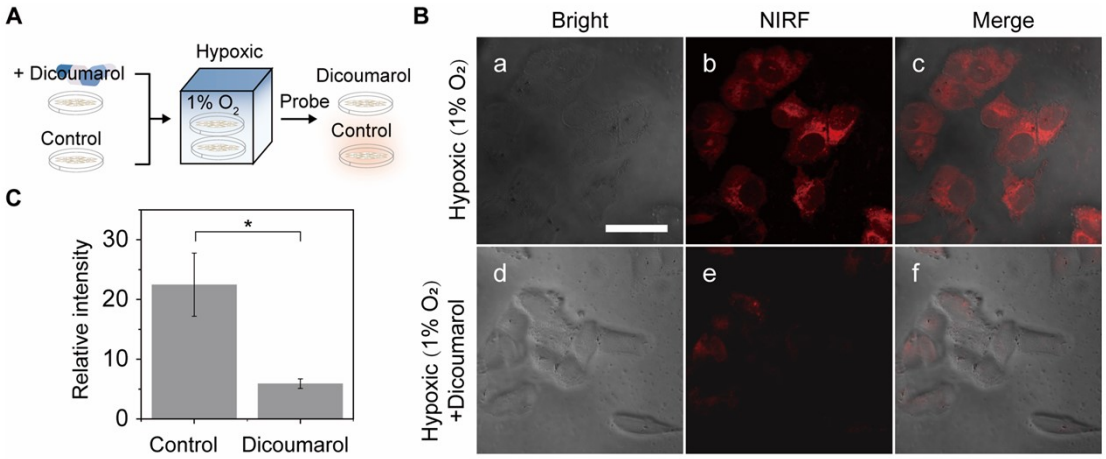

Figure S10 (A) Imaging schematic and (B) Fluorescence imaging of A549 cells under hypoxic (1% O_2, a-c) and hypoxic with 100 μM dicoumarol (1% O_2, d-f). (C) Relative fluorescence intensity of the corresponding fluorescence images in panel B. λ_{ex} = 635 nm, λ_{em} = 663-738 nm. Scale bar: 50 μm.

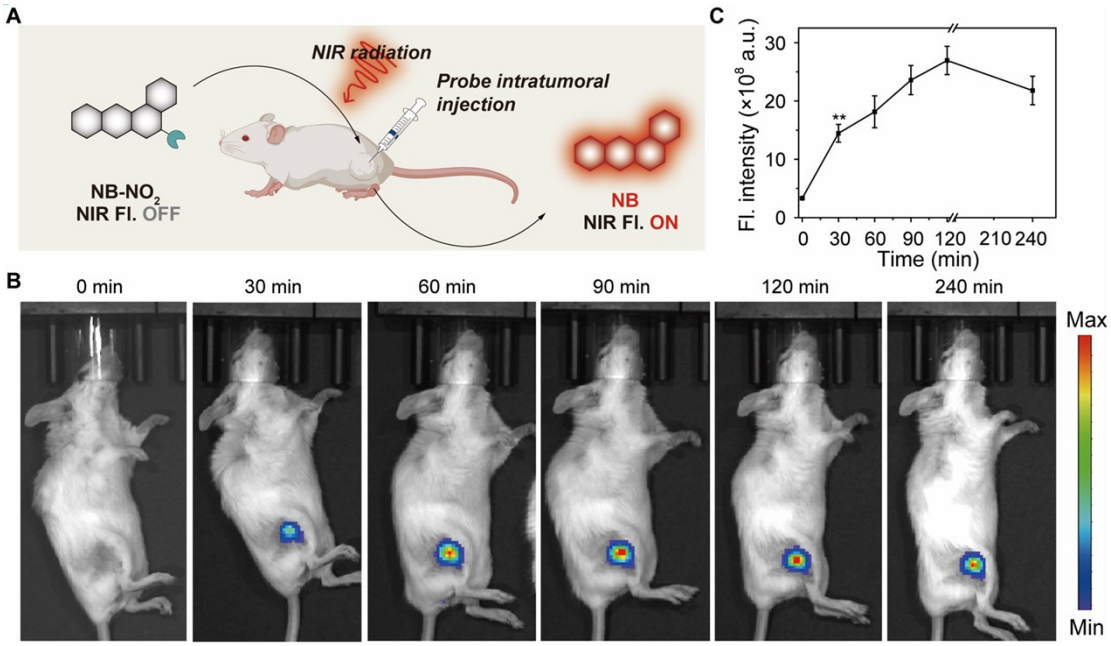

Figure S11 (A) Schematic and (B) time-dependent in vivo fluorescence images of A549 tumor-bearing mice after intratumoral injection of **NB-NO_2** (100 μM, 50 μL). (C) Quantification of the fluorescence signal in B. λ_{ex} = 640 nm, λ_{em} = 680-700 nm.

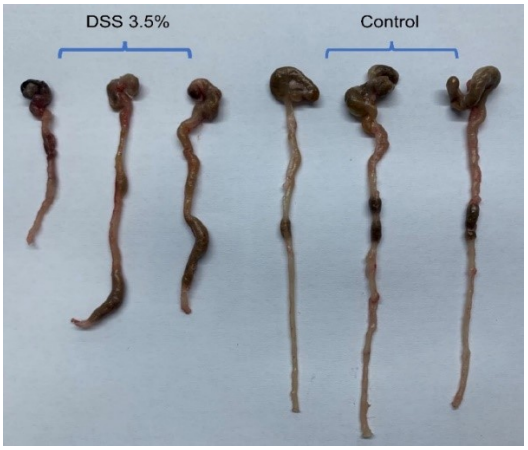

Figure S12 Difference of colonic length of the mice in two group after 7 days of DSS- or water treated.

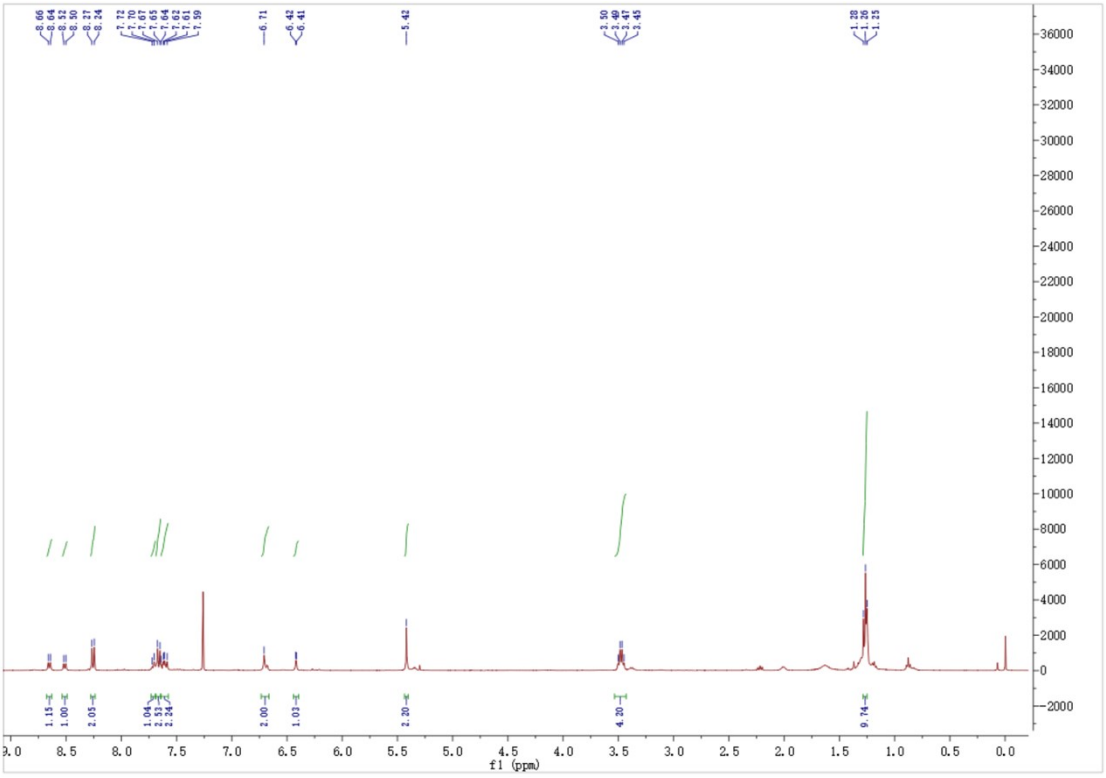

Figure S13 ^{1}H NMR spectrum of **NB-NO_2** in $CDCl_3$.

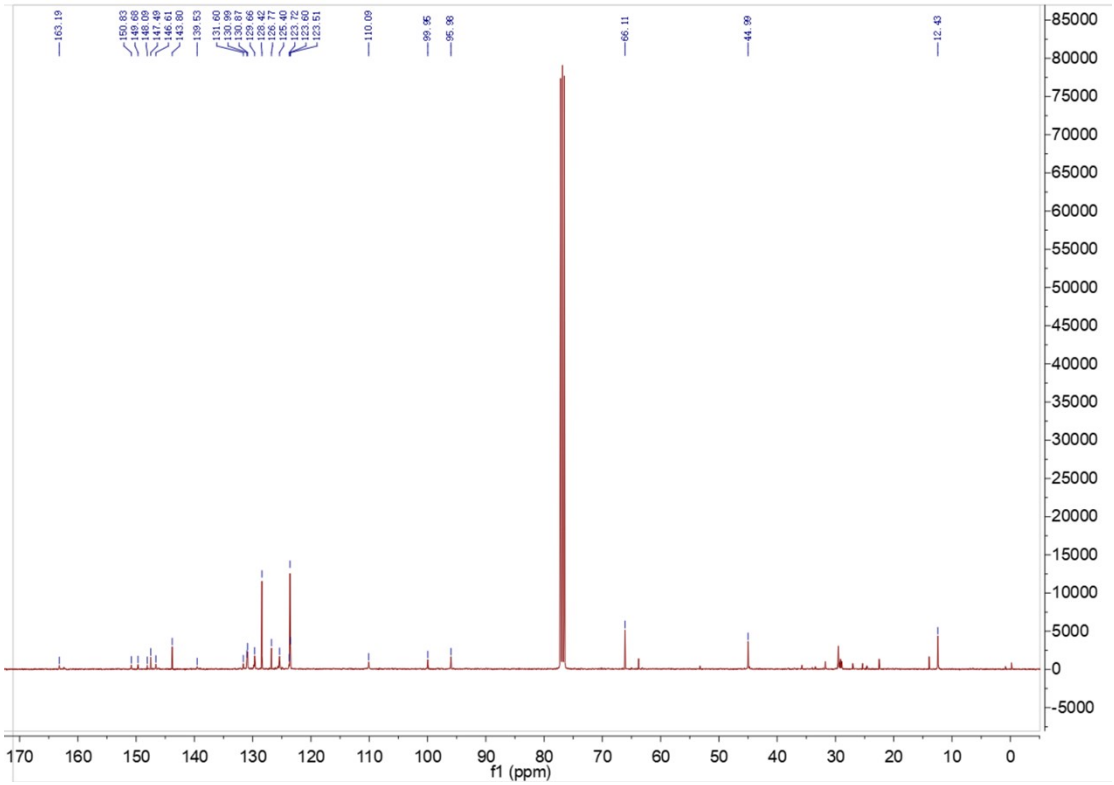

Figure S14 ^{13}C NMR spectrum of **NB-NO_2** in $CDCl_3$.