Electronic Supplementary Material (ESI) for New Journal of Chemistry.

Supporting Information for

A novel fluorescent probe for the detection of Golgi nitroreductase under hypoxic conditions

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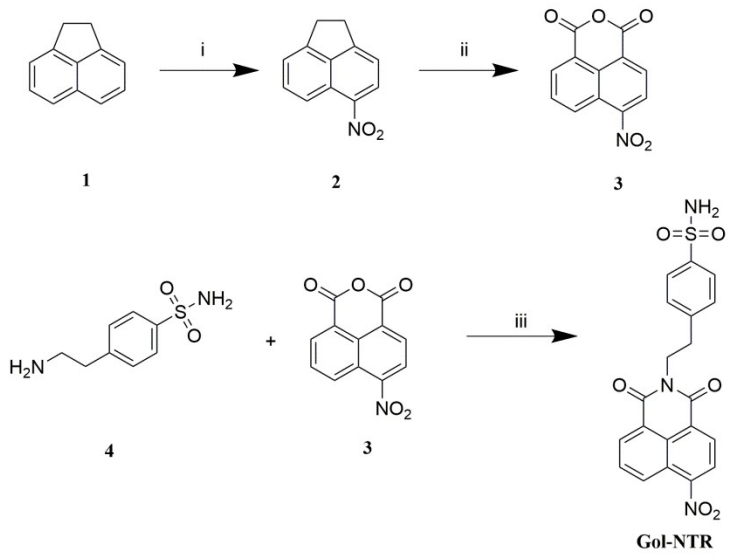
Materials and apparatus

Unless otherwise stated, all reagents and solvents were purchased from commercial sources and can be used without further purification. The metal ion solution is prepared with phosphate dissolved in deionized water. The equipment used in the experiment is the same as the equipment in the article we reported earlier. TLC analysis is performed using pre-coated silica plates. Using DMSO-d_6 as the solvent and Tetramethylsilane (TMS) as the internal reference, respectively, ^{1}H and ^{13}C NMR spectra were measured in a 500 MHz NMR spectrometer. The abbreviations are as follows: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet. The UV spectrum was measured by UV-2700 UV-Vis spectrophotometer from Shimadzu Corporation, Japan. The fluorescence spectrum is measured by the F-4700 fluorescence spectrophotometer from Japan Hi-Tech Corporation. The pH value is obtained from the pH meter of PHS-3E of *Shanghai INESA Scientific Instrument Co., Ltd*. Cell imaging using TCS-SP8 DIVE confocal microscope from Leica.

Synthesis



Scheme S1. Synthesis of probe **Gol-NTR**. Reaction conditions: (i) acetic anhydride, -15℃, 3h, 85%. (ii) glacial acetic acid, $K_2Cr_2O_7$, refulx, 4h, 90%. (iii) 85℃, refulx, 3h, 80%.

Synthesis of compound 2

Add 0.32 mL (4.6 mmol) of concentrated nitric acid slowly to a solution of 0.61 g (3.96 mmol) of acenaphthene in 8 mL of acetic anhydride at -15 °C. Stir the reaction mixture for 3 h at this temperature. Treat the mixture with water and extracted three times with EtOAc. Separate the organic layers and wash with saturated solution of Na_2CO_3 and brine for two times. Dry the mixture over anhydrous $MgSO_4$. Filter the reaction mixture and concentrate to obtain compound **1**.

Synthesis of compound 3

Add the compound **1** (3 mmol) and glacial acetic acid (7 mL) into a round-bottomed flask for stirring. Reflux the reaction mixture for 4 h. Add potassium dichromate (26 mmol) to the mixture in three portions and each interval for 30 minutes. Pour the reaction mixture into ice water (25 mL). Filter the reaction mixture. Dissolve the filter cake with 5% boiling sodium carbonate solution and filter while hot. Adjust the mixture the pH to 2 then filter to obtain compound **2.**

Synthesis of Gol-NTR

4-(2-Aminoethyl)benzenesulfonamide (400 mg, 2 mmol)and compound **2** (242.86 mg, 0.998 mmol) were dissolved in ethanol (7 mL) at a 1:1 equivalent ratio, heated to reflux at 85 °C for more than 3 h, cooled to room temperature after the reaction was completed, and purified by column chromatography (PE: DCM =50: 1) **Gol-NTR** can be obtained (89%). ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.71 (d, J = 8.7 Hz, 1H), 8.62 (dd, J_1 = 12.2 Hz, J_2 = 7.6 Hz, 2H), 8.55 (d, J = 8.0 Hz, 1H), 8.10 (dd, J_1= 8.6 Hz, J_2 = 7.4 Hz, 1H), 7.77 (d, J = 8.2 Hz, 2H), 7.49 (d, J = 8.2 Hz, 2H), 7.32 (s, 2H), 4.34 – 4.24 (m, 2H), 3.11 – 2.98 (m, 2H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 163.3, 162.5, 149.7, 143.3, 142.8, 132.2, 130.6, 130.1, 129.6, 129.3, 128.8, 127.0, 126.4, 124.7, 123.2, 123.2, 41.4, 33.6. HRMS m/z calcd for $C_{20}H_{15}N_3O_6S$ $[M+H]^+$: 426.0682, found 426.0759.

Preparation of test solutions

Without further instructions, all tests were performed according to the following procedure.

Dissolve phosphate buffered saline powder (1x PBS, calcium and magnesium free) in a certain amount of ultrapure water in a beaker, stir until completely dissolved, and make up to volume in a 2 L volumetric flask. At this point the pH was 7.42 and it was sealed for use.

Prepare Gol-NTR stock solution (1.0 mM) in DMSO. Adjust the final volume to 5 mL with 0.1 M PBS buffer. Transfer 2 mL to a 1 cm quartz cell to measure absorbance or fluorescence. The final Gol-NTR concentration was 5 μm, the solution was dimethyl sulfoxide (5%), and the pH was 7.4 PBS buffer (95%). The excitation light wavelength was 440 nm and the fluorescence emission spectrum at 450-750 nm was recorded.

Optical properties test

1. Absorption Spectroscopy

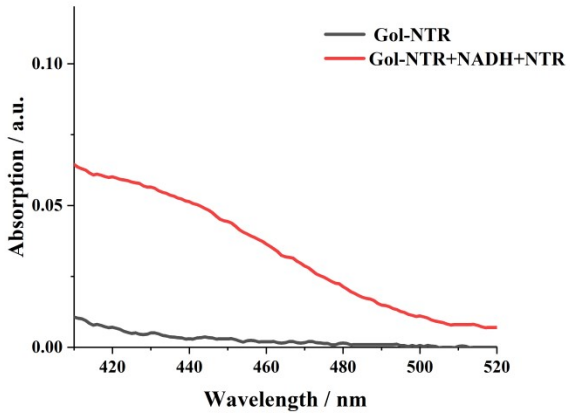


Fig. S1. UV-Vis absorption titration spectra of **Gol-NTR**; UV-Vis absorption titration spectra of **Gol-NTR** solutions (5% DMSO, 95% PBS, pH=7.4) were added with NTR and the same concentration of NADH, and incubated at 37 °C for 1 h.

2. Titration experiment

In the fluorescence titration experiment, several groups of parallel **Gol-NTR** solutions (5% DMSO, 95% PBS, pH=7.4) were added with different concentrations of NTR and the same

concentration of NADH, and incubated at 37 °C for 1 h. Measurements of fluorescence spectra were performed.

3. Kinetic experiment

Reaction-time profiles of **Gol-NTR** (5 μM) treated with the varied concentrations of NTR in the presence of NADH (500 μM): 0-5 μg/mL. The fluorescence intensities at 544 nm were continuously monitored at time intervals for 60 min in PBS buffer (pH 7.4, 5% DMSO).

4. pH experiment

In the pH experiment, multiple groups of parallel **Gol-NTR** solutions (5 μm, 5% DMSO, 95% PBS) were added with the same concentrations of NTR (3 μg/mL) and NADH (500 μm), without pH PBS buffer solution, and incubated at 37 °C for 1 h. Fluorescence spectra were measured.

5. Stability test

Photostability test: Spectra of **Gol-NTR** (5.0 μm) were measured in the presence or absence of UV radiation (440 nm) for 1 h in PBS (10 mM, pH 7.4, 5% DMSO) for 5 min as the fluorescence intensity at 511 nm was continuously monitored at time intervals.

6. selective experiment

To evaluate the selectivity of **Gol-NTR**, a series of common ions and biomolecules, such as representative amino acids (Cys, Hcy, GSH), cations, anions, reactive oxygen species (ROS), and NTR solutions, were selected for interference experiments. The concentration of **Gol-NTR** was 5 μm, the concentration of amino acid analytes was 100 μm, the concentration of inorganic salt ion analytes was 50 μm, and the wavelength of the test excitation light was 440 nm.

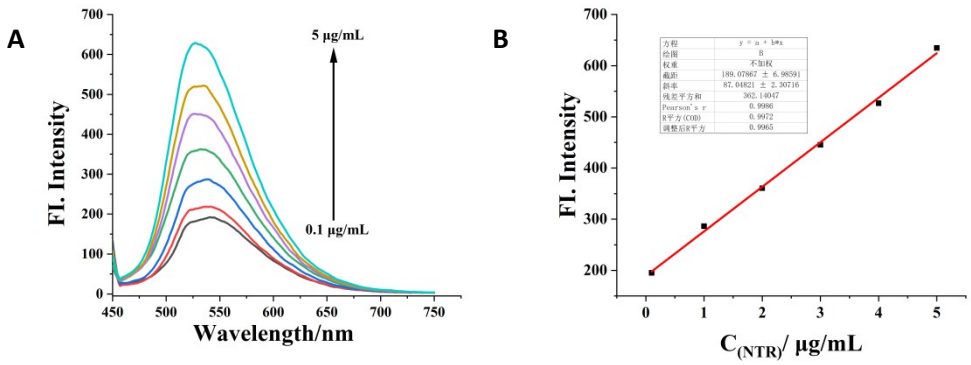


Fig. S2. A) The fluorescence response of the probe **GoI-NTR** (5 μM) to NTR at the different concentrations in PBS buffer (pH 7.4, 5% DMSO). λ_{ex} =440 nm. The spectra were recorded upon treatment of the probe with NTR (0.1-5.0 μg/mL) in the presence of NADH (500 μM) for 60 min. **B)** Linear fitting graph of fluorescence intensity changing with NTR concentration.

HRMS Spectra

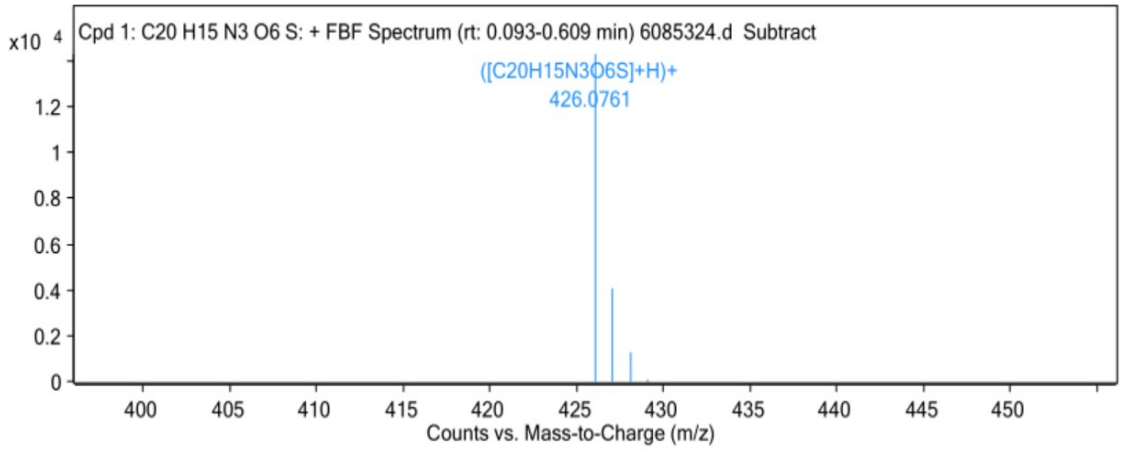


Fig. S3. HRMS (ESI) spectrum of **GoI-NTR**, $[M+H]^+$, 426.0761

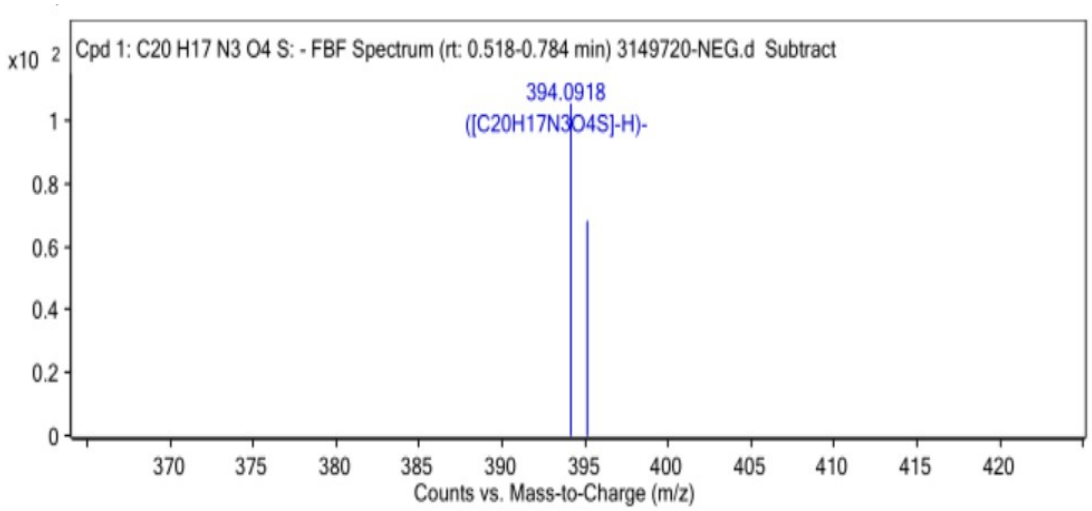


Fig. S4. HRMS (ESI) spectrum of **GoI-NTR-NH_2**, $[M-H]^-$, 394.0918

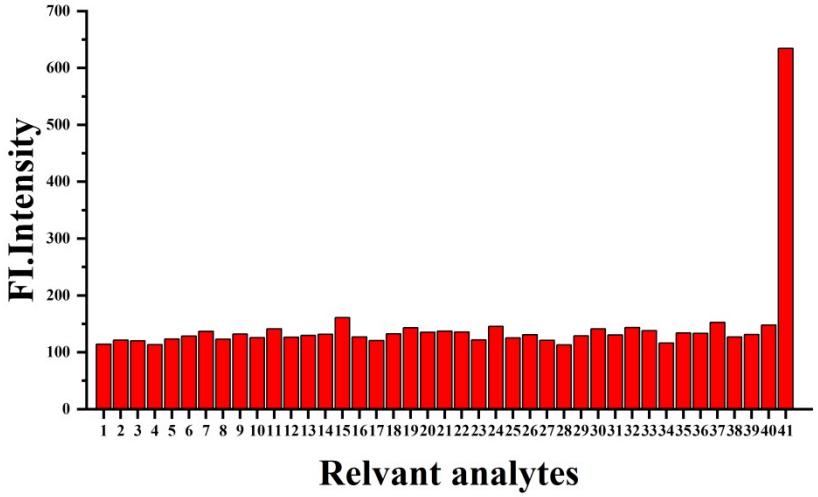


Fig. S5. Fluorescence intensities of the probe system (**GoI-NTR**, 5μM each) at 544nm for various analytes (70 μM): (1) free probe, (2) probe+NADH (500 μm) (3) Cys, (4) L-Phe, (5) L-Ala, (6) L-Met, (7) D-Hcy, (8) Gly, (9) GSH, (10) L-Arg, (11) L-Aa, (12) L-Lys, (13) L-Tyr, (14) L-Leu, (15) L-Pro, (16) Glu, (17) L-Trp, (18) L-Ser, (19) L-Asn, (20) L-Val, (21) L-Ile, (22) L-His, (23) Al^{3+}, (24) Ca^{2+}, (25) Cu^{2+}, (26) Fe^{2+}, (27) Fe^{3+}, (28) H_2O_2, (29) K+, (30) Mg^{2+}, (31) NaF, (32) NaCl, (33) $NaNO_2$, (34) NaSCN, (35) CH_3COONa, (36) Na_2SO_4, (37) Na_3PO_4, (38) $Na_2Fe(CN)_5NO$, (39) $NaHS{\cdot}XH_2O$, (40) Na_2CO_3, (41) Probe+ NADH (500μm)+NTR(3μg/mL).

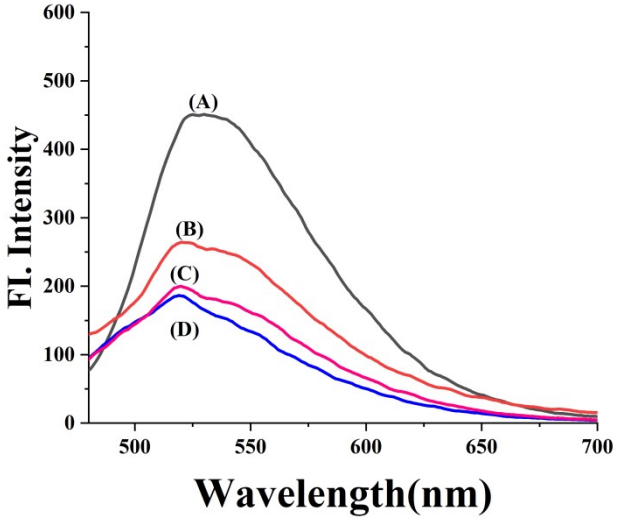


Fig. S6. Fluorescence emission spectra of different reaction systems: (A) The probe **GoI-NTR** (5 μM) treated with NTR (3 μg/mL) in the presence of NADH (500 mM); (B) The probe **GoI-NTR** (5 μM) treated with 0.5 mM dicoumarin and NTR (3 μg/mL) in the presence of NADH (500 mM); (C) The probe **GoI-NTR** (5 μM) in the presence of NADH (500 mM); (D) The free probe **GoI-NTR** (5 μM). Incubation time: 60 min. λex = 440 nm. (b) Fluorescence intensity histogram of four situations (A, B, C, D), λ_{ex} = 440 nm, λ_{em}=544 nm.

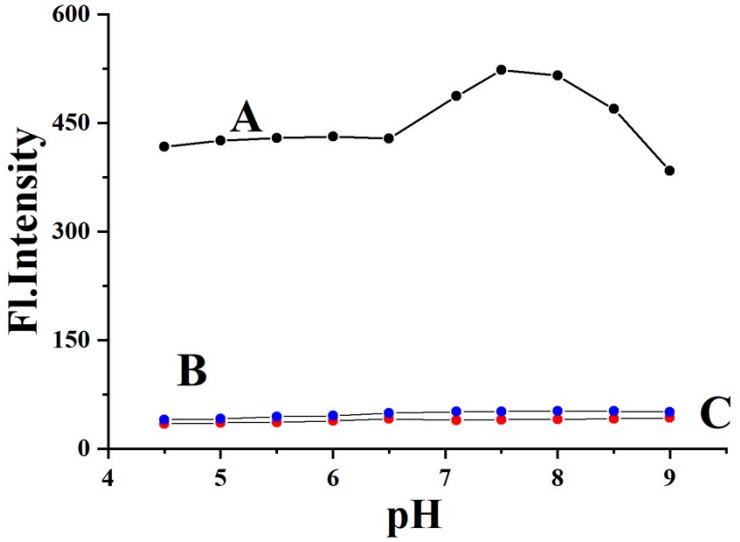


Fig. S7. The relationship curve of fluorescence intensity and pH value before and after the reaction of probe **GoI-NTR**(5μM) and NTR (3 μg/mL). A) **GoI-NTR** (5 μm)+NADH (500 μm)+NTR (3 μg/mL). B) **GoI-NTR** (5 μm)+NADH (500 μm) C) **GoI-NTR** (5 μm).

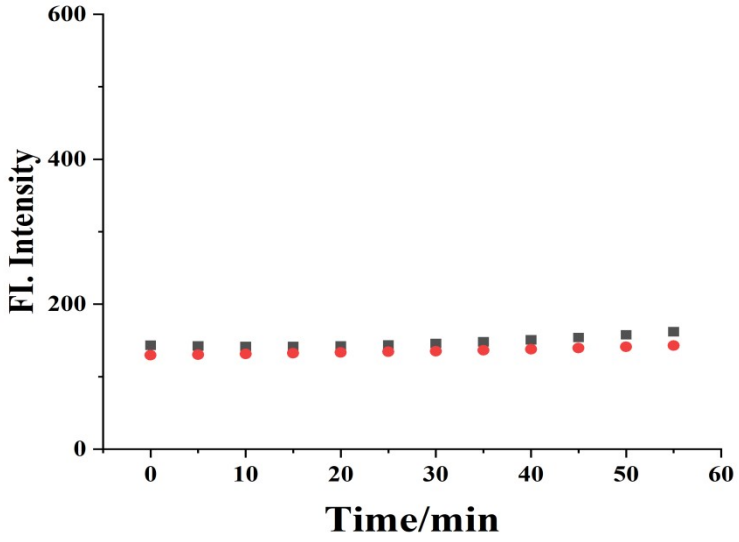


Fig. S8. Light stability curve of **GoI-NTR** (5.0 μm)[●]or in the presence of ultraviolet radiation [■] (440 nm). The fluorescence intensity at 511 nm was continuously monitored at time intervals in PBS (10 mM, pH 7.4, 5% DMSO). Time points represent 0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50 and 55 min.

Cell culture and cell staining

This article uses HeLa cells. All HeLa cells were stored in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Cells were cultured at

37°C in a humidified atmosphere of 5% CO2 and different oxygen levels (1% and 20%) and grown on 18mm coverslips for 1-2 days. Cells are ready to use after reaching 70% to 90% confluency. Remove the HeLa cell culture medium, then add 1 mL of DMEM containing the probe **GoI-NTR** (5 μM), incubate for 30 min at 37°C in 95% air, 5% CO2, and visualize staining under a confocal microscope.

Cytotoxicity assay

In a 96-well plate, about 2×10^5 HeLa cells were seeded per well. After 24 h of culture, the cells have adhered to the wall. Place the 96-well plate in an incubator with different oxygen（1%，5%，10%，15%，20%） levels for 12 hours. After adding probes with different concentration gradients (0, 1, 2, 5, 10, 20, 30, 40, 50μm), the HeLa cells were placed in a cell incubator and incubated for 24 hours. Then, remove the supernatant and add 100 μL MTT solution to each well. After incubating for 4 h, add 100 μL DMSO to each well to dissolve the purple crystalline product formed. Shake in a shaker for 15 minutes, and measure the absorbance at 492 nm with a microplate reader.

$$Cell\ survival\ rate(100\%) = \frac{OD_{492}sample - OD_{492}blank}{OD_{492}control - OD_{492}blank} \times 100\%$$

In the above calculation formula, OD_{492}sample represents the absorbance of cells incubated with different concentrations of probes at 492 nm, OD_{492}control represents the absorbance of cells incubated without probes at 492 nm, and OD_{492} blank indicates the absorbance at 492 nm of each medium-only well.

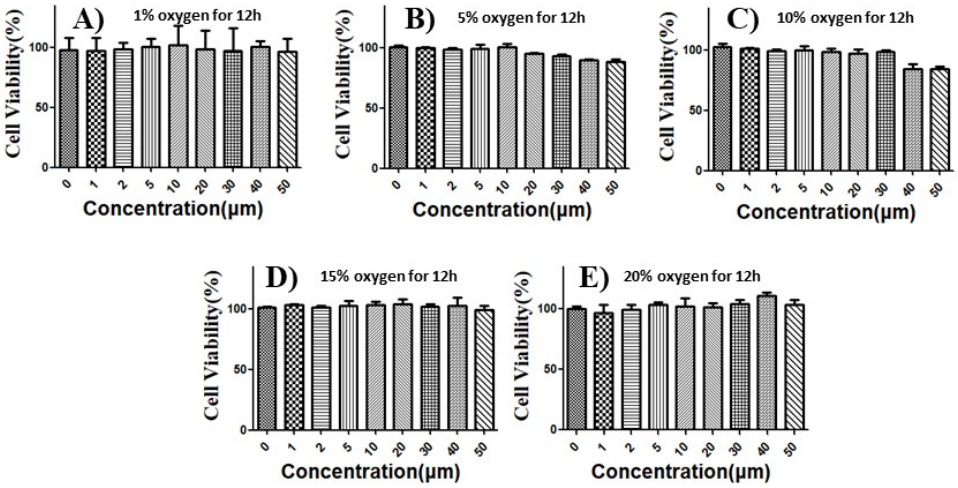


Fig. S9. The cytotoxicity of the probe **Gol-NTR** on HeLa cells was determined at different concentrations (0 μM; 5 μM; 10 μM; 20 μM; 30 μM; 40 μM; 50 μM). (A) Cytotoxicity test with oxygen content of 1%. (B) Cytotoxicity test with oxygen content of 5%. (C) Cytotoxicity test with oxygen content of 10%. (D) Cytotoxicity test with oxygen content of 15%. (E) Cytotoxicity test under normoxia.

Colocalization experiments

In colocalization experiments, we incubated HeLa cells with the commercially available fluorescent probe Golgi-Tracker-Red (GTR) for 30 min and added **Gol-NTR** for another 30 min. Wash twice with PBS buffer and replace with fresh DMEM medium, and then test **Gol-NTR** in green channel ($\lambda_{ex}/\lambda_{em}$=440/450-600 nm) and red channel ($\lambda_{ex}/\lambda_{em}$=589/600-750 nm) Perform cellular imaging.

Construction of tumor mice

Female balb/c mice (about 4 weeks aged) were purchased from Guangxi Medical University. The farming system of animals was under standard laboratory conditions. All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Guangxi University and approved by the Animal Ethics Committee of Guangxi University (China). The well-growing B16 cells were digested by trypsin, centrifuged, and evenly mixed with 150 μL PBS (pH=7.4) solution, injected into the front thigh of 4-week-old nude mice. Tumor mice were obtained after one week.

Two-photon imaging of muscle and tumor tissue

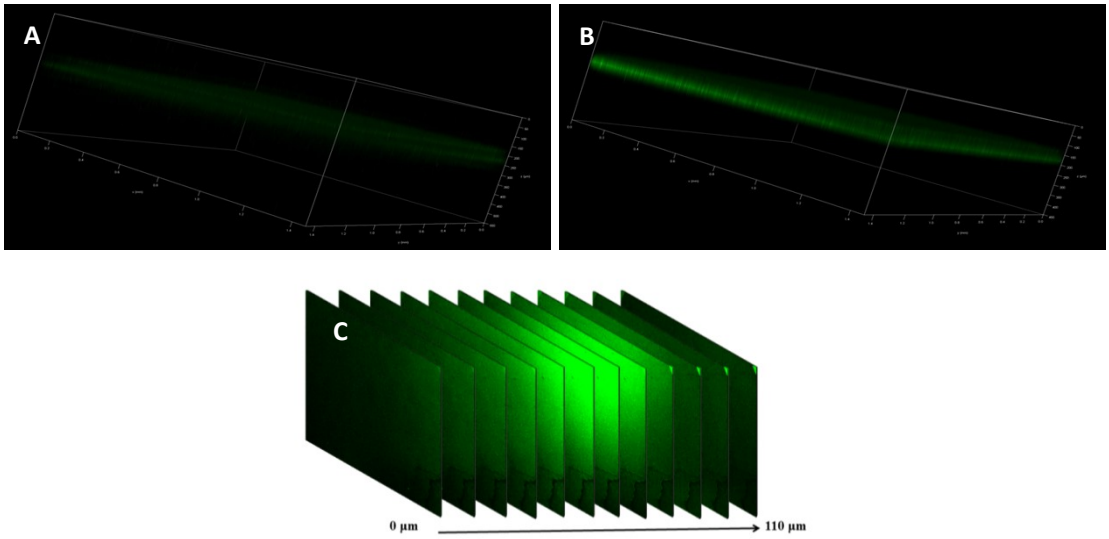


Fig. S10. Two-photon imaging of normal muscle tissue and tumor tissue. (A) Two-photon imaging of normal muscle tissue with **GoI-NTR**. (B) Two-photon imaging of tumor tissue with **GoI-NTR**. (C) Two-photon fluorescence imaging of NTR in live tumor tissue sections. Fluorescence image of tumor tissue sections incubated with probe **GoI-NTR** (20 μM) for 4 hours. The femtosecond laser is excited at 800 nm and the emission collection range is 500 to 550 nm.

NMR Spectra

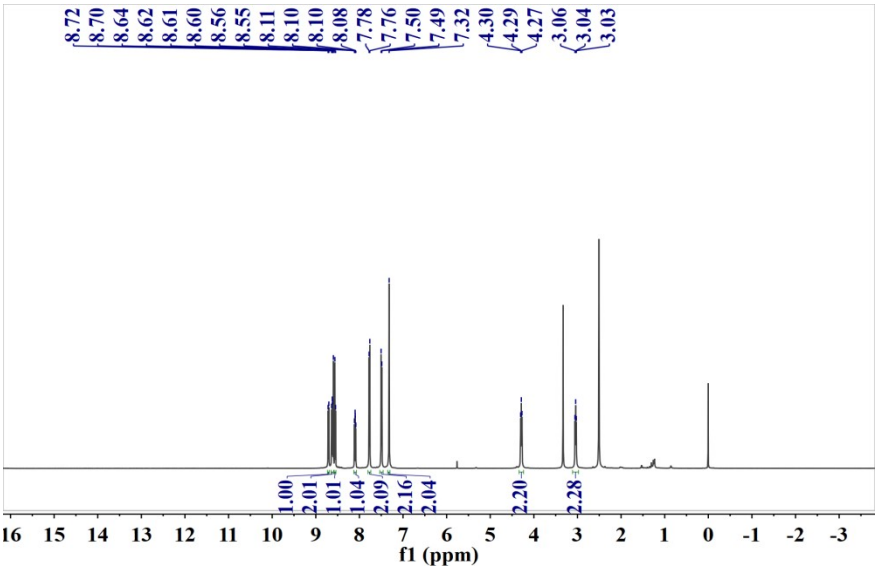


Fig. S11. ^{1}H-NMR (500MHz, DMSO-d_6) spectrum of **GoI-NTR**.

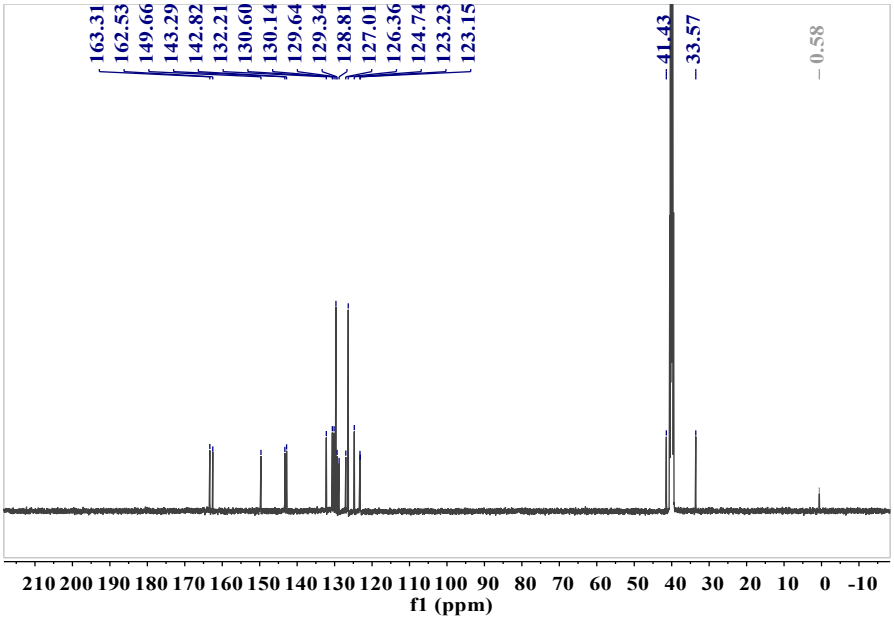


Fig. S12. ^{13}C NMR (125MHz, DMSO-d_6) spectrum of **Gol-NTR.**