Supporting Information

A high selectivity fluorescent probe for hypoxia imaging in cells and tumor-bearing mice model

Yue Wang,[a,d] Xiaoyue Han,[a] Xia Zhang, [a,d] Li Zhang,[a,d] Lingxin Chen*[a,b,c]

[a] *CAS Key Laboratory of Coastal Environmental Processes and Ecological Remediation, Yantai Institute of Coastal Zone Research, Chinese Academy of Sciences, Yantai 264003, China. E-mail: lxchen@yic.ac.cn (L Chen)*

[b] *School of Pharmacy, Binzhou Medical University, Yantai 264003, China.*

[c] *Center for Ocean Mega-Science, Chinese Academy of Sciences, Qingdao 266071, China.*

[d] *University of Chinese Academy of Sciences, Beijing 100049, China.*

Contents:

1. Materials and instruments

Materials: The solution of the probe hTP-NNO_2 (1 mM) was dissolved in dimethyl sulfoxide (DMSO) as stock solution and maintained in refrigerator at 4 °C. All the reagents were obtained from Aladdin (USA), unless indicated otherwise. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma-Aldrich, and the nucleic acid stain (Hoechst 33342) was purchased from Invitrogen Corporation. DMEM (Dulbecco's Modified Eagle Medium, #SH30022.01) was purchased from HyClone, and DMEM (no Glucose, #90113-500) was purchased from Solarbio. Fetal Bovine Serum (FBS, #C0252) was purchased from Beyotime. The stock solution of Fe^{3+}, Ca^{2+}, Na^{+}, K^{+}, Mg^{2+}, vitamin C, H_2O_2, H_2S_n, glutathione, homocysteine, tyrosine, serum albumin (BSA), dithiothreitol, NADH, and cysteine were prepared and used when needed. CCK-8 kit was provided by Dojindo.

Instruments: Absorption spectra were determined by UV-vis spectrometer evolution 200 (Thermo Scientific). Fluorescence spectra were collected on a HORIBA Scientific Fluoromax - 4 spectrofluorometer. All pH measurements were performed by a basic pH-Meter PH-3C digital pH-meter (Lei Ci Device Works, Shanghai, China) with a combined glass-calomel electrode. ^{1}H and ^{13}C NMR spectra were taken on a Bruker spectrometer. The one-photon fluorescence images of cells were acquired using a LTE confocal laser scanning microscope (Olympus FV1000 confocal laser-scanning microscope). The two-photon fluorescence images of cells were collected using a two-photon laser confocal microscope (Zeiss LSM 880). Flow cytometry data were collected by BD Biosciences FACSAria. Absorbance was measured in a TECAN infinite M200 PRO microplate reader in the MTT assay. Mass spectra were taken on LCQ Fleet LC-MS System (Thermo Fisher Scientific). ^{1}H NMR spectra was obtained on a Bruker spectrometer. Mice fluorescence images were imaged by Bruker In-vivo Imaging System.

2. General methods

Spectrophotometric Measurements: Fluorescence and absorption spectra were measured in 10 mM HEPES. The pH gradient of HEPES buffer solution from 4.0 to 10.0 was achieved by adding different volumes of HCl or NaOH solution. Absorption spectra were collected with 1.0-cm glass cells. Spectral detection was performed as following: 1 mL various volumes of NTR was added into a 10.0 mL color comparison tube and diluted to 10.0 mL (0 - 20 μg / mL) with HEPES buffer. The probe (10 μM) was added. All spectroscopic experiments were carried out at room temperature.

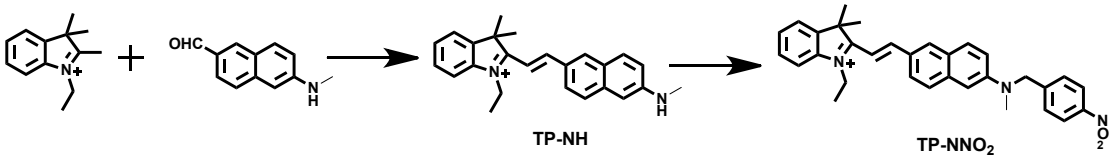
MTT assay: Cells were grown in an atmosphere of 5% CO_2 and 95% air at 37 ^oC. Then the cells were seeded into 96-well plates at a density of 1×10^5 cells/mL in 100 μL medium and allowed to adhere for 24 hours. Then the cells were incubated for 24 h at 37 °C in a 5 % CO_2 / 95 % air upon different concentrations probe of 0 μM to 70 μM respectively. Then MTT solution was added to each well and the plate was shaken and the absorbance was measured at 450 nm using a microplate reader (TECAN infinite M200pro).

Flow Cytometry Analysis: The cells were cultured at 2.0×10^5 cells / well in 6-well plates, and then the cells were treated as described in the paper. After harvest, cells were washed for three times with PBS, and then analyzed by flow cytometry.

Imaging Mice *in vivo*: A Bruker In-vivo Imaging System was employed to image tumor. The mice were anesthetized prior to imaging. After *in vivo* imaging, the organs (lung, heart, liver, kidney and spleen) and tumors were excised to perform *ex vivo* imaging.

3. Synthesis steps of Compounds

Scheme S1. Synthetic routes for probe **hTP-NNO$_2$**.



Synthesis of compound TP-NH. The synthesis of compound **TP-NH** was reported by our lab and shown in the reference.[1]

4. Effect of pH values on probe

The experiments were performed in 10 mM HEPES solution, and the concentrations of compounds were 10 μM. As shown in Fig. S1, hTP-NNO_2 and TP-NH were stable under different pH range from 4.0 to 9.0. On the basis, our probe hTP-NNO_2 was stable for NTR detection.

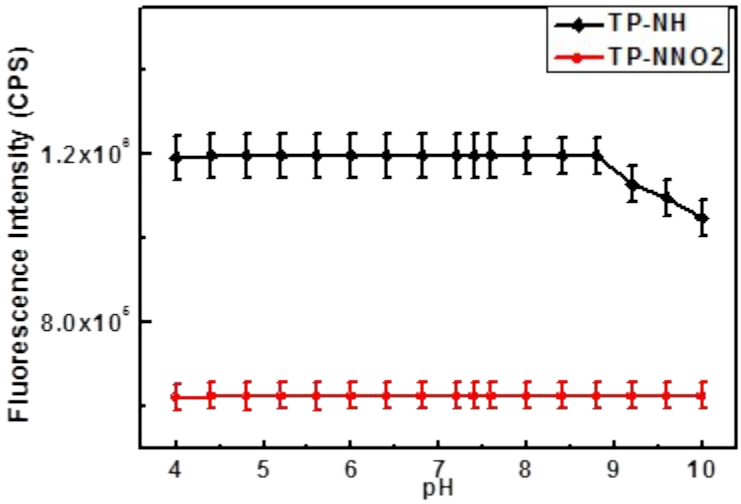


Fig. S1. Effect of pH values. The fluorescence emission of hTP-NNO_2 and TP-NH under different pH. pH: 4.0, 4.4, 4.8, 5.2, 5.6, 6.0, 6.4, 6.8, 7.2, 7.4, 7.6, 8.0, 8.4, 8.8, 9.2, 9.6, 10.0.

5. MTT assays for probe

To access the potential toxicity of hTP-NNO_2 and TP-NH, MTT assays were carried out. A549 cells and SH-SY5Y cells (10^6 cells/mL) were planted into 96-well microtiter plates with 10% fetal bovine serum (FBS). Plates were maintained at 37°C in a 5 % CO_2 / 95 % air incubator for 12 h. Then the cells were incubated for 24 h at 37 °C with different concentrations probe of 0 - 70 μM respectively. MTT solution was added to each well. Then the absorbance was measured in a TECAN infinite M200pro microplate reader.

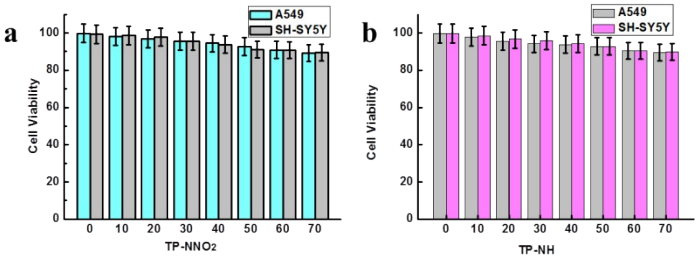


Fig. S2. Cytotoxicity assay. A549 cells and SH-SY5Y cells were treated with different concentrations of hTP-NNO_2 and TP-NH. The experiments were repeated five times and the data were shown as mean (±S.D.). The data were shown as mean (± s.d.) (n = 5).

6. Bright-field and nuclear Images of Fig. 2

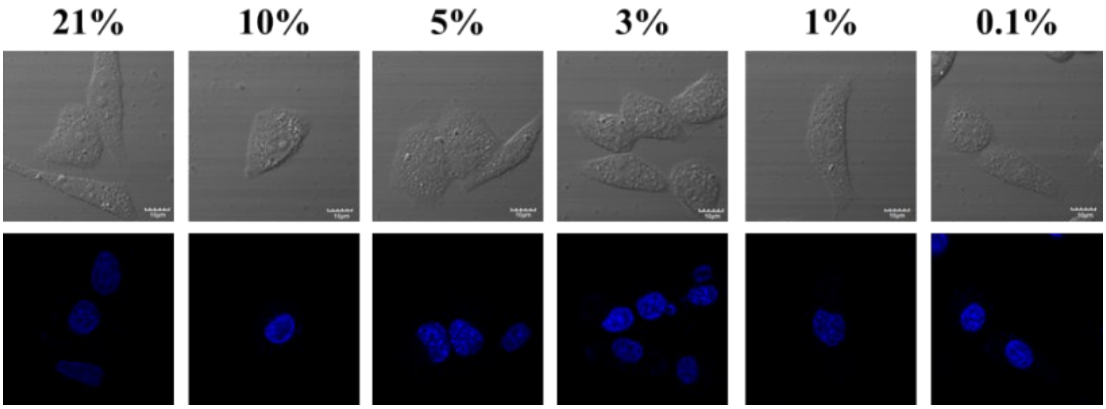


Fig. S3. Bright-field and nuclear images of Fig. 2.

7. Bright-field and nuclear Images of Fig. 3

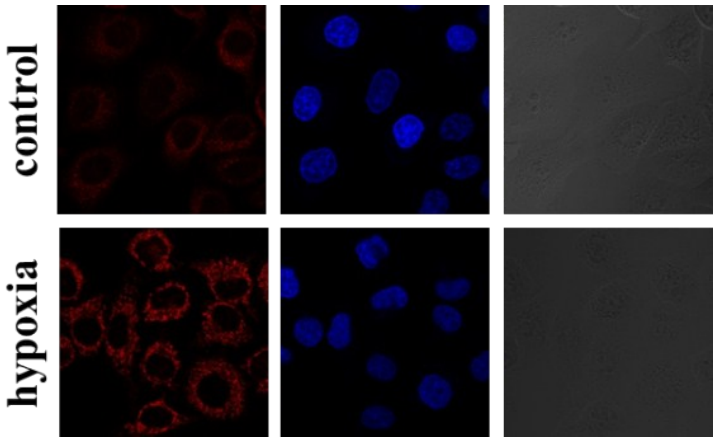


Fig. S4. Bright-field and nuclear images of Fig. 3.

8. Bright-field Images of Fig. 4

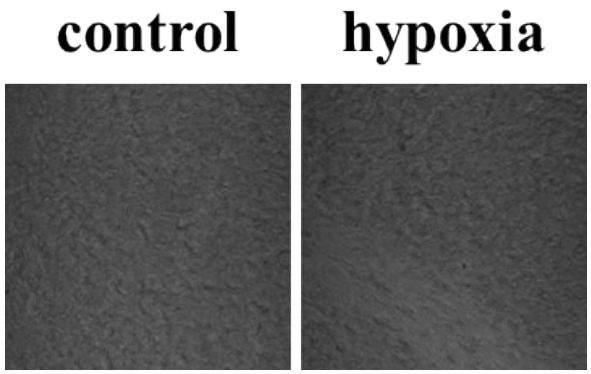
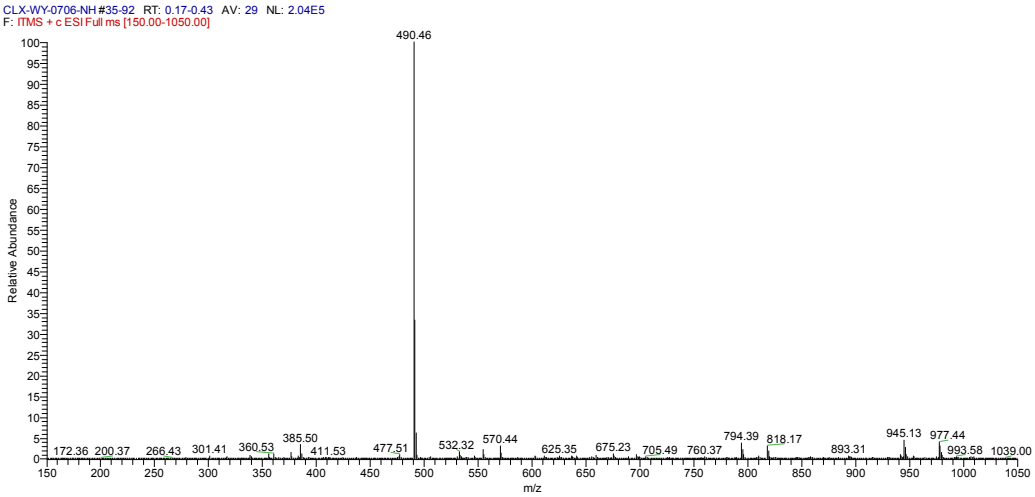
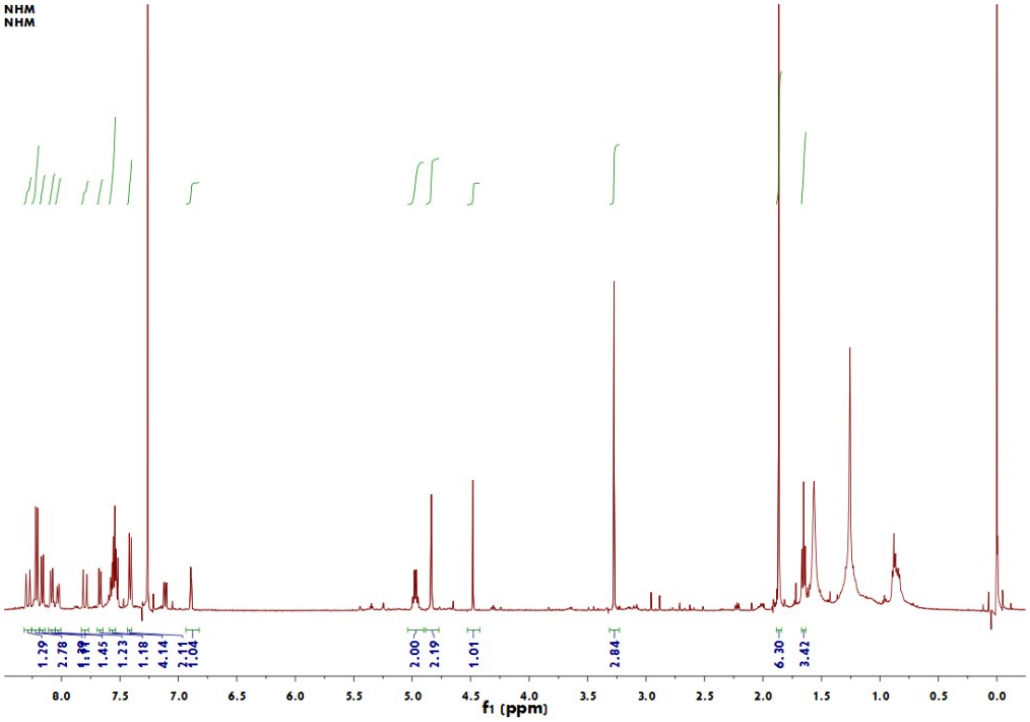


Fig. S5. Bright-field images of Fig. 4.

9. LC-MS and ^{1}H NMR of hTP-NNO$_2$





10. Reference

(1) Y. Wang, L. Zhang, S. Zhang, Z. Liu, L. Chen. *Anal. Chem.* 2019, **91**, 7812-7818