Electronic Supplementary Information

Monitoring mitochondrial nitroreductase activity in tumors and a hind-limb model of ischemia in mice using a novel activatable NIR fluorescent probe

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1. Materials

All chemical reagents can be bought in the market and can be used without further purification. Nitroreductase from Escherichia coli and NADH purchased from Sigma-Aldrich. In order to keep the enzyme activity unchanged as far as possible, a milligram of nitroreductase freeze-dried powder was dissolved in a milliliter of ultrapure water, packed into 20 parts, and immediately cryopreservation at 20 degrees celsius. Only part of the thaw was used in each experiment. Configured 0.5 mM NADH solution with HEPES (10 mM pH=7.4) before each spectral experiment.

2. Instrumentation

^{1}H NMR and ^{13}C NMR spectra were characterized by Bruker 500 MHz, electrospray ionization mass spectra (ESI-MS) were recorded in LTQ mass spectrometry.

3. Syntheses and characterizations

Synthesis of 2. Compound 2 was synthesized according to the previous literature [1].

HRMS (ESI, *m/z*): $[M]^+$ calcd. for $C_{24}H_{26}NO_3^+$: 376.1907, found: 376.1924

Synthesis of 1. Synthesis of 4-(4-Nitro-benzyloxy)-benzaldehyde(1). To a stirred solution of 4-hydroxy benzaldehyde (169.6mg, 1.39mmol) in N,N-dimethylformamide (5 ml) at room temperature was added potassium carbonate (480mg, 3.44mmol) and 4-nitrobenzyl bromide (300mg , 1.39mmol). Reaction mixture was heated at 60 °C for 2 h. Reaction mixture was cooled to room temperature and poured in cold water (15 ml). The precipitated solid was filtered, washed with cold water and dried under vacuo, Compound 1 was received as off-white solid (325.9 mg, 1.27mmol), yield: 91.23%. HRMS (ESI, *m/z*): $[M+H]^+$ calcd. for $C_{14}H_{12}NO_4^+$: 258.0766, found: 258.0760.

Synthesis of CS-OH. Compound 4-hydroxybenzaldehyde (181.4mg, 1.49 mmol) and compound 2 (508.2mg, 1.07 mmol), acetic acid (15 mL). The mixture was heated to 90 °C for 24 h, and the solvent was removed by the evaporation under the reduced pressure. The crude product was purified by silica gel chromatography (CH_2Cl_2/ MeOH = 20:1), **CS-OH** was received as dark purple solid (433.6 mg, 0.75 mmol), yield: 70%. ^{1}H NMR (500 MHz, MeOD) 8.26 (dd, *J* = 7.6, 1.6 Hz, 1H), 8.15 (s, 1H),

7.78 (td, *J* = 7.6, 1.5 Hz, 1H), 7.71 (td, *J* = 7.6, 1.4 Hz, 1H), 7.60 (s, 1H), 7.58 (d, *J* = 2.2 Hz, 1H), 7.28 (dd, *J* = 7.5, 1.5 Hz, 1H), 7.22 (d, *J* = 2.3 Hz, 1H), 7.19 (dd, *J* = 9.4, 2.4 Hz, 1H), 7.10 (d, *J* = 9.4 Hz, 1H), 6.92 (d, *J* = 2.1 Hz, 1H), 6.90 (d, *J* = 2.1 Hz, 1H), 3.70 (q, *J* = 7.2 Hz, 4H), 2.98 (m, 2H), 2.43 (t, *J* = 6.2 Hz, 2H), 1.83 (m, 2H), 1.32 (t, *J* = 7.1 Hz, 6H). ^{13}C NMR (126 MHz, DMSO-d$_6$) δ 166.63, 158.98, 147.07, 144.57, 133.30, 132.71, 130.21, 129.46, 129.34, 128.55, 128.29, 126.88, 123.64, 115.21, 95.61, 68.21, 45.27, 43.98, 40.42, 40.10, 26.45, 25.09, 21.08, 12.47. HRMS (ESI, *m/z*): $[M]^+$ calcd. for $C_{31}H_{30}NO_4^+$: 480.2169, found: 480.2177.

Synthesis of CS-NO$_2$. Compound 1 (254.7mg, 0.99 mmol) and compound 2 (338.8g, 0.71 mmol), acetic acid (15 mL). The mixture was heated to 90 ^{o}C and stirred overnight, the solvent was removed by the evaporation under the reduced pressure. The crude product was purified by silica gel chromatography (CH_2Cl_2/ MeOH = 20:1), **CS-NO$_2$** was received as dark purple solid (98.9 mg, 0.14 mmol), yield: 20%. ^{1}H NMR (500 MHz, DMSO-d$_6$) 13.31 (s, 1H), 8.28 (dd, *J* = 8.7, 1.7 Hz, 2H), 8.19 (d, *J* = 7.9 Hz, 1H), 8.08 (s, 1H), 7.87 (t, *J* = 7.6 Hz, 1H), 7.77 (t, *J* = 8.4 Hz, 3H), 7.67 (d, *J* = 8.2 Hz, 2H), 7.39 (d, *J* = 7.7 Hz, 1H), 7.26 (s, 1H), 7.20 (d, *J* = 7.3 Hz, 3H), 6.92 (d, *J* = 9.5 Hz, 1H), 5.39 (s, 2H), 3.65 (d, *J* = 7.3 Hz, 4H), 3.46 (dd, *J* = 7.0, 1.7 Hz, 1H), 3.43 (dd, *J* = 6.9, 1.6 Hz, 1H), 1.77 (d, *J* = 6.5 Hz, 1H), 1.69 (d, *J* = 6.6 Hz, 1H), 1.23 (t, *J* = 7.1 Hz, 6H), 1.06 (td, *J* = 7.0, 1.7 Hz, 2H). ^{13}C NMR (126 MHz, DMSO-d$_6$) δ 166.69, 158.93, 147.06, 144.58, 133.36, 132.66, 130.21, 129.42, 129.27, 128.57, 128.29, 126.92, 123.64, 115.19, 95.64, 68.21, 55.99, 45.23, 40.01, 39.93, 39.76, 26.45, 25.03, 21.10, 18.52, 12.47. HRMS (ESI, *m/z*) $[M]^+$ calcd. for $C_{31}H_{30}NO_4^+$: 615.2490, found: 615.2492.

4. General procedure for in vitro detection

Therefore, unless otherwise specified, all the spectral measurements were performed in HEPES (10 mM, pH = 7.4): DMSO = 9:1 solution. A certain amount of probe **CS-NO$_2$** was dissolved in DMSO and configured it to 1 mM stock solution. Preserved at 4 °C and dilute to required concentration when used. All the UV-vis spectra and fluorescence spectra were measured with the following procedure. In a 3 mL tube, 30 μL **CS-NO$_2$** stock solution and 300 μL NADH were mixed, and then an

appropriate volume of nitroreductase solution was added. The final solution volume was adjusted to 3 mL with appropriate volume of HEPES (10 mM, pH = 7.4) and DMSO. The solution was quickly mixed, incubated in a constant temperature incubator at 37 °C, and then transferred to a 3 mL quartz colorimetric dish to measure the absorbance or fluorescence spectrum.

5. Selective test

The probe was incubated with the following different substances at 37°C for one hour, and the fluorescence intensity at 670 nm was measured: NaCl (50 mM); KCl (50 mM); $CaCl_2$ (50 mM); $MgCl_2$ (50 mM); Na_2SO_3 (50 mM); Vc (1 mM); GSH (10 mM); fructose (10 mM); glucose (10 mM); galactose (10 mM); Arg (1 mM); Lys (1 mM); Cys (1 mM); Leu (1 mM); Phe (1 mM); His (1 mM); H_2O_2 (1 mM); H_2S (1 mM); $ONOO^-$ (0.1 mM); NADH (0.5 mM) + NTR (10 μg/mL)

6. The test of fluorescence quantum yield

A solution of DMSO: HEPES=1:9 was configured as a blank solution, and the absolute quantum yield of **CS-NO_2** and **CS-OH** at a concentration of 10 μmol/L was measured by the absolute fluorescence quantum yield instrument.

7. Cell culture and cytotoxicity assay

4T1 cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 incubator within 1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin-streptomycin. MCF-7 cells were cultured in DMEM with 10% (v/v) FBS and 1% (v/v) penicillin-streptomycin in a similar manner.

The cytotoxicity was tested by the MTT assay. In brief, 4T1 cells were seeded in a 96-well plate at a density of 1×10^5 cells per well, followed by an incubation with different concentrations of **CS-NO_2** at 37°C for 24 h, and set up blank group without probe. Next, MTT solution (0.5 mg/mL) was added to each well for incubation for another 4 h. The supernatant was removed, and DMSO (100 μL) was added to dissolve the formed formazan. The 96-well plate was gently shaken for 10 min, and the absorbance values were measured at 570 nm and 630 nm with a microplate reader. The cell survival rate of each group was calculated according to the following formula:

Cells viability (%) = $(OD_{dye}-OD_{Kdye})/(OD_{control}-OD_{Kcontrol}) \times 100\%$

In the above formula, OD_{dye} refers to the absorbance value of the dye molecular pore, OD_{Kdye} refers to the blank absorbance value of the dye molecular pore, $OD_{control}$ refers to the absorbance value of the control pore, and $OD_{Kcontrol}$ refers to the absorbance value of the control pore blank. The cytotoxicity for MCF-7 cells was evaluated by a similar procedure.

8. Confocal fluorescence imaging for cells

4T1 (mouse breast cancer cells) and MCF-7 (human breast cancer cells) were seeded in a confocal culture dish and incubated overnight for adherence. Then replace fresh medium, the cells were then kept under normoxic (20% O_2) or hypoxic conditions (2% O_2), generated for another 8 h at 37 °C. After culture, the probe solution was added to the confocal dish under two conditions to make its final concentration 5μM. The cells without probes were set as the blank control group, and the cells with nitroreductase inhibitors were set as the negative control group. After 30 min of culture under two conditions, the cells were removed from the incubator, and the cell culture medium was discarded. The cells were rinsed with PBS for three times. Finally, fluorescence photographs of cells cultured under two conditions were taken by laser confocal microscope. The channel for **CS-NO_2** was excitation at 559 nm and emission collected at 640-740 nm.

9. Immunofluorescence imaging of HIF-1α protein expression

After 4T1 cells were incubated in normoxia and hypoxia, PBS was used to clean the dishes three times, then 1 mL 4 % paraformaldehyde was added to soak the adherent cells for 10 min, and then the paraformaldehyde was discarded. PBS was used to clean the dishes three times, and then 0.03 % Triton was added to soak the adherent cells for 10 min. After discarding the solution, PBS was used to clean the dishes three times. Finally, antibodies were added to the dishes for confocal imaging after dark incubation. MCF-7 cells were treated with the same method. The only difference was that the soaking time of paraformaldehyde and 0.03 % triton was shortened to 5 min.

10. Establishment of mice model

Tumor-bearing mice model. Balb/c mice were purchased from Liaoning Changsheng Biotechnology Co., Ltd. Subcutaneous injection of 2×10^6 4T1 cells into 4~6-week-old Balb/c mice. Observation of tumor growth, and 20 days later, it was used for fluorescence imaging.

Hind limb ischemic model. The hindlimb ischemia model of Balb/c mice was established by surgical method to evaluate the imaging effect of **CS-NO_2** on the local hypoxic region caused by ischemia. The 4~6week-old female Balb/c was anesthetized. Afterwards, an incision of the skin was made from the medial thigh to the knee and the membranes covering the muscle were dissected away. The neurovascular bundle was exposed by piercing the membranous femoral sheath, and the external iliac artery, femoral artery, and peroneal artery were separated, respectively. Ligations were performed in proximal and distal femoral artery with 6/0 polypropylene suture and a cut out was made between two ligations. Then, animals were sutured, 3 days later, it was used for fluorescence imaging.

11. In vivo imaging of mice

The mice were imaged using the Night OWL LB 983 in vivo imaging system (Berthold). Mice were anesthetized before fluorescence imaging. After an intratumor or lower limb hypoxia injection of **CS-NO_2** (0.5 mM) in 0.1 mL of HEPES (pH 7.4), the mice were placed in the in vivo imaging system (Berthold) for in vivo fluorescence imaging (($\lambda_{ex}/\lambda_{em}$ =550 nm/760 nm).

All the animal experiments involved in this study were conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (8th edition, 2011), and approved by the local research ethics review board of the Animal Ethics Commolittee of Dalian University of Technology.

12. Supporting tables and figures

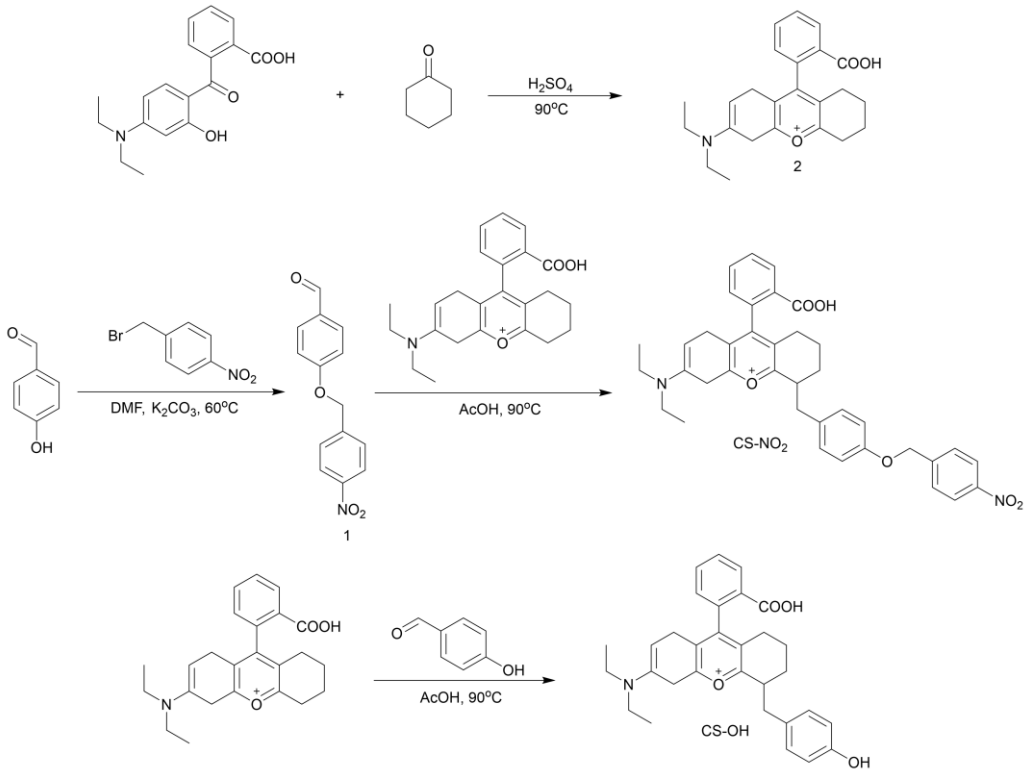

Figure S1. Syntheses of **CS-OH** and **CS-NO_2.**

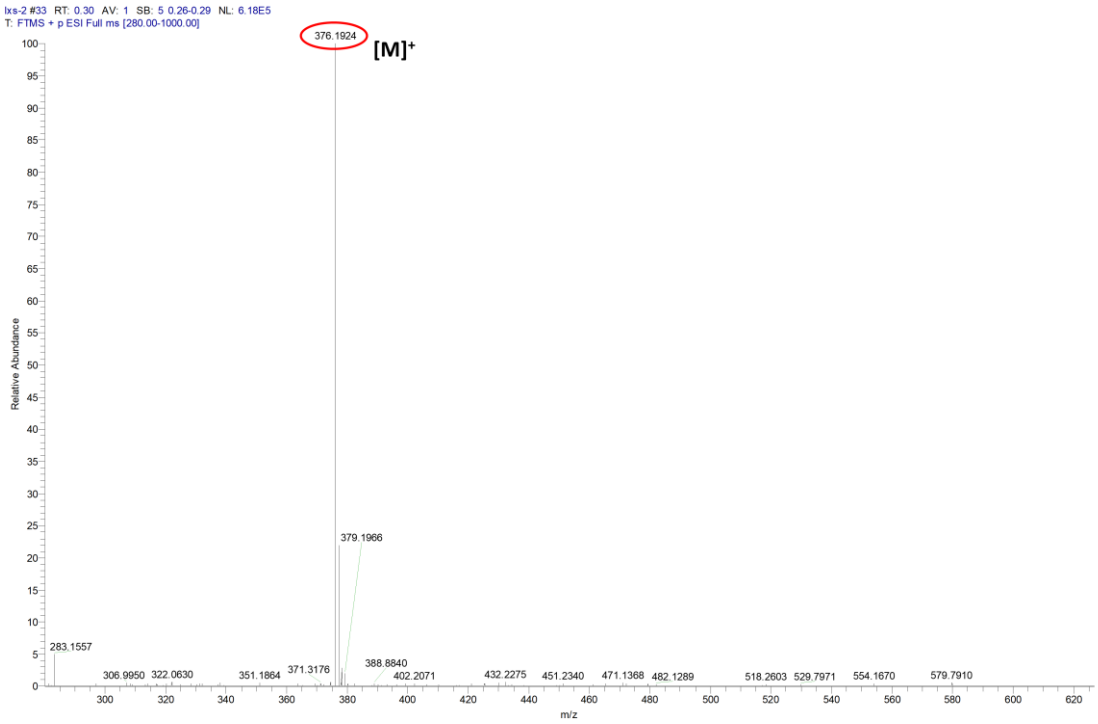

Fig. S2. HRMS spectrum of compound 2.

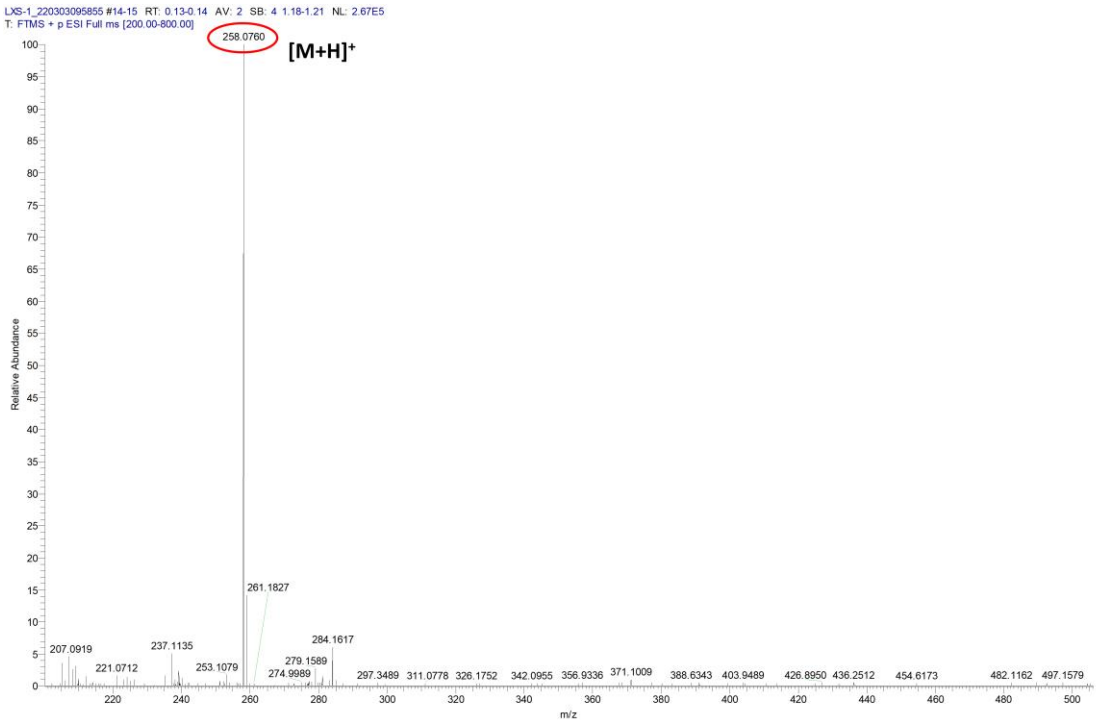

Fig. S3. HRMS spectrum of compound 1.

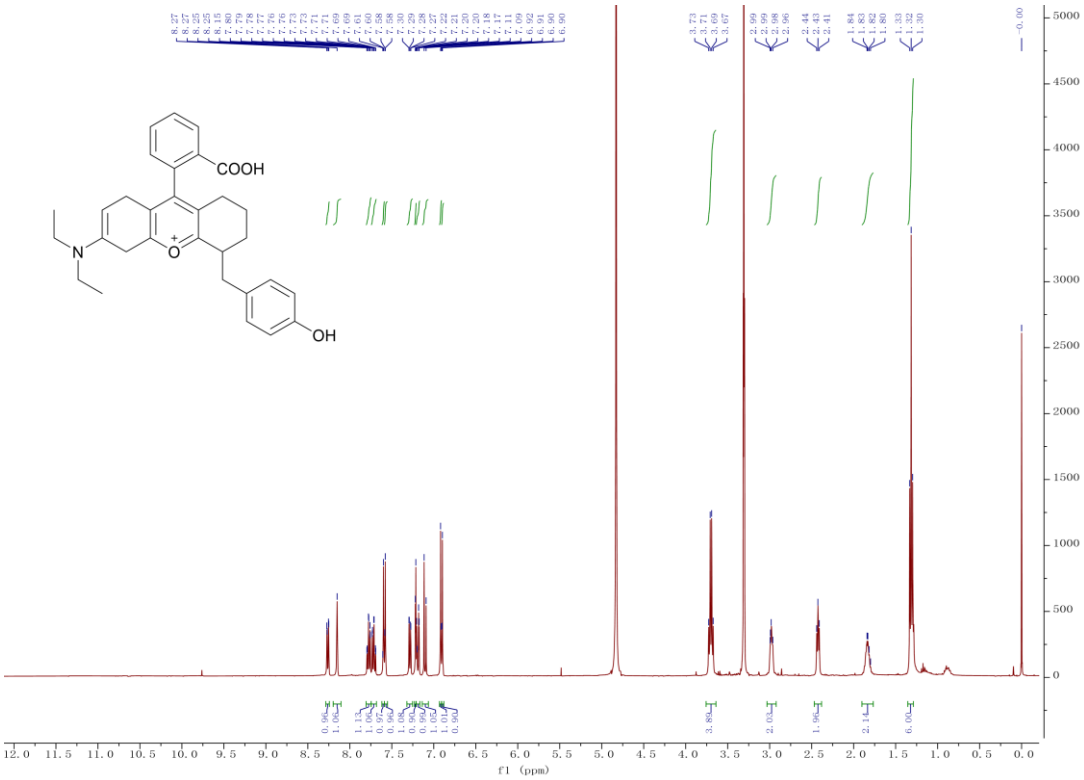

Fig. S4 ^{1}H NMR spectrum of **CS-OH** (500 MHz, 298 K, CD_3OD).

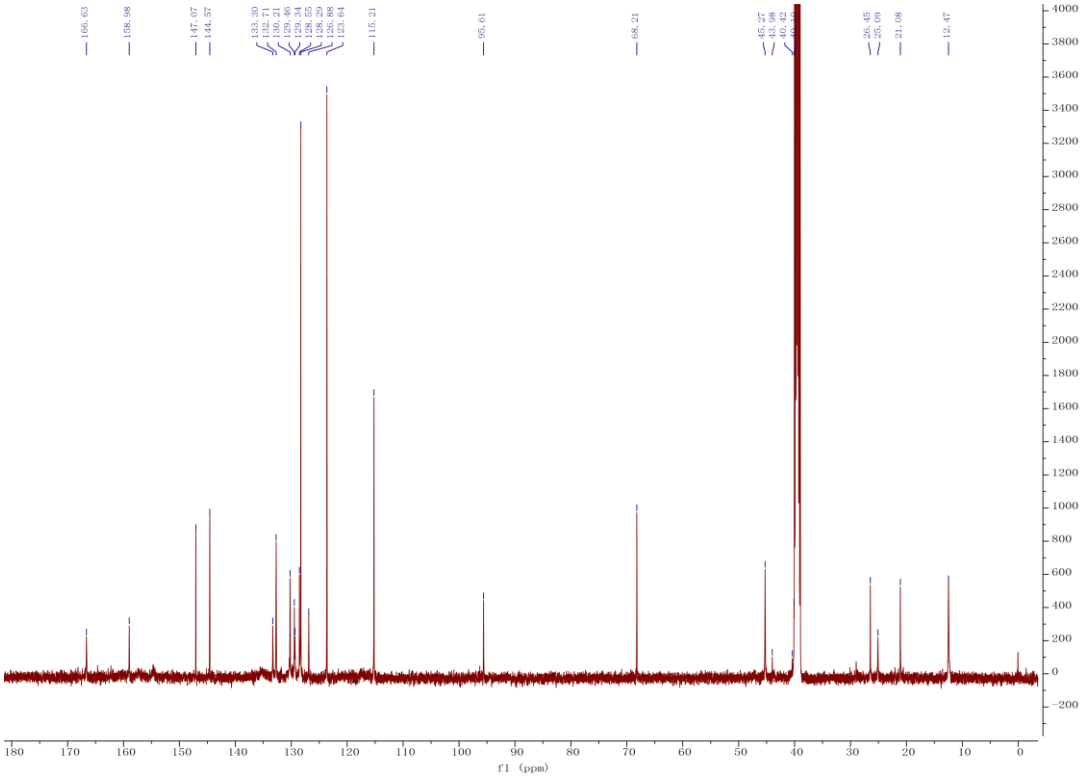

Fig. S5 ^{13}C NMR spectrum of **CS-OH** (500 MHz, 298 K, DMSO-d_6).

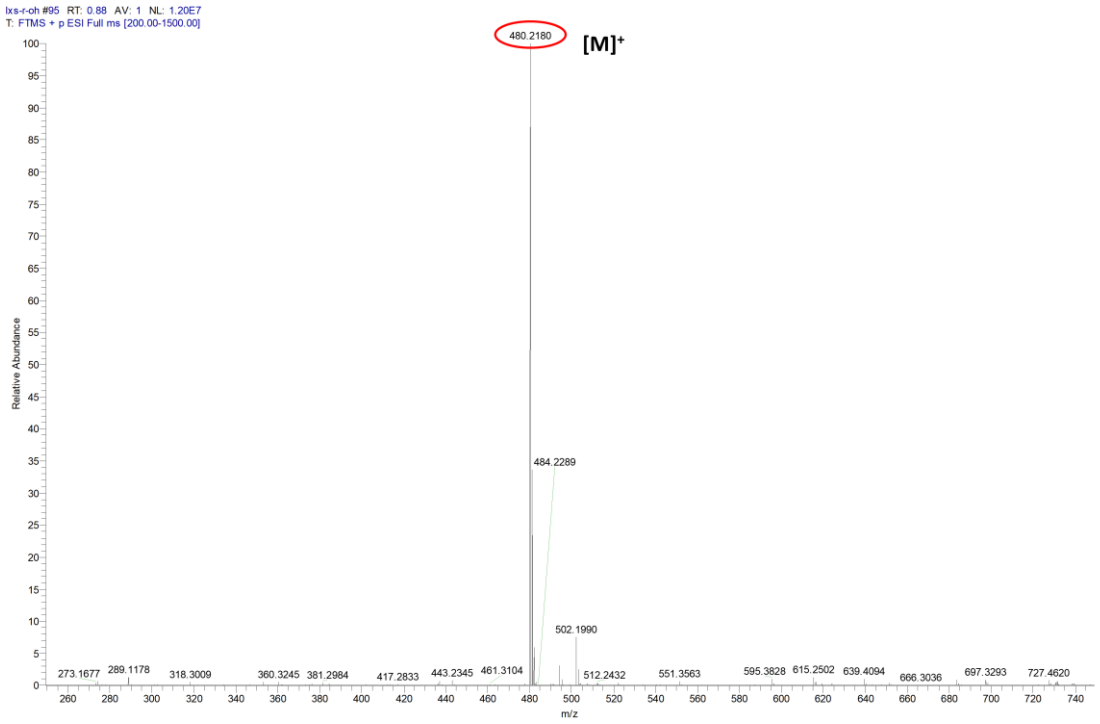

Fig. S6. HRMS spectrum of compound **CS-OH**.

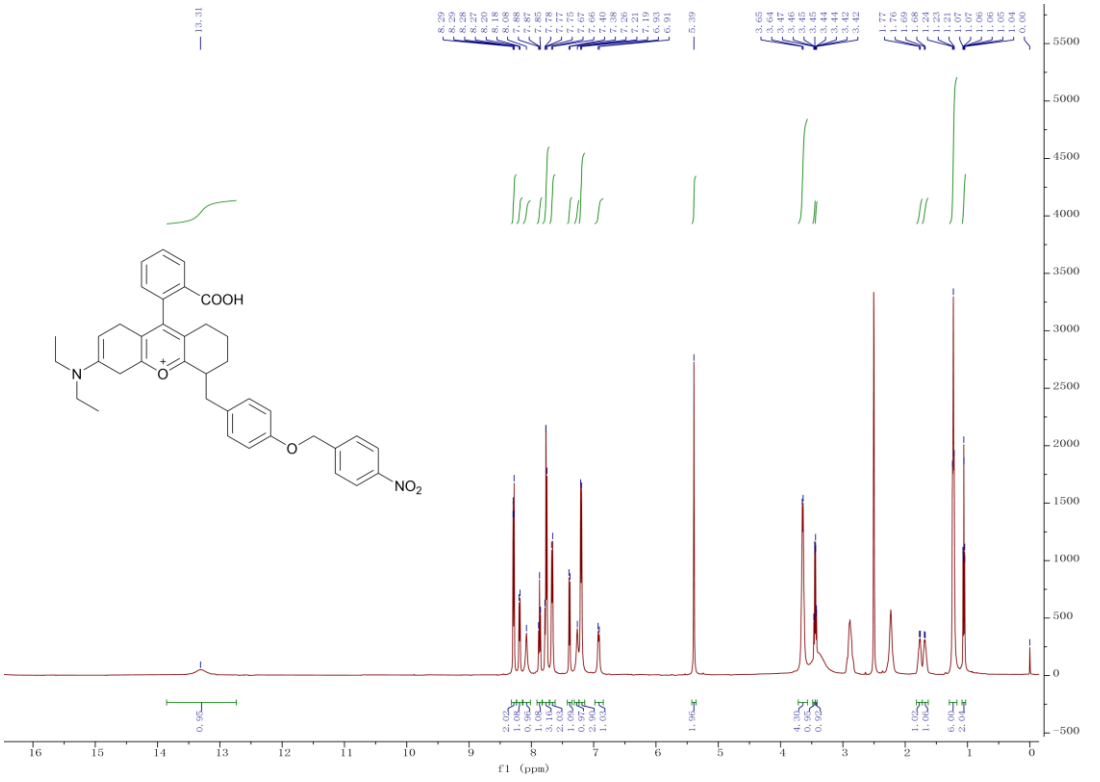

Fig. S7 ^{1}H NMR spectrum of **CS-NO$_2$** (500 MHz, 298 K, CD_3OD).

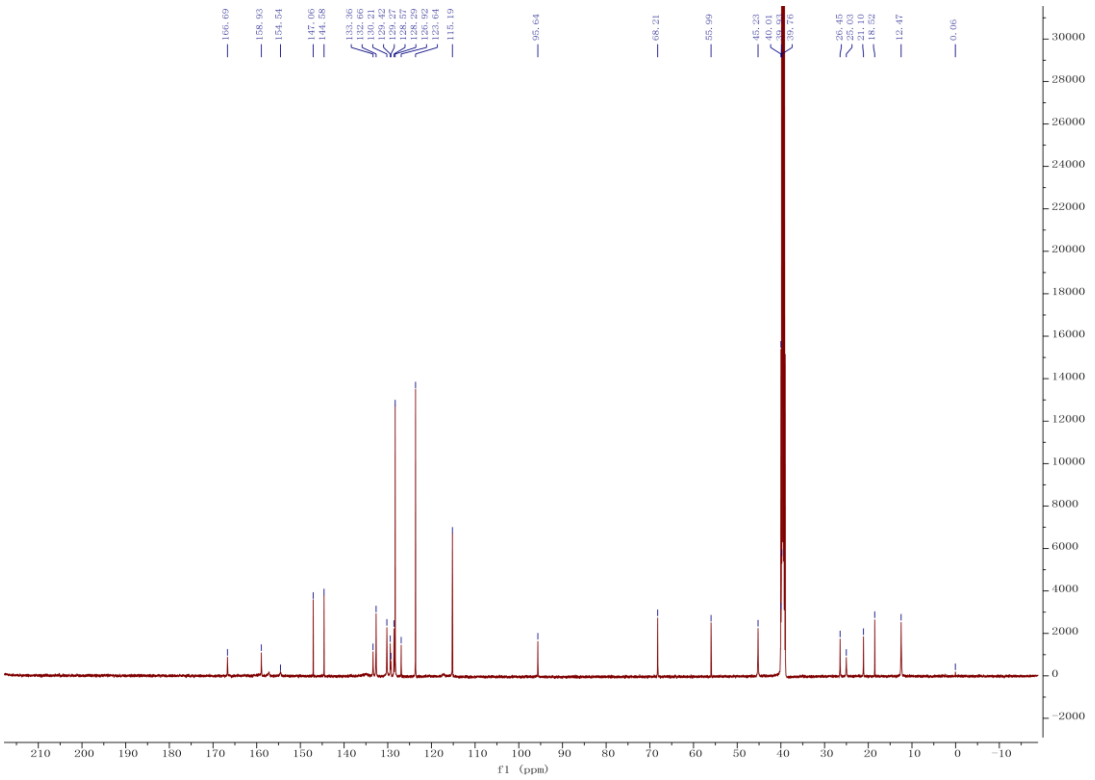

Fig. S8 ^{13}C NMR spectrum of **CS-NO$_2$** (500 MHz, 298 K, CD_3OD).

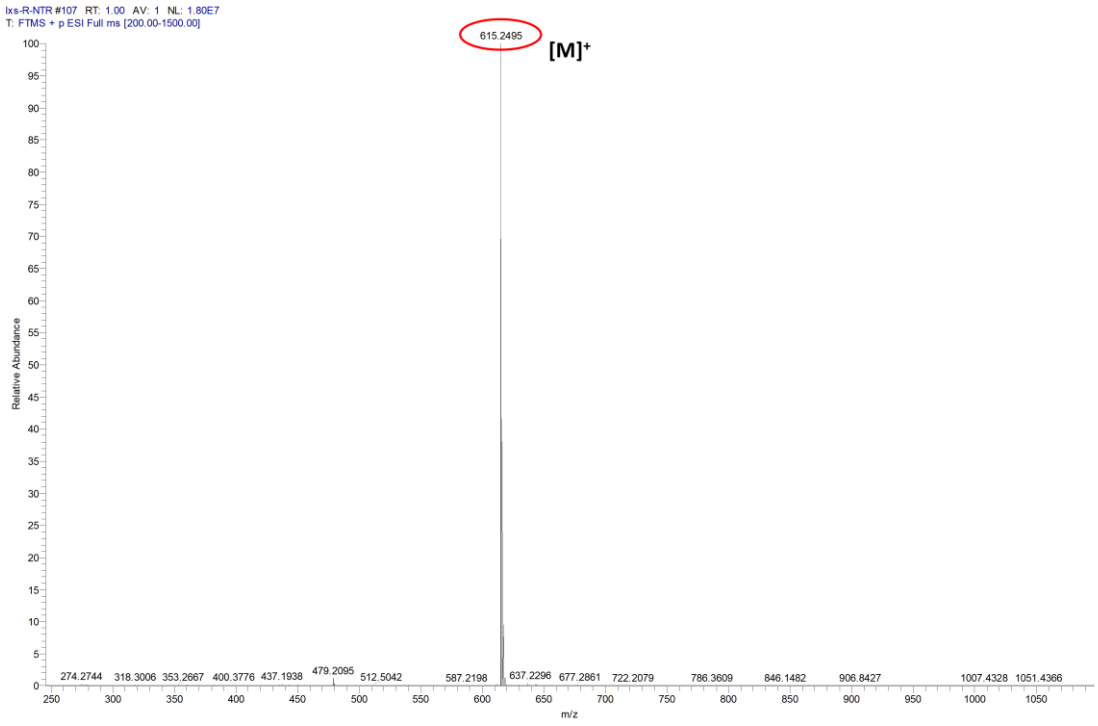

Fig. S9. HRMS spectrum of compound **CS- NO$_2$**.

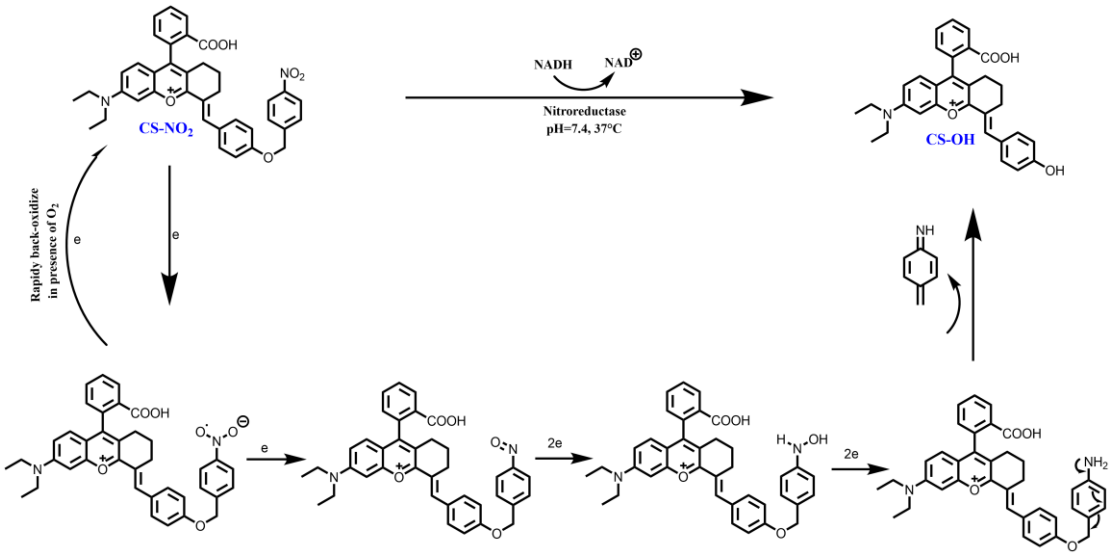

Fig. S10. Schematic of type II (oxygen-dependent) NTR reduction of **CS-NO$_2$**

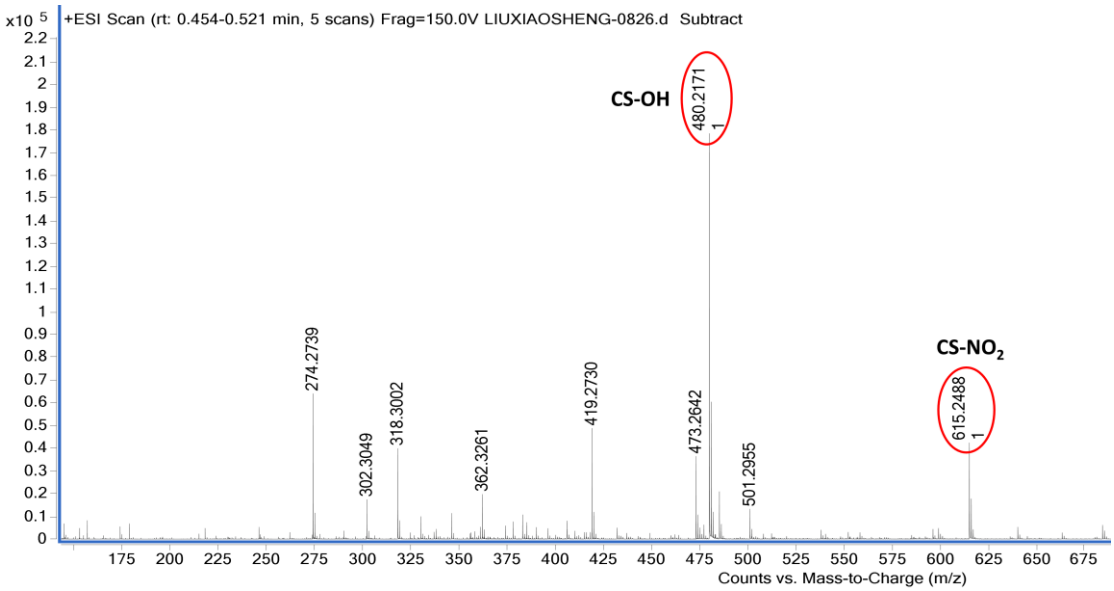

Fig. S11. HRMS spectrum of **CS-NO$_2$** after reaction with NTR

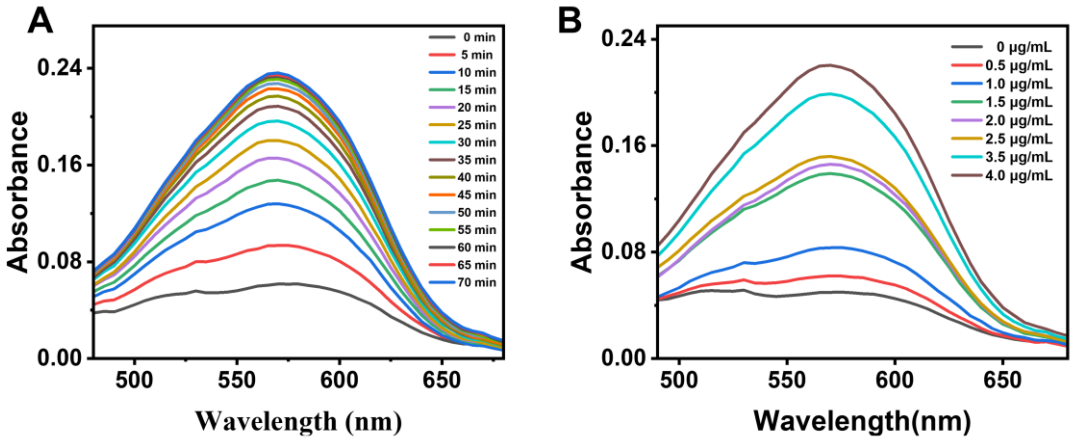

Fig. S12. Spectral properties of **CS-NO$_2$** towards NTR. (A) Time-dependent absorbance changes of **CS-NO$_2$** (10 μM) in the presence of nitroreductase (NTR) (10 μg/mL). (B) After an hour of incubation at 37 ºC, the changes of absorbance of **CS-NO$_2$** to different concentrations of NTR (0-4 μg/mL).

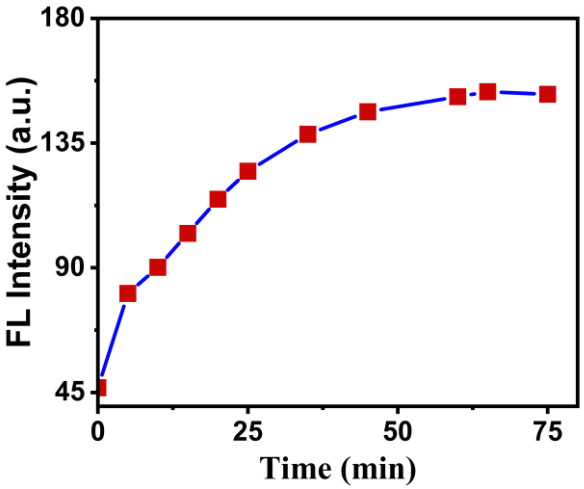

Fig. S13. The corresponding time-dependent changes of fluorescence intensity at 670 nm.

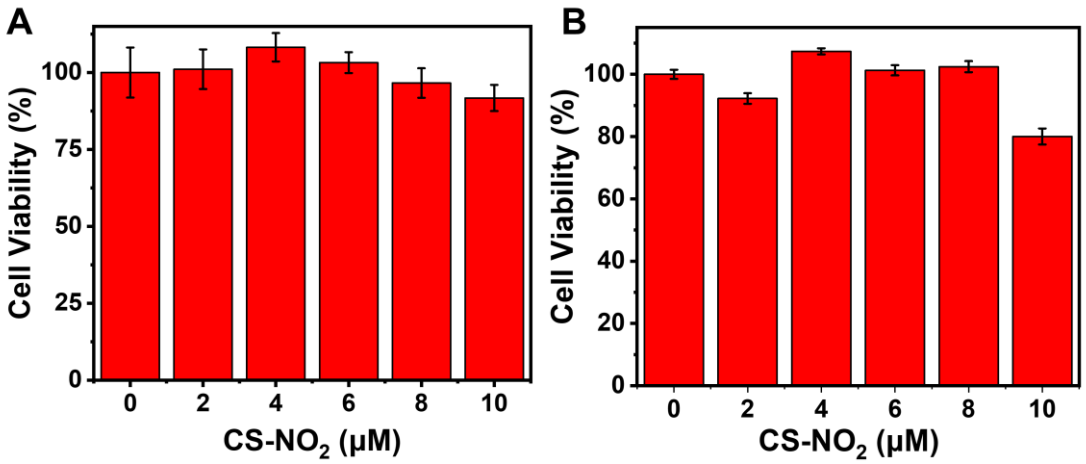

Fig. S14. Cytotoxicity of **CS-NO$_2$** for 4T1 (A) and MCF-7 (B) cells. Cells were incubated with the probe at corresponding concentrations for 24 h. Cell viability was measured by MTT assay and the results are reported as percentage relative to untreated cells.

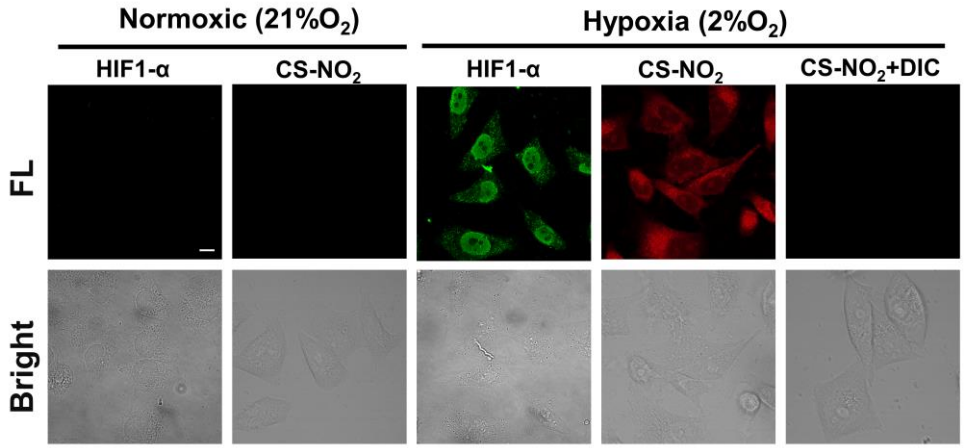

Fig. S15. Immunofluorescence imaging of HIF-1α protein expression and confocal fluorescence microscopy imaging of MCF-7 cells incubated with **CS-NO$_2$** under different conditions. Scale bar: 10 μm.

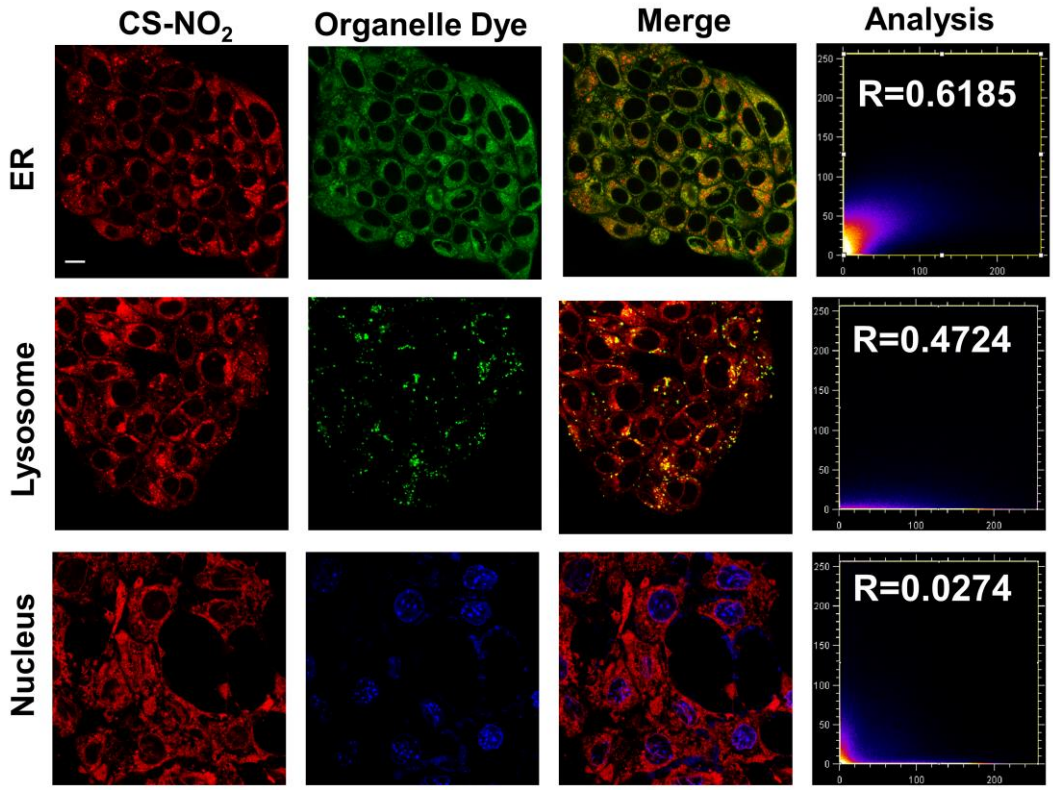

Fig. S16. Co-localization of **CS-NO$_2$** (red, λ_{ex} = 580 nm, λ_{em} = 600-700 nm) and different commercial dyes (green, λ_{ex} = 488 nm, λ_{em} = 510-530 nm. blue, λ_{ex} = 405 nm, λ_{em} = 450-470 nm) for organelles in 4T1 cells. Scale bar: 10 μm.

12. References

1. X. J. Zhao, Y. T. Li, Y. R. Jiang, B. Q. Yang, C. Liu and Z. H. Liu, *Talanta*, 2019, **197**, 326-333.