Electronic Supplementary Information for:

Azo-based near-infrared fluorescent theranostic probe for tracking hypoxia-mediated cancer chemotherapy in vivo

Ning Ding\textsuperscript{a}, Zhao Li\textsuperscript{a} \textsuperscript{,*}, Xinwei Tian\textsuperscript{a}, Jiahang Zhang\textsuperscript{a}, Kaili Guo\textsuperscript{b} and Pan Wang\textsuperscript{b}

\textsuperscript{a} Shannxi Engineering Laboratory for Food Green Processing and Safety Control, College of Food Engineering and Nutritional Science, Shaanxi Normal University, Xi'an 710062, China.

\textsuperscript{b} Ministry of Education Key Laboratory of Medicinal Resources and Natural Pharmaceutical Chemistry, National Engineering Laboratory for Resource Developing of Endangered Chinese Crude Drugs in Northwest of China, College of Life Sciences, Shaanxi Normal University, Xi’an 710062, China.

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1. Apparatus and reagents

Apparatus. Electrospray ionization mass spectrum (ESI-MS) were carried out on a Shimadzu LC-MS 2010A instrument (Kyoto, Japan). $^1$H NMR and $^{13}$C NMR were obtained by Bruker DMX-600 spectrometer using CD$_3$OD as solvent. Ultraviolet-visible (UV-Vis) absorption spectra were measured by a Hitachi U-3010 spectrophotometer (Kyoto, Japan). And fluorescence spectra were performed witha HITACHI F-7000 fluorescence spectrophotometer (Hitachi Limited Ltd., Japan). Fluorescence imaging of the cells was performed with a confocal laser scanning microscope (Leica, Germany) at the excitation wavelength of 635 nm. In vivo imaging was subjected to IVIS Spectrum imaging (Xyngon, Americ) with the mean fluorescence flux (p/sec/cm$^2$/sr).

Reagents. IR-780 iodide was purchased from Sigma-Aldrich Co. Ltd. 3-Nitrophenol, N-phenyldiethanolamine, trifluoroacetic acid, sulfamic acid, tin (II) chloride and sodium dithionite were purchased from Acros Organics Co. Ltd. Dulbecco's modified eagle's medium (DMEM), fetal bovine serum (FBS), phosphate buffered saline buffer solution (PBS), penicillin and streptomycin were obtained from HyClone (South Logan, UT, U.S.A.). The stock solution of probe 1 (1 mM) was prepared with deoxygenated dimethyl sulfoxide (DMSO). Flash column chromatography and thin lay chromatography (TLC) were carried out with 200-300 mesh silica gel. All other chemicals used were of analytical grade without purification. The 7-8 weeks old female BALB/c mice were obtained from the Experimental Animal Center of the Air Force Medical University (Xi’an, China). All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Shaanxi Normal University and approved by the Animal Ethics Committee of Shaanxi Normal University.

2. Synthesis of probe 1

2.1 Synthesis of compound 2. The fluorophore AXPI was synthesized by a previous reported method. To a stirred solution of AXPI (30 mg, 0.073 mmol) in CH$_3$CN/CH$_2$Cl$_2$/TFA (v/v/v, 1/4/0.01, 5 mL) at 0 °C under N$_2$ atmosphere, NaNO$_2$ (8 mg, 0.12 mmol) was added and the solution was
stirred for 15 min after which amidosulfonic acid (12 mg, 0.12 mmol) was added and the solution was stirred for 7 min. Subsequently, N-phenyldiethanolamine (40 mg, 0.22 mmol) dissolved in CH$_3$CN (1 mL) was added to the reaction mixture and the solution was stirred at 0 °C under N$_2$ atmosphere for 1.5 h. The mixture was diluted with CH$_2$Cl$_2$ and then washed with pure water, the organic layer was dried over sodium sulfate anhydrous and concentrated under reduced pressure. The residue was separated by column chromatography using CH$_2$Cl$_2$/Acetone/MeOH (v/v/v, 100/1/0~60/40/1) as eluent, obtaining the blue-green solid product (25.6 mg, 0.042 mmol, yield 58.2%). The $^1$H NMR, $^{13}$C NMR and LC-MS spectra of intermediate compound 2 is given in Figure S1-S3, respectively. $^1$H NMR (600 MHz, CD$_3$OD) δ 8.36 (d, J = 15.0 Hz, 1H), 7.71-7.66 (m, 2H), 7.59 (d, J = 8.9 Hz, 2H), 7.52-7.44 (m, 3H), 7.38 (dd, J = 5.7, 2.7 Hz, 1H), 7.33 (s, 1H), 7.13 (s, 1H), 6.71 (d, J = 9.0 Hz, 2H), 6.13 (d, J = 15.0 Hz, 1H), 4.06-3.99 (m, 2H), 3.82 (t, J = 5.9 Hz, 4H), 3.68 (dd, J = 12.1, 6.2 Hz, 4H), 2.63-2.58 (m, 2H), 2.38-2.32 (m, 2H), 1.80 (d, J = 8.9 Hz, 6H), 1.77 (dd, J = 14.8, 7.4 Hz, 6H), 1.26 (dt, J = 14.3, 6.3 Hz, 6H), 1.00 (dt, J = 10.0, 6.8 Hz, 3H). $^{13}$C NMR (151 MHz, CD$_3$OD) δ 210.14, 179.40, 173.02, 161.04, 155.88, 154.55, 153.43, 146.74, 144.41, 143.47, 142.59, 140.02, 131.83, 130.37, 129.51, 129.02, 126.89, 124.11, 116.19, 114.42, 113.03, 108.26, 106.39, 61.57, 60.36, 54.91, 52.26, 47.98, 40.25, 30.74, 28.41, 22.31, 11.65. ESI-MS: C$_{38}$H$_{43}$N$_4$O$_3$+ M*, m/z Calculated: 603.33, Found: 603.3325.

2.3 Synthesis of probe 1. To a stirred solution of intermediate compound (15 mg, 0.025 mmol) in CH$_2$Cl$_2$ (5 mL), SOCl$_2$ (30 μL, 0.26 mmol) was added to the reaction mixture and the reaction solution was heated at 70 °C under reflux for 1.5 h. The solution was cooled to room temperature and MeOH (5 mL) was added, after which the mixture was concentrated under reduced pressure. The reaction solution was separated by column chromatography using MeOH/CH$_2$Cl$_2$ (v/v, 1/100~1/10) as eluent, affording 2-((E)-2-6-((Z)-(4-(bis(2-chloroethyl)amino)phenyl)diazenyl)-2,3-dihydro-1H-xanthen-4-yl)vinyl)-3,3-dimethyl-1-propyl-3H-indolium (probe 1) as a blue-green solid product (14.8 mg, 0.023 mmol, yield 93.1%). The $^1$H NMR, $^{13}$C NMR and LC-MS spectra of probe 1 are
given in Figure S4-S6, respectively. $^1$H NMR (600 MHz, CD$_3$OD) δ 8.60 (d, J = 15.1 Hz, 1H), 7.77 (t, J = 8.3 Hz, 3H), 7.71 (d, J = 6.7 Hz, 1H), 7.57 (s, 2H), 7.54-7.47 (m, 3H), 7.28 (s, 1H), 6.82 (d, J = 8.5 Hz, 2H), 6.39 (d, J = 14.9 Hz, 1H), 4.21 (t, J = 7.0 Hz, 2H), 3.91 (d, J = 6.1 Hz, 4H), 3.79 (t, J = 6.3 Hz, 4H), 2.73 (s, 2H), 2.54 (s, 2H), 1.88 (t, J = 7.0 Hz, 2H), 1.29 (s, 6H), 1.26-1.20 (m, 2H), 1.06 (t, J = 7.1 Hz, 3H). $^{13}$C NMR (151 MHz, CD$_3$OD) δ 210.09, 180.00, 154.70, 151.79, 147.28, 145.48, 143.72, 142.74, 132.85, 132.30, 131.31, 130.35, 129.54, 129.08, 126.94, 124.55, 123.95, 122.03, 116.39, 114.47, 113.20, 108.62, 106.65, 61.55, 54.18, 52.43, 48.03, 41.68, 30.70, 28.28, 24.24, 22.41, 11.62. ESI-MS: C$_{38}$H$_{41}$Cl$_2$N$_4$O$^+$ M$^+$, m/z Calculated: 639.27, Found: 639.2643.

Fig. S1 $^1$H NMR spectrum of intermediate compound 2 in CD$_3$OD.
**Fig. S2.** $^{13}$C NMR spectrum of intermediate compound 2 in CD$_3$OD.

**Fig. S3** Electrospray ionization mass spectrum (ESI-MS) of intermediate compound 2.
Fig. S4 $^1$H NMR spectrum of probe 1 in CD$_3$OD.

Fig. S5 $^{13}$C NMR spectrum of probe 1 in CD$_3$OD.
3. Spectroscopic evaluations

The sodium dithionite (20 mM) mediated azoreductuction of probe 1 (10 μM) was investigated in PBS buffer solution (10 mM, pH 7.4, 1% DMSO) at 37 °C. Then fluorescence spectra of probe 1 were recorded at the excitation of 670 nm (slit ex/em 10/10) and mission was detected at 705 nm.

In order to optimize the reaction time of probe 1, we evaluated its fluorescence spectra at various times (0, 4, 8, 12, 16, 20, 24, 28, 32, 36 and 40 min) and the results showed that probe 1 reached its maximum peak and fluorescence saturation at 40 min.

Fig. S6 Electrospray ionization mass spectrum (ESI-MS) of probe 1.
4. Electrospray ionization mass spectrum of the reaction solution of probe 1

**Fig. S7** ESI-MS of probe 1 (10 μM) was reaction with sodium dithionite (20 mM) at 37 °C for 40 min.
5. Selectivity of probe 1

![Fluorescence responses of probe 1 (10 µM) to various reductants: control (blank), 1 mM of MgCl₂, CaCl₂, ZnSO₄, FeCl₂, FeCl₃, glucose, arginine (Arg), vitamin C (Vc), 100 µM of NO₂⁻, ClO⁻, ·OH, hydrogen peroxide (H₂O₂), Serine (Ser), phenylalanine (Phe), glutathione (GSH), cysteine (Cys), homocysteine (Hcy), tyrosinase (Tyr), Nicotinamide adenine dinucleotide (NADH), and 20 mM of sodium dithionite (Na₂S₂O₄). The results are the mean ± standard deviation of three separate measurements. λex/em= 670/705 nm.](image)

**Fig. S8** Fluorescence responses of probe 1 (10 µM) to various reductants: control (blank), 1 mM of MgCl₂, CaCl₂, ZnSO₄, FeCl₂, FeCl₃, glucose, arginine (Arg), vitamin C (Vc), 100 µM of NO₂⁻, ClO⁻, ·OH, hydrogen peroxide (H₂O₂), Serine (Ser), phenylalanine (Phe), glutathione (GSH), cysteine (Cys), homocysteine (Hcy), tyrosinase (Tyr), Nicotinamide adenine dinucleotide (NADH), and 20 mM of sodium dithionite (Na₂S₂O₄). The results are the mean ± standard deviation of three separate measurements. λex/em= 670/705 nm.

6. Fluorescence imaging in living cells

4T1 and HepG2 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37 °C in 5% CO₂ incubator. Before fluorescence imaging, the cells were subcultured into confocal plates (20 mm) and incubated for 12 h for cells adherence, and then medium was changed and cultured for 4 h under various oxygen conditions (21%, 15%, 10%, 5% and 1% O₂) at 37 °C. Afterward, the cells and probe 1 (10 µM) were incubated at 37 °C for 40 min and then rinsed with PBS to remove the remaining probe. The averaged fluorescence intensity of image was quantitatively measured by ImageJ software [version 1.37c, National Institutes of Health (NIH)] at least 10 cells were imaged in each cell lines.
**Fig. S9** Relative pixel intensity measurements obtained from the images of 4T1 and HepG2 cells: the two kinds of cells were incubated with the 10 μM probe 1 under normoxic (21% O₂) and hypoxic (15%, 10%, 5% and 1% O₂) conditions. The strongest fluorescence intensity of HepG2 cells from the image (j) is defined as 1.0. The results are the mean ± standard deviation of three separate measurements.

**Fig. S10** Confocal fluorescence images of 4T1 and HepG2 cells under hypoxic (1% O₂) conditions. (a, c) 4T1 and HepG2 cells were incubated with only 10 μM probe 1. (b, d) 4T1 and HepG2 cells were pretreated with 20 mM Na₂S₂O₄, then incubated with 10 μM probe 1. Scale bar: 30 μm (4T1 cells); Scale bar: 20 μm (HepG2 cells).
7. Cytotoxicity analysis

![Graph showing cell viability percentages under normoxic and hypoxic conditions for BJ, 4T1, and HepG2 cells with varied concentrations of probe 1.](image)

**Fig. S11** Effects of probe 1 with varied concentrations (0, 10, 20 μM) on the viability of BJ, 4T1 and HepG2 cells under normoxic (21% O₂) and hypoxic (1% O₂) conditions. The viability of the BJ cells without probe 1 under normoxic conditions is defined as 100%. The results are the mean ± standard deviation of six separate measurements.

8. Fluorescence imaging in 4T1-bearing mouse models

In order to establish the 4T1 breast cancer model, six- to seven-week-old female BALB/c mice were inoculated 100 μL of suspension containing 4T1 cells (approximately 8×10⁵/mL) directly into the left axilla of the mice. Under the guidelines of the Institutional Animal Care and Use Committee, BALB/c mice were nursed and normally fed for 14-16 days and subsequently carried out fluorescence imaging in living mice.

For fluorescence imaging *in vivo*, mice were fasted for 12 h to avoid the effect of food on imaging results. During the imaging process, the mice were anesthetized with isoflurane gas by inhaling. Instantly, probe 1 (500 μM in 100 μL PBS) was injected into 4T1-bearing mice via tail vein. Fluorescence images were collected 0.5 h, 1 h, 3 h, 6 h, and 12 h after administration of the final does of probe 1 by the IVIS imaging system.
Fig. S12 Relative pixel intensity measurements obtained from the images of tumor site at time point of 0.5, 1, 3, 6 and 12 h after injection.

9. Histochemical analysis.

After 14 days of chemotherapy, the mice were dissected, and the main organs (heart, liver, spleen, stomach and kidney) were collected and fixed with 4% paraformaldehyde to prepare samples for H&E staining. Before H&E staining, these tissues were embedded in paraffin and then stained with H&E using standard procedures. Finally, stained images were collected under an optical microscope.

Fig. S13 The weight of dissected solid tumor at termination. The significance was assessed by a two sided t-test: **P < 0.01, n = 6.