Supporting information for

High Selectively Imaging for Nitroreductase by a Near-infrared Fluorescence Probe in Hypoxic Tumor

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1. Apparatus and Materials

Apparatus. Fluorescence measurements were performed on a Cary Eclipse fluorescence spectrophotometer (Varian, Inc., USA) with a 1.0 cm quartz cells at the slits of 10/10 nm. Absorption spectra were measured on a pharmaspect UV-1700 UV-visible spectrophotometer (SHIMADZU). All pH measurements were performed with a pH-3c digital pH-meter (Shanghai Lei Ci Device Works, Shanghai, China) with a combined glass-calomel electrode. $^1$H and $^{13}$C spectra were taken on Bruker 300-MHz spectrometer. The fluorescence images of cells were taken using a LTE confocal laser scanning microscope (Germany Leica Co., Ltd) with an objective lens (×40). Absorbance was measured in a TRITURUS microplate reader in the MTT assay. Hypoxic environment was made by triple-gases MCO-5M incubator (Sanyo, Japan).

Materials. Unless stated otherwise, solvents were dried by distillation. All reagents were of commercial quality and used without further purification. glutathione (GSH), dithiothreitol (DTT), Cysteine (Cys), arginine (Arg), ascorbic acid (Vc), propidium iodide (PI), 4',6-diamidino-2-phenylindole (DAPI), Mito Tracker Green FM, $\text{H}_2\text{O}_2$ and 3-(4, 5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Company. 2-nitroimidazole and N-(2-bromoethyl) phthalimide were purchased from Alfa Aesar Chemical Company. Ascorbic acid and dithiothreitol (DTT) were obtained from Sinopharm. Chemical Regent Co., Ltd. Sartorius ultrapure water (18.2 MΩ cm) water was used throughout the analytical experiments. Anti-E-cadherin, anti-α-smooth muscle actin and the second antibody were purchased from Santa Cruz Biotechnology (CA, USA). HepG2 cells were purchased from the Committee on Type Culture Collection of the Chinese Academy of Sciences. Male Wistar rats were purchased from New Drug Evaluation Center of Shandong University.

2. Synthesis and Characterization of Cy-NO$_2$

2-(2-nitro-lH-imidazolyl) ethylamine: According to a reported literature $^{[1]}$ with some modification, we synthesized the compound of 2-(2-nitro-lH-imidazolyl) ethylamine, as shown in Scheme S1. A stirred solution of 2-nitroimidazole (3.0 g,
26.5 mmol), 2-bromoethylphthalimide (7.08 g, 27.9 mmol), and K₂CO₃ (3.85 g, 27.9 mmol) in DMF (75 mL) was heated at 110 °C for 2 h. The solvent was removed under reduced pressure, and the residue was poured into water (200 mL). The precipitate was collected, washed with water (100 mL), and dried to give N-[2-(2-nitro-1H-imidazolyl)ethyl] phthalimide (5.10 g, 75%): mp 208-210 °C; ¹H NMR (DMSO-d₆, 300 MHz): δ(ppm) 7.83 (m, 4 H), 7.59 (s, 1 H), 7.05 (s, 1 H), 4.62-4.60 (t, 2 H), 4.06-4.04 (t, 2 H). ESI-HRMS: m/z Calcd 287.0702, found 287.0765 [M+H]+.

A stirred solution of this phthalimide (3.65 g, 12.8 mmol) and hydrazine monohydrate (1.24 mL, 25.5 mmol) in EtOH (70 mL) was heated under reflux for 2 h. The resulting suspension was cooled to 0 °C and filtered, and the filtrate was evaporated to dryness under reduced pressure. The residue was dissolved in 1 N HCl (50 mL) and filtered, the solvent was removed under reduced pressure, and the residue was crystallized from MeOH / EtOAc to give 2-(2-nitro-1H-imidazolyl) ethylamine hydrochloride (1.49 g, 70%): ¹H NMR (DMSO-d₆, 300 MHz): δ(ppm) 8.35 (m, 2H, N-H), 7.78 (d, 1H), 7.21 (d, 1H), 4.70-4.66 (t, 2H), 3.29-3.15 (m, 2H). ESI-HRMS: m/z Calcd 157.0647, found 157.0655 [M+H]+.

Scheme S1. Synthesis of compound 1

2-[4-Chloro-7-(1-ethyl-3,3-dimethyl(indolin-2-ylidene)]-3,5-(propane-1,3-diyl)-1,3,5-heptatrien-1-y1]-1-ethyl-3,3-dimethyl-3H-indolium (Cy.7.Cl): According to a reported protocol,[²] with some modification, Cy.7.Cl was synthesized, to give 3.2 g of pure product, with yield of 91%. ¹H NMR (CDCl₃, 300 MHz): δ(ppm) 1.47 (t, 6H), 1.72-1.78 (t, 12H), 1.99 (m, 2H), 2.79 (t, 4H), 4.26-4.28 (t, 4H), 6.23-6.30 (d, 2H), 7.17-7.41 (d, 8H), 8.21-8.38 (d, 2H). ESI-HRMS: m/z Calcd 511.2875, found 511.2845
Synthesis of Cy-NO2: According to the synthesis of a series of near-infrared cyanine dyes proposed by Xiaojun Peng,\textsuperscript{3} with some modification, we synthesized the Cy-NO2. Firstly, Cy.7.Cl (0.64 g), 2-(2-nitro-lH-imidazoly1) ethylamine hydrochloride (0.20 g) and Et3N (0.22 g) were dissolved in 20 mL DMF. The mixture was heated to 55 °C slowly under Ar atmosphere for 3 h in a 50-mL round bottom flask. Then the solvent was evaporated on a rotary evaporator. The solid was purified on silica gel chromatography eluted with ethyl acetate / methanol (3:1 v/v) to give Cy-NO2 as a violet solid (55% yield).

1H-NMR (300 MHz, DMSO-d6): \(\delta\) (ppm) 1.19 (t, 6H), 1.31-1.38 (m, 2H), 1.58 (s, 12H), 1.89 (s, 1H), 2.35 (t, 4H), 3.96-3.98 (q, 4H, J = 6 Hz), 4.18-4.22 (t, 2H, J = 6 Hz), 4.76 (t, 2H, J = 6 Hz), 5.65-5.69 (d, 2H, J = 12 Hz), 7.02-7.07 (m, 4H), 7.12-7.30 (m, 2H), 7.44-7.56 (m, 4H), 7.64-7.69 (d, 2H, J = 12 Hz) ppm. 13C-NMR (300 MHz, DMSO-d6): 169.55, 167.40, 166.92, 145.10, 142.83, 140.48, 137.54, 132.14, 131.96, 129.09, 128.60, 122.93, 122.54, 119.85, 109.36, 94.50, 65.46, 50.37, 47.69, 37.88, 31.26, 31.24, 29.21, 25.56, 19.00 ppm. MS: m/z Calcd 631.37 [M-I]+, found 631.36 [M-I]+.

3. Preparation of Rat Liver Microsomes

Seven-week-old male Wistar rats were executed by cervical dislocation. The liver was washed with water and phosphate-buffered saline (PBS) containing 0.15 M KCl, pH 7.4, then was homogenized in 3 volumes of the same buffer. Microsomes were prepared according to method of Omura and Sato.\textsuperscript{4}

4. General Procedure for Nitroreductase Detection

Unless otherwise stated, all the fluorescence measurement were made in 10 mM PBS (pH = 7.4) according to the following procedure. In a 5 mL tube, 4 mL of PBS and 10 μM probe were mixed. The solution was degassed with nitrogen for 30 min to remove oxygen, followed by addition of an appropriate volume of liver microsomes and NADPH. The final volume was adjusted to 5 mL with PBS. After incubation at 37 °C for 15 min, the spectra were measured (\(\lambda_{ex}/\lambda_{em} = 695/750\) nm) with 10/10 nm slit widths.
5. Absorption and Emission Spectra of Cy-NO₂ in the presence and absence of nitroreductase

![Absorption and Fluorescence Emission Spectra](image)

**Fig. S1** (a) Absorption and (b) fluorescence emission spectra ($\lambda_{ex}/\lambda_{em} = 695/750$ nm). a and a': NADH (50 μM) and liver microsomes (15 μg/mL) were added into the probe solution (10 μM); b and b': the mixture of NADH (50 μM), liver microsomes (15 μg/mL) and nitroimidazole (30 μg/mL) at 37 °C for 15 min in 10 mM PBS (pH 7.4) was added into the probe solution (10 μM); c and c': the probe solution (10 μM) as a control. Spectra were measured after reaction of probe with nitroreductase for 15 min at 37 °C in 10 mM PBS (pH 7.4).

6. The pH Dependence of the Probe Reaction

![Fluorescence Intensity vs pH](image)

**Fig. S2.** Effects of pH on the reaction between Cy-NO₂ (10 μM) and nitroreductase: a: Cy-NO₂ (10 μM) + rat liver microsomes (13 μg/mL) + NADPH (50 μM) in 10 mM PBS for 15 min at 37 °C. b: Cy-NO₂ (10 μM) + NADPH (50 μM) in 10 mM PBS for 15 min at 37 °C.
7. Fluorescence Quantum Yield ($\Phi_F$) of Cy-NO$_2$

For measurement of the quantum yield of Cy-NO$_2$, the solution of the probe was adjusted to an absorbance of $\sim$ 0.05. The emission spectra were recorded using a maximum excitation wavelength and the integrated areas of the fluorescence-corrected spectra were measured. Relative fluorescence quantum yield ($\Phi_F = 0.03$) were obtained by comparing the area under the emission spectrum of the test samples with that of a solution of IR-786 in methanol ($\Phi_F = 0.159$).[5]

8. Detecting mechanism for Cy-NO$_2$ to nitroreductase

Cy-NO$_2$ and the reaction product (Cy-NH$_2$) of Cy-NO$_2$ with nitroreductase were characterized by ESI-MS in Figure S3. A proposed detecting mechanism was shown in Scheme S2 according to reported literatures.[6,7]

Fig. S3. Mass spectrum of Cy-NO$_2$ and Cy-NH$_2$
Scheme S2. A proposed detecting mechanism for Cy-NO$_2$ to nitroreductase

9. Fluorescence Imaging of Hypoxia in HepG2 Cells by Probe

HepG2 cells were grown on glass-bottom culture dishes at 37 °C using RPMI 1640 culture medium supplemented with 10%(v/v) fetal bovine serum (FBS) and 1% penicillin-streptomycin in a humidified incubator under normoxic (95% air and 5% CO$_2$) and hypoxic. In our experiments, these cells were incubated under normoxic and hypoxic conditions at 37 °C for 6h, followed by the addition of 2 μM Cy-NO$_2$ to PBS solution, and incubated for 5 min, and then to observe the fluorescence imaging with a fluorescence microscope. The fluorescence emission signals from Cy5 channel (650-800 nm) were collected by irradiation with 633 nm light.

10. Cytotoxicity Assay

The hypoxia probe toxicity was determined by MTT assay *in vitro*. HepG2 cells were seeded in 96-well flat bottom microtiter plates at a density of 1×10$^4$ cells/mL with 100 μL per well, incubated in a humidified 5% CO$_2$ atmosphere at 37°C for 24h, then exposed to different concentrations (0-64 μM) of hypoxia probe for 48h. After treatment, 20 μL MTT solution (5 mg/mL) was added to each well and continued to incubate for 4h. The crystals were then dissolved in 100 μL dimethyl sulfoxide per well. The absorbance was measured at 490 nm with a microtiter plate reader (Becton Dickinson). Cell viability was calculated according to the following formula: Cell viability (%) = $A_{490}$ (sample)/$A_{490}$ (control) ×100. The experiments were performed at least three times.
Fig. S4. Effect of hypoxia probe on the viability of HepG2 cells: Cell viability was determined by MTT assay after treatment with different concentrations of Hypoxia probe for 48h.

11. $^1$H-NMR and $^{13}$C-NMR spectra of intermediates and Cy-NO$_2$

$^1$H-NMR of N-[2-(2-nitro-1H-imidazolyl)ethyl] phthalimide
$^1$H-NMR of 2-(2-nitro-1H-imidazolyl)ethamine hydrochloride

$^1$H-NMR of Cy-NO$_2$
$^{13}$C-NMR of Cy-NO$_2$

References


