

## Supplementary Information for

### **Hypoxia-triggered single molecule probe for high-contrast NIR II/PA tumor imaging and robust photothermal therapy**

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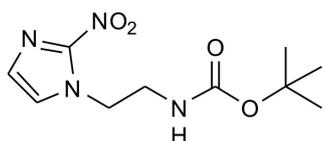
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#### **Experimental section**

**Materials and apparatus:** N,N-Dimethylformamide (DMF), dichloromethane, 2-nitro-1H-imidazole, tert-butyl N-(2-bromoethyl)carbamate, NADH and other chemical reagents were purchased from J&K scientific Reagent Ltd (Beijing, China). IR-1048, Pimonidazole antibody (PIMO) Nitroreductase from *Escherichia coli* were purchased from Sigma-Aldrich Co. Ltd. (St.

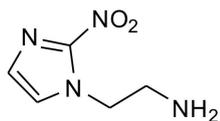
Louis, MO, USA). DMEM medium, fetal bovine serum (FBS) and kanamycin sulfate were purchased from Invitrogen Co. Ltd. Deionized water was used to prepare all aqueous solutions. All reagents were purchased from commercial suppliers and used without further purification. Solvents used were purified by standard methods prior to use. A549 cells were kindly provided by the Cell Center of our institute. UV-Vis spectra were obtained using a Scinco 3000 spectrophotometer (1 cm quartz cell) at 37 °C. NIR II fluorescence spectra were recorded on FSP920 spectrofluorometer (Edinburgh Instruments, the UK). HPLC was performed on an Ultimate 3000 HPLC system (Dionex, USA) with a Hypersil ODS2 column (250 × 4.6 mm, 5 μm, ThermoFisher). The NMR were recorded on a JMS-HX 110A/110A Tandem High Resolution Mass Spectrometer (JEOL). The <sup>1</sup>H NMR spectra were obtained on a Bruker AM-400 spectrometer. UV-Vis spectra were obtained using a Scinco 3000 spectrophotometer (1 cm quartz cell) at 37 °C. NIR II fluorescence images were taken by a Maestro<sup>TM</sup> *in vivo* fluorescence imaging system (Cambridge Research & Instrumentation, Inc. USA). 3D PA imaging was detected with preclinical photoacoustic computerized tomography scanner (Endra Nexus 128, Ann Arbor, MI). The real-time temperature change of tumors region and infrared thermographic maps were obtained using a Ti27 infrared thermal imaging camera (Fluke, USA)



### Synthesis and characterization of tert-butyl

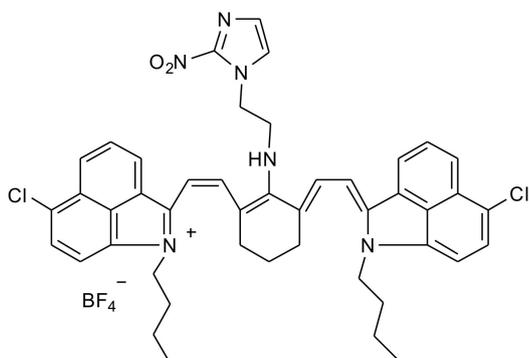
**2-(2-nitroimidazolyl)ethyl carbamate (MZ-BOC).** K<sub>2</sub>CO<sub>3</sub> (0.915 g, 6.63 mmol) was added to a stirring solution of 2-nitro-1H-imidazole (0.5 g,

4.42 mmol) in DMF (2 mL). Tert-butyl N-(2-bromoethyl)carbamate (0.99 g, 4.42 mmol) was then added dropwise, and the reaction mixture was stirred at room temperature overnight. The reaction mixture was then filtered, the solid obtained was washed with MeOH, and residual solvent was evaporated. The solid obtained was dissolved in water, extracted with ethyl acetate, and the organic layer was evaporated in vacuo to obtain the solid. Finally the crude compound was recrystallized from ethyl acetate to afford pure compound MZ-BOC as a dark yellow solid (0.10 g, 89%). <sup>1</sup>H NMR (400 MHz, Methanol-d<sub>4</sub>) δ 7.37 (s, 1H), 7.14 (s, 1H), 4.58–4.53 (t, J = 5.6 Hz, 2H), 3.51 (t, J = 5.6 Hz, 2H), 1.38 (s, 9H). <sup>13</sup>C NMR (101 MHz, Methanol-d<sub>4</sub>) δ 127.67, 127.04, 125.54, 122.79, 78.99, 49.85, 39.51, 29.62, 28.64, 27.34. HRMS (ESI<sup>+</sup>): m/z calcd for C<sub>10</sub>H<sub>16</sub>N<sub>4</sub>O<sub>4</sub>: 279.1064 [M + Na<sup>+</sup>], found 279.1060.



### Synthesis and characterization of 2-(2-nitroimidazolyl)ethylamine (MZ).

A sample of 1.25 M HCl in MeOH (2 mL) was added to a solution of MZ-BOC (0.85 g, 3.3 mmol) in MeOH (2 mL) was stirred at room temperature under Nitrogen atmosphere. After 5 h the resulting product MZ was filtered and washed with MeOH and evaporated in vacuo. The solid obtained was recrystallized from MeOH to afford pure compound MZ as a pale yellow solid (0.64 g, 89%).  $^1\text{H}$  NMR (400 MHz, Methanol-d<sub>4</sub>)  $\delta$  7.50 (s, 1H), 7.17 (s, 1H), 4.53 (t,  $J = 6.3$  Hz, 2H), 3.09 (t,  $J = 6.3$  Hz, 2H).  $^{13}\text{C}$  NMR (101 MHz, Methanol-d<sub>4</sub>)  $\delta$  162.87, 144.28, 127.21, 51.67, 41.01. HRMS (ESI<sup>+</sup>):  $m/z$  calcd for C<sub>5</sub>H<sub>8</sub>N<sub>4</sub>O<sub>2</sub>: 157.0720 [M + H<sup>+</sup>], found 157.0725; 179.0539 [M + Na<sup>+</sup>], found 179.0537.



### Synthesis and characterization of IR1048-MZ.

The IR-1048 (37 mg, 0.05 mmol) was dissolved in anhydrous DMF (10 mL). Then, 2-(2-nitroimidazolyl)ethylamine (112.6 mg, 0.5 mmol) was added to it immediately and stirred dramatically. The mixture was stirred at 40 °C for 5h under Nitrogen atmosphere. Finally, the solvent was evaporated under reduced pressure to give crude products, which was further purified by silica gel column chromatography with the dichloromethane and methanol to afford pure product IR1048-MZ. Yield: 6.44 mg (23%).  $^1\text{H}$  NMR (400 MHz, Methanol-d<sub>4</sub>)  $\delta$  8.63 (d,  $J = 7.5$  Hz, 1H), 8.17 (d,  $J = 7.6$  Hz, 1H), 8.11 (d,  $J = 8.2$  Hz, 1H), 8.01 (d,  $J = 12.8$  Hz, 1H), 7.96–7.84 (m, 5H), 7.72 (s, 1H), 7.51 (d,  $J = 7.8$  Hz, 1H), 7.14 (s, 1H), 7.10 (d,  $J = 8.4$  Hz, 1H), 7.06 (s, 1H), 6.90 (d,  $J = 7.8$  Hz, 1H), 6.15 (d,  $J = 12.9$  Hz, 1H), 4.29–4.23 (m, 2H), 4.08–4.03 (m, 2H), 1.82–1.77 (m, 2H), 1.47 (td,  $J = 15.3, 14.4, 7.8$  Hz, 4H), 1.12 (dq,  $J = 14.5, 7.7$  Hz, 4H), 1.01 (t,  $J = 7.4$  Hz, 4H), 0.92 (dt,  $J = 18.6, 7.2$  Hz, 6H), 0.76–0.70 (m, 1H), 0.59 (t,  $J = 7.2$  Hz, 3H).  $^{13}\text{C}$  NMR (101 MHz, MeOD)  $\delta$  156.41, 156.07, 148.49, 144.62, 141.97, 141.62, 141.17, 137.59, 132.32, 131.60, 130.90, 129.68, 129.53, 129.45, 128.78, 128.72, 128.67, 128.62, 128.33, 127.12, 126.59, 125.17, 124.87, 123.08, 122.63, 121.92, 110.00, 104.02, 99.89, 54.45, 42.41, 31.67, 30.96, 29.67, 29.35, 26.71, 25.80, 22.34, 19.88, 19.78, 17.33, 15.89, 12.88, 12.74, 11.78. HRMS (ESI<sup>+</sup>):  $m/z$  calcd for C<sub>45</sub>H<sub>45</sub>BCl<sub>2</sub>F<sub>4</sub>N<sub>6</sub>O<sub>2</sub>: 771.2976 [M – BF<sub>4</sub><sup>-</sup>]; found 771.2975.

### **Synthesis and characterization of IR1048-MZ.**

Step 1: To a flask containing 2-aminoimidazole sulfate (1 g, 3.8 mmol) in DMF (20 mL) was added sodium hydride (0.65 g, 16.3 mmol) in small portions with stirring. The temperature was maintained below 30°C during the addition by means of an ice bath. After stirring for 30 minutes at 25-30°C, the solution was cooled to 0 °C and (2-bromo-ethyl)-carbamic acid tert-butyl ester (1.7 g, 7.6 mmol) in DMF (1 mL) was added. After stirring for 1 min the cooling bath was removed and the solution was stirred toward rt for 16 h. The reaction was carefully quenched with water (10 mL), extracted with EtOAc (4x10 mL), dried over sodium sulfate, filtered, and concentrated to a residue. The residue was stored at < 0.5 mm Hg for 24 h to remove residual DMF, then purified by normal phase chromatography to give compound MZH-BOC.

Step 2: To a flask containing MZH-BOC (11.3 mg, 0.05 mmol) in dichloromethane (2 mL) at 30 °C was added TFA (2 mL). The the solvent was evaporated under reduced pressure then concentrated to a residue, stored at a vacuum oven at 30 °C for 24 h, and used directly without further purification.

Step 3: The IR-1048 (37 mg, 0.05 mmol) was dissolved in anhydrous DMF (10 mL). Then, MZH (63.1 mg, 0.5 mmol) was dropped to it and stirred dramatically. The mixture was stirred at 30 °C for 4 h under Nitrogen atmosphere. Finally, the solvent was evaporated under reduced pressure to give crude products, which was further purified by silica gel column chromatography with the dichloromethane and methanol to afford pure product IR1048-MZH. Yield: 4.56 mg (11%). HRMS (ESI<sup>+</sup>): m/z calcd for C<sub>45</sub>H<sub>47</sub>BCl<sub>2</sub>F<sub>4</sub>N<sub>6</sub>: 741.3234 [M – BF<sub>4</sub><sup>-</sup>]; found 741.3216.

### **Spectroscopic study of the probe to NTR**

The UV-vis and NIR II fluorescence spectra were acquired in 10 mM HEPES buffer solution at 37 °C. The NIR II fluorescence spectra were obtained on a FSP920 spectrofluorometer. Xe900 was selected as light source. Emission1 mono was set to NIR grating. Em1 Detector was set to Ge. The excitation and emission slits were fixed at 20 nm and 25 nm, respectively. The excitation wavelength ( $\lambda_{ex}$ ) was 980 nm, and the emission spectra were scanned over the spectra range of 1010–1200 nm. Fluorescence responses of 5  $\mu$ g/mL IR1048-MZ to different concentrations (0, 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10  $\mu$ g/mL) of NTR (500  $\mu$ M NADH as a coenzyme) were recorded respectively. All samples were measured in PBS in the presence of 1% DMSO as a co-solvent.

### **HPLC for Nitroreductase Detection Assay.**

HPLC was performed on an Ultimate 3000 HPLC system (Dionex, USA) with a Hypersil ODS2 column (250 × 4.6 mm, 5 μm, ThermoFisher). The conditions were as follows: volume ratio of methanol/H<sub>2</sub>O = 100:0 (0 min) to 20:80 (20 min); flow rate 1 mL min<sup>-1</sup>; detection under UV light at 254 nm.

### **Fluorescence quantum yield (ΦF) of IR1048-MZ**

For the determination of the quantum yield of IR1048-MZ, the solution of the probe was adjusted to an absorbance of ~ 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 (ΦF = 1.88E-05) were obtained by using the solution of IR-1048 in ethanol as a reference solution (ΦF = 0.001) [1-2].

### **Photothermal Effect and Calculation of the Photothermal Conversion Efficiency**

The solutions of 5 μg/mL IR1048-MZ with different concentrations of NTR (0, 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 μg/mL) and 500 μM NADH as a coenzyme in 0.5 mL glass vials were irradiated by 980 nm laser (0.1 W/cm<sup>2</sup>) for 2 min, respectively. Meanwhile, the temperature of solutions were recorded using a thermometer at an interval of 10 s. To assess the photothermal conversion efficiency, the solutions of 5 μg/mL IR1048-MZ with 10 μg/mL NTR and 500 μM NADH in quartz cuvettes were irradiated at 980 nm laser (0.1 W/cm<sup>2</sup>). When the temperature reached a plateau, the irradiation was removed for cooling down to room temperature. The temperature of the solutions was recorded at an interval of 30 s, and then their photothermal conversion efficiencies were calculated according to the calculation method in previous literature [3-4].

### **Test for probe selectivity**

The test solutions for bioactive small molecules selectivity were: 5 μg/mL IR1048-MZ and 500 μM NADH in HEPES (10 mM, pH 7.40) with various species: blank (IR1048-MZ + NADH), NTR (10 μg/mL), Vc (vitamin C, 2 mM), glucose (50 mM), GSH (1 mM), DTT (DL-Dithiothreitol, 2 mM), Cys (cysteine, 2 mM), Arg (arginine, 2 mM), <sup>-</sup>OC1 (10 μM), H<sub>2</sub>O<sub>2</sub> (10 μM), KCl (20 mM), CaCl<sub>2</sub> (2 mM), NaCl (20 mM), and MgCl<sub>2</sub> (1 mM). After incubation at 37 °C for 30 min, the test solutions were subjected to fluorescence measurement.

## **Cell Culture.**

Cells were cultured in DMEM medium containing 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin in a humidified atmosphere with 5% CO<sub>2</sub> at 37 °C. The cells were maintained in an exponential growth phase by periodic subcultivation. The cell density was determined using a hemocytometer, and this was performed prior to any experiments. A549 cells for imaging were initially seeded in 35 mm glass bottom dishes (P35G-0-10-C, MatTek Corp.) at a density of 5000 cells/well with medium containing 10 % FBS overnight.

## ***In Vitro* Cytotoxicity**

The cytotoxicity of IR1048-MZ was evaluated by the standard MTT assay. Briefly, A549 cells were seeded in 96-well U-bottom plates at a density of 7000 cells/well, and incubated with IR1048-MZ at varied concentrations (0–100 µg/mL) at 37 °C for 24 h. Then, the culture media were discarded, and 100 µL of the MTT solution (0.5 mg/mL in DMEM) was added to each well, followed by incubation at 37 °C for 4 h. The supernatant was abandoned, and 100 µL of DMSO was added to each well to dissolve the formed formazan. After shaking the plates for 10 min, absorbance values of the wells were read on a microplate reader at 490 nm. The cell viability rate (VR) was calculated according to the equation:  $VR = A/A_0 \times 100\%$ , where A is the absorbance of the experimental group (i.e., the cells were treated by IR-PY) and A<sub>0</sub> is the absorbance of the control group (i.e., the cells were untreated by IR1048-MZ).

## **Tumor xenograft models**

All 5–6 week Balb/c nude mice were purchased from Medical Experimental Animal Center of Guangdong Province. The A549 tumors were generated by subcutaneous injection of  $4 \times 10^6$  A549 cells suspended in 200 µL PBS into the right hind legs of Balb/c mice. All animals received care in compliance with the Guidance Suggestions for the Care and Use of Laboratory Animals. All animal

operations were approved by Shenzhen Institutes of Advanced Technology, Chinese Academy of Sciences Animal Care and Use Committee.

### **Immunofluorescence staining**

The A549 tumor-bearing mice were intraperitoneally injected with pimonidazole hydrochloride (60 mg kg<sup>-1</sup>). After 1 h, the mice were sacrificed and the tumor samples were excised. After snap-frozen, the tumor samples were cut into 8 μm sections, post-fixed with cold acetone for 20 min, washed with ice-cold PBS and blocked with 10% BSA overnight. For the double-staining of Pimonidazole antibody and DAPI, the frozen sections were stained with anti-pimonidazole mouse monoclonal antibody conjugated to FITC (FITC-Mab1, diluted 200 times, Hypoxyprobe-1 Plus Kit, Hypoxyprobe Inc., Burlington), and then stained with DAPI. The images of the frozen sections were captured with Laser Scanning Confocal Microscope (LSCM, Leica TCS SP5).

### ***In vivo* NIR II fluorescence imaging.**

To evaluate biodistribution *in vivo* and tumor NIR II fluorescence imaging, the A549 tumor bearing Balb/c mice were anesthetized by intraperitoneal (IP) injection of 40 mg/kg pentobarbital sodium. The nude mice were sacrificed at 14 h after tail vein injection of 200 μL 40 μg/mL IR1048-MZ PBS-DMSO solution (99%, v/v). NIR II fluorescence imaging of freshly removed organs (heart, liver, spleen, lung, kidney and tumor) from treated mice was collected and analyzed. Whole body NIR II fluorescence imaging of living mice was taken at different time-points (0 h, 5 h, 10 h, 14 h, 20 h, 24 h and 48 h) after IR1048-MZ injection. The NIR II fluorescence imaging was performed with a 640 × 512 pixel two-dimensional InGaAs/SWIR VGA standard camera (Photonic Science, UK) equipped with a 1000 nm longpass filter (Thorlabs FEL1000 nm). A NIR lens pair SWIR-35 (Navitar, Rochester, NY, USA) was used to focus the image onto the photodetector. The excitation light was provided by an 980 nm laser (MDL-III-980R, Changchun New Industries Optoelectronics Technology Co., Ltd, China). The excitation power density at the imaging plane was 25 mW/cm<sup>2</sup>. The NIR II images were taken at a fixed exposure time of 200 ms. The display range of fluorescence intensity was set as 2000–65000 a.u. And Matlab 7 was used to process the images for any necessary flat-field correction. The falsecolor was set as jet. All the images were obtained without any further processing.

### ***In vivo* 3D PA imaging.**

3D PA imaging was detected with preclinical photoacoustic computerized tomography scanner (Endra Nexus 128, Ann Arbor, MI). The impulse excitation wavelength was 880 nm. Then the tumor region on mice was observed at 0 h, 5 h, 10 h, 14 h, 20 h, 24 h and 48 h post-injection. The PA imaging intensity at tumor was analyzed at different time. The penetration depth of PA imaging was measured at longitudinal section (L) after 14 h post injection.

### **Bio-Distribution Assay.**

To evaluate biodistribution *in vivo* and tumor NIR II fluorescence imaging, the A549 tumor bearing Balb/c mice were anesthetized by intraperitoneal (IP) injection of 40 mg/kg pentobarbital sodium. Then the nude mice were injected with 200  $\mu$ L 40  $\mu$ g/mL IR1048-MZ PBS-DMSO solution (99%, v/v). At each time point post injection (0, 5, 10, 14, 20, 24, 48, and 72 h), the blood samples from five mice were collected. Then the mice were sacrificed and major organs and tissues including heart, liver, spleen, lung, kidney, and tumor were dissected.

### ***In Vivo* PTT Treatment.**

The nude mice bearing A549 tumors (five per group) were injected intravenously with 200  $\mu$ L IR1048-MZ (40  $\mu$ g/mL), and control mice were treated with 200  $\mu$ L of saline. At 14 h, the tumors of mice were irradiated under the laser (980 nm, 0.1 W/cm<sup>2</sup>) for 2 min. The real-time temperature change of tumors region and infrared thermographic maps were obtained using a Ti27 infrared thermal imaging camera (Fluke, USA), of which the detection sensitivity is  $\pm 2$  °C or 2 % (at 25 °C nominal, whichever is greater). The growth of the tumor was measured with a caliper every 3 days during the period of treatment (30 d) and the volume was calculated (Volume = Length  $\times$  Width<sup>2</sup>  $\times$  0.5). The percentage of surviving mice was determined by monitoring tumor growth-related events. The mice were assumed to be death when tumor size over 1000 mm<sup>3</sup>. To further detect the effect of photothermal therapy *in vivo*, 48 h after treatment, tumors in different groups were stained with hematoxylin and eosin (H&E).

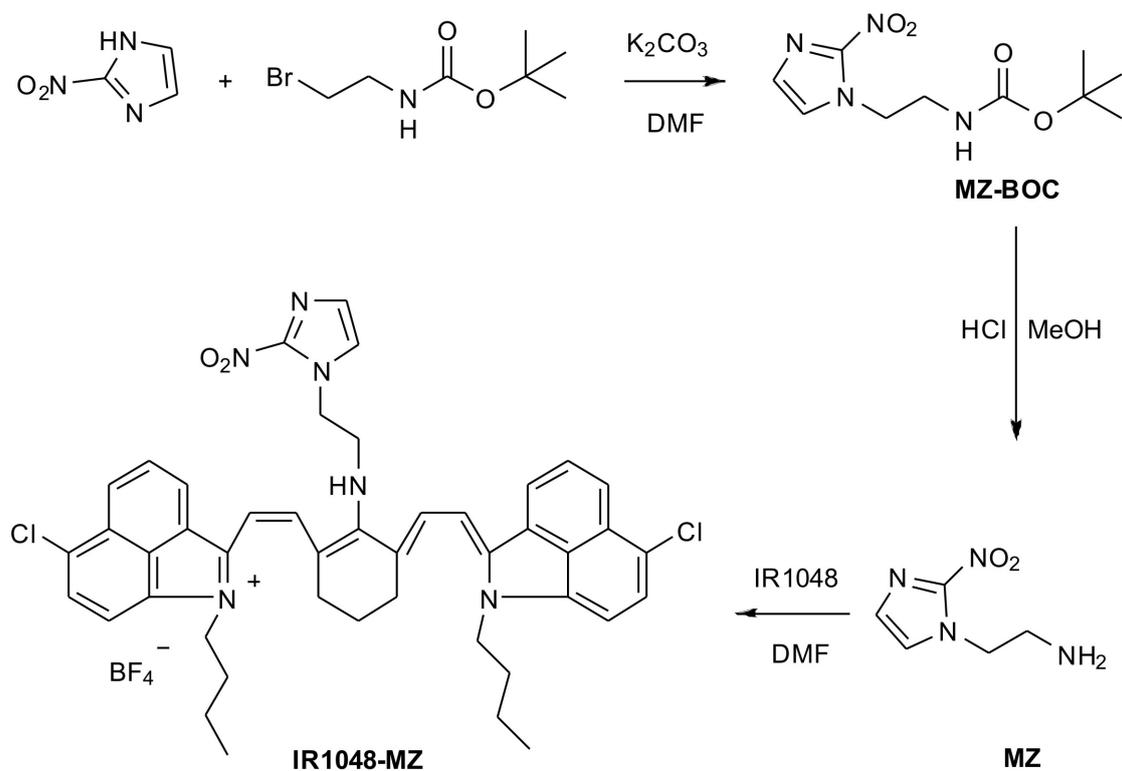
### ***In Vivo* Toxicity Assay.**

Twenty healthy 5–6 week Balb/c nude mice were randomly divided into two groups (n = 10), which received the intravenous administration of 200  $\mu$ L saline or IR1048-MZ (40  $\mu$ g/mL). At the 30th day after intravenous administration, the blood samples were collected to conduct serum biochemistry

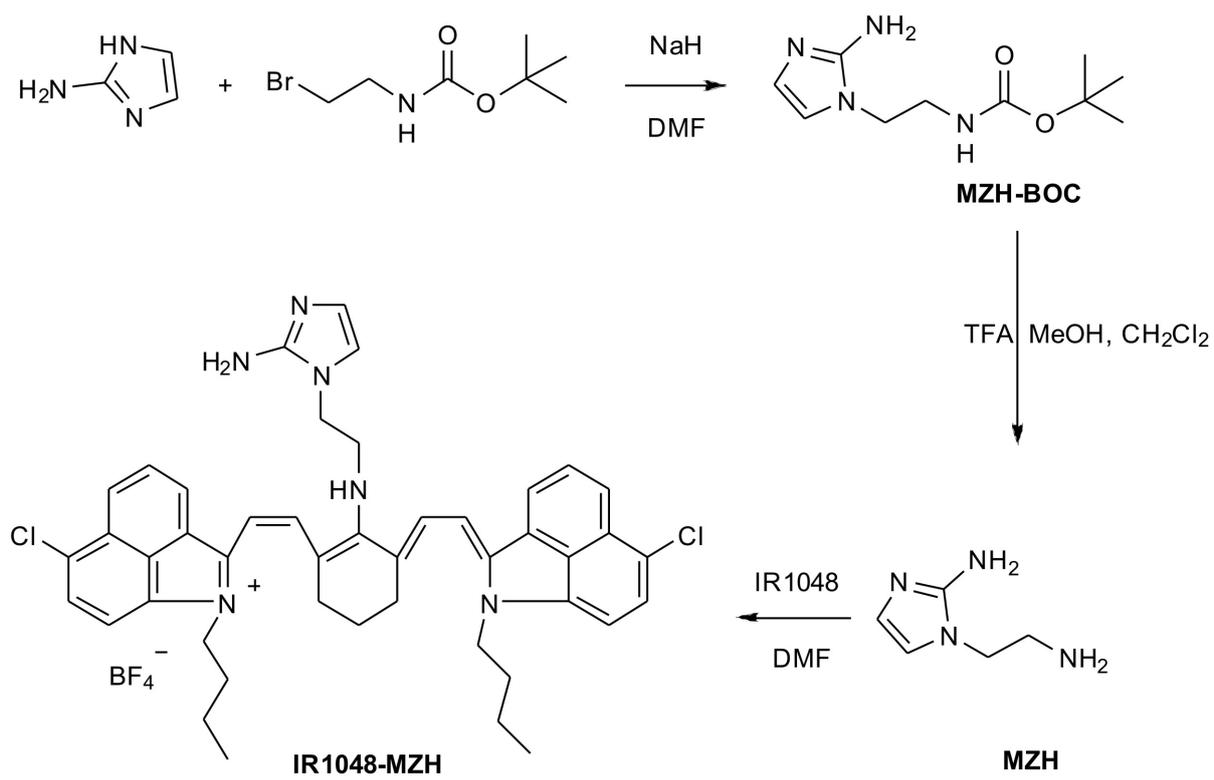
assay and complete blood panel test. The major organs of mice including heart, lung, liver, kidney, and spleen were harvested, fixed in a 10% formalin, embedded into paraffin, and stained with hematoxylin and eosin (H&E) for histological analysis.

### Statistical analysis

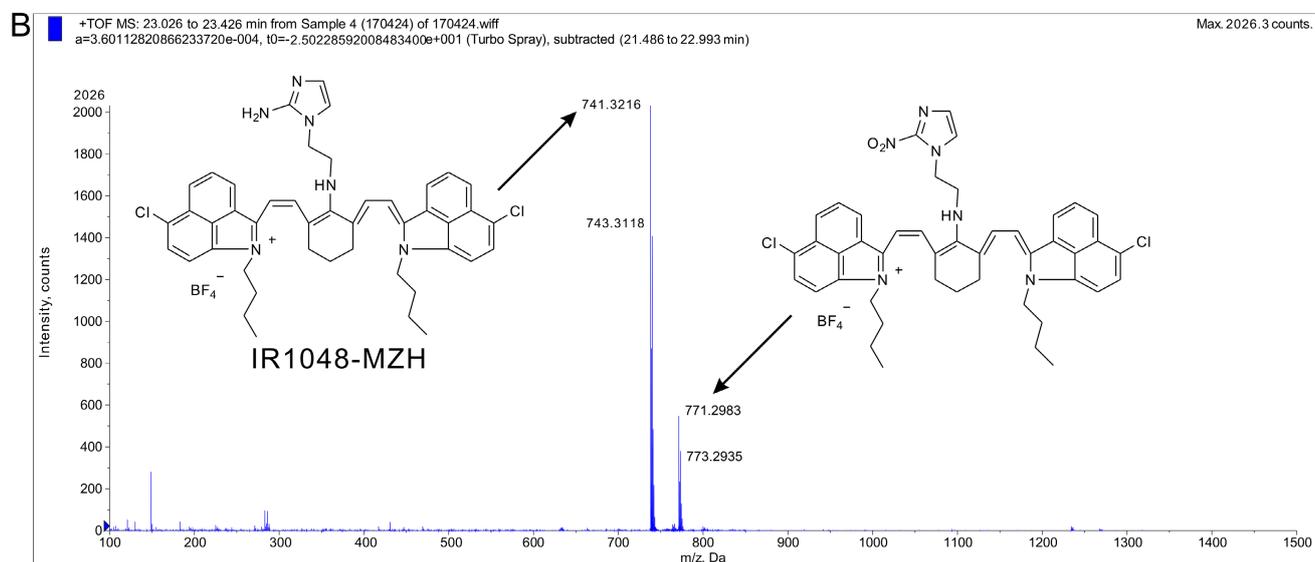
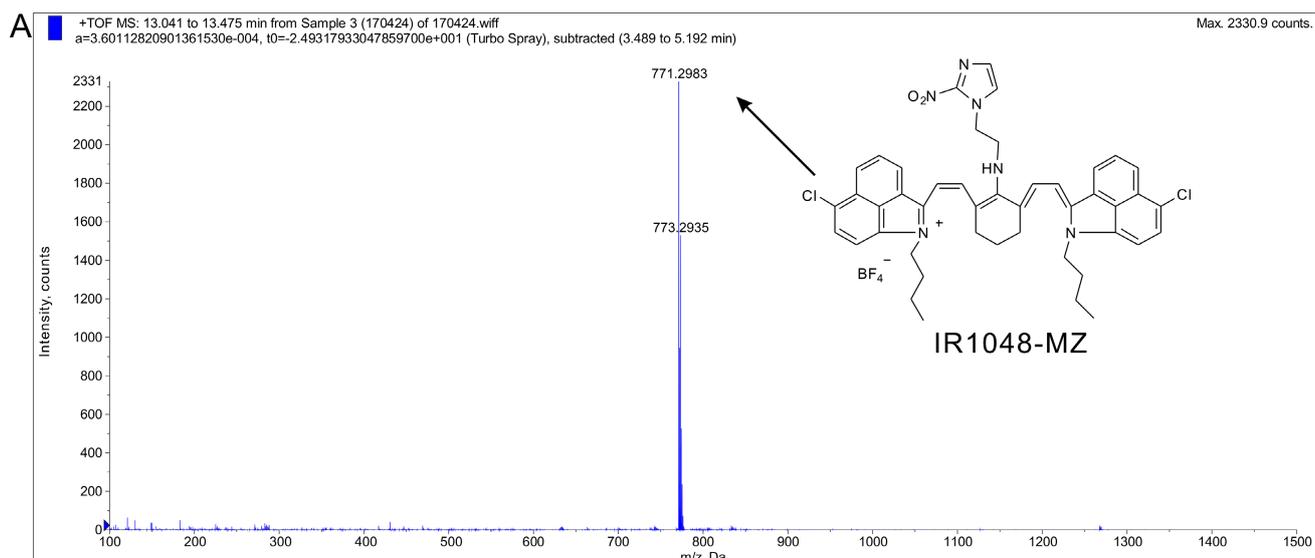
Data of independent experiments were presented as mean  $\pm$  SD. Statistical differences between the indicated groups were conducted by one-way analysis of variance (ANOVA) and Tukey's post-test for multiple comparisons; (\*)  $P < 0.05$ , (\*\*)  $P < 0.01$ .



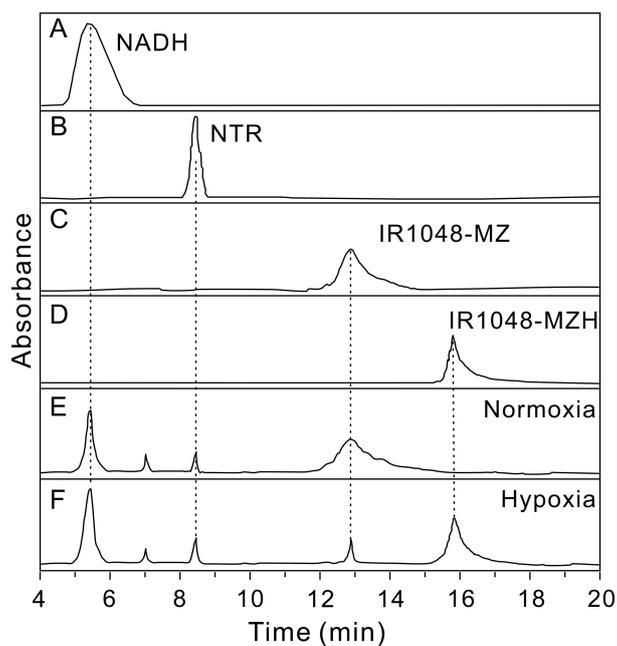
**Figure S1. Synthetic routine of the single molecular probe of IR1048-MZ.**



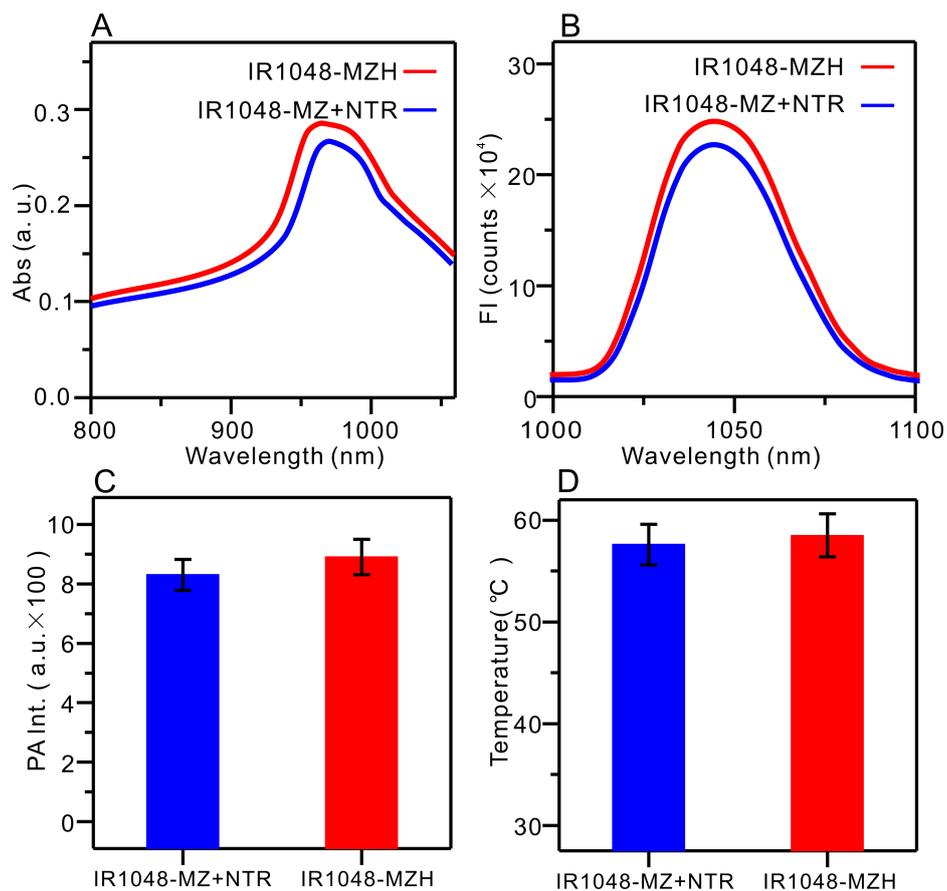
**Figure S2. Synthetic routine of IR1048-MZH.**



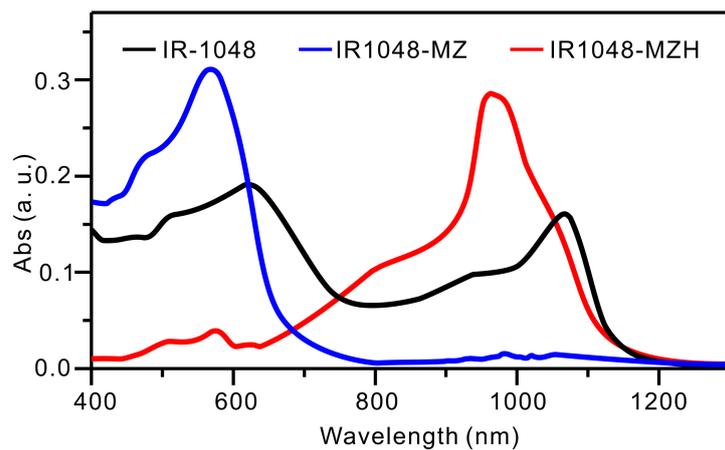
**Figure S3. The mass spectra after treating probe IR1048-MZ with NTR and NADH (A) normoxic (20% pO<sub>2</sub>) or (B) under hypoxic (0% pO<sub>2</sub>) for 5 min.**



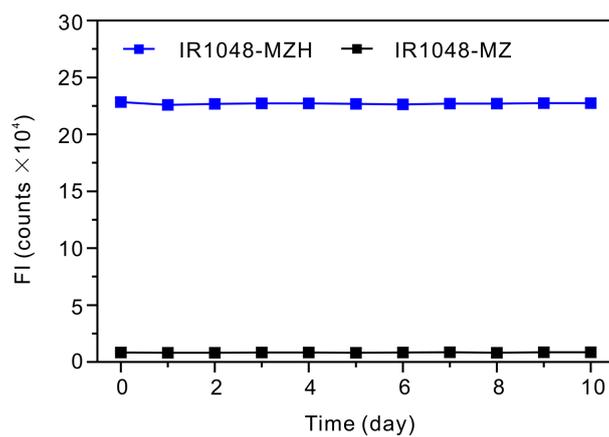
**Figure S4. IR1048-MZH was verified as a major final product by HPLC analysis.** HPLC chromatogram of (A) 500  $\mu\text{M}$  NADH, (B) 10  $\mu\text{g mL}^{-1}$  NTR, (C) 50  $\mu\text{M}$  IR1048-MZ, (D) 50  $\mu\text{M}$  IR1048-MZH, (E) 50  $\mu\text{M}$  IR1048-MZH mixed with 1  $\mu\text{g mL}^{-1}$  NTR in the presence of 200  $\mu\text{M}$  NADH under normoxic (20%  $\text{pO}_2$ ) for 5 min and (F) 50  $\mu\text{M}$  IR1048-MZH mixed with 1  $\mu\text{g mL}^{-1}$  NTR in the presence of 200  $\mu\text{M}$  NADH under hypoxic (0%  $\text{pO}_2$ ) for 5 min.



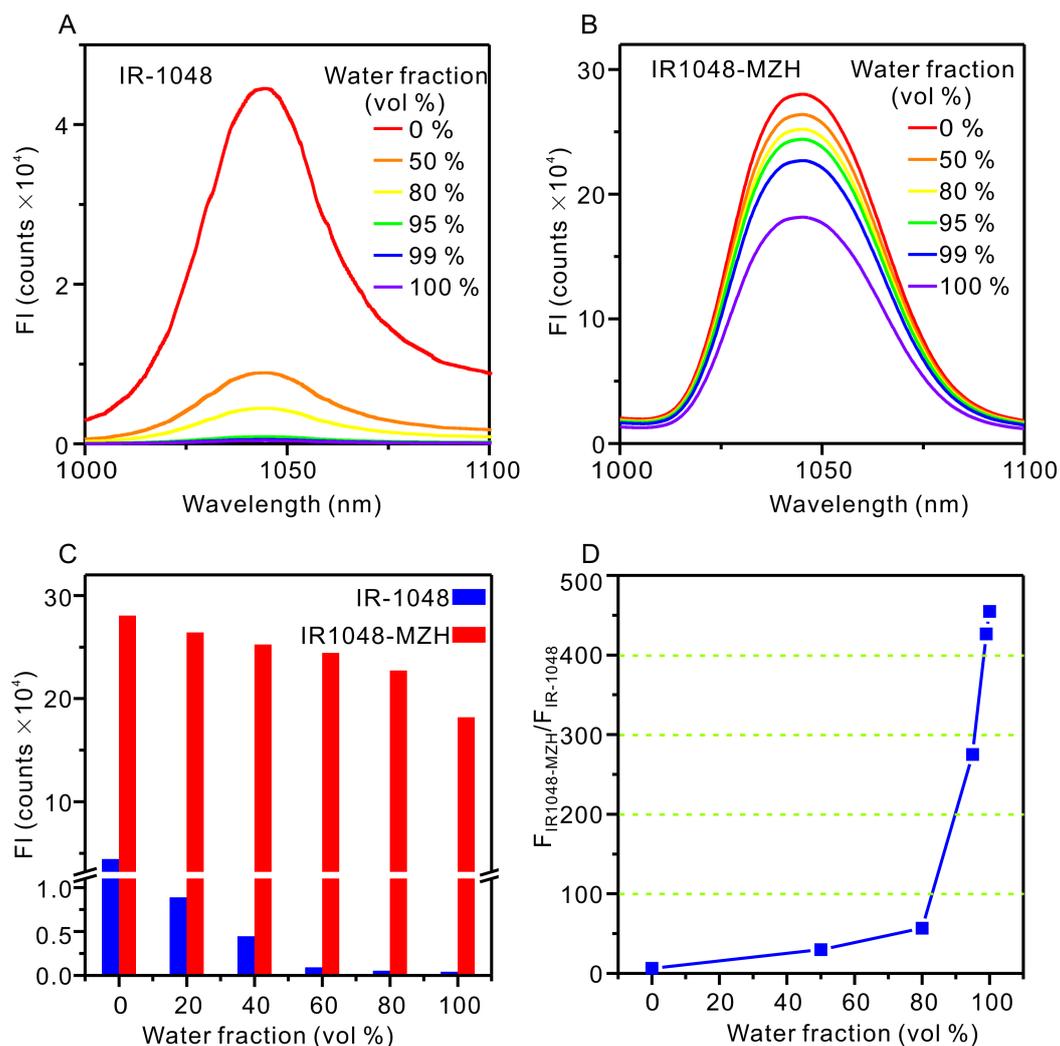
**Figure S5. The optical, photoacoustic and photothermal characteristics of 5  $\mu\text{g/mL}$  IR1048-MZH and NTR-reduced IR1048-MZ (5  $\mu\text{g/mL}$ ).** (A) The absorbance spectrum, (B) fluorescence spectrum, (C) photoacoustic intensity of IR1048-MZH and IR1048-MZ added 10  $\mu\text{g/mL}$  NTR with 500  $\mu\text{M}$  NADH as a coenzyme under normoxic. (D) The temperature of 5  $\mu\text{g/mL}$  IR1048-MZH or 5  $\mu\text{g/mL}$  IR1048-MZ added 10  $\mu\text{g/mL}$  NTR after 980 nm NIR laser irradiation (0.1  $\text{W/cm}^2$ ) for 2 min.



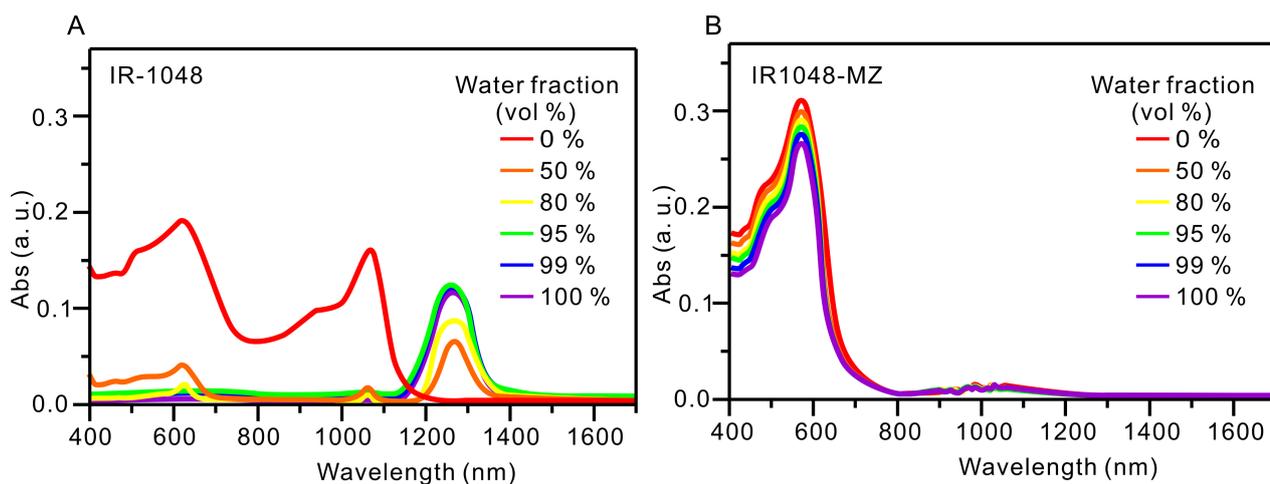
**Figure S6. The absorption spectra of 5  $\mu\text{g/mL}$  IR1048, IR1048-MZ and IR1048-MZH.**



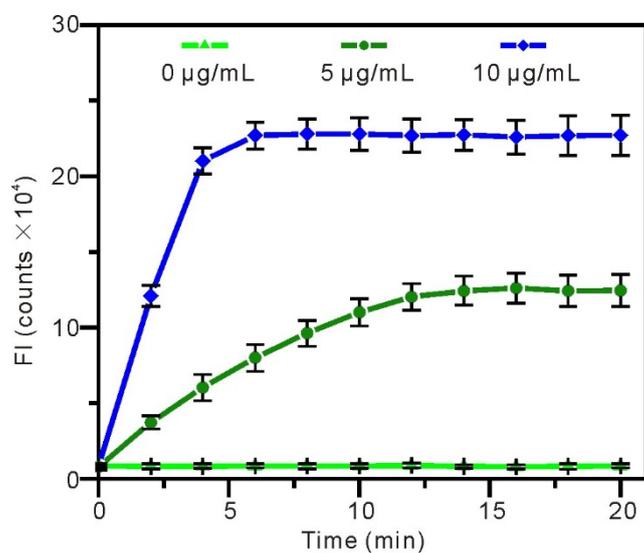
**Figure S7. The stability of IR1048-MZ and IR1048-MZH within 10 days.**



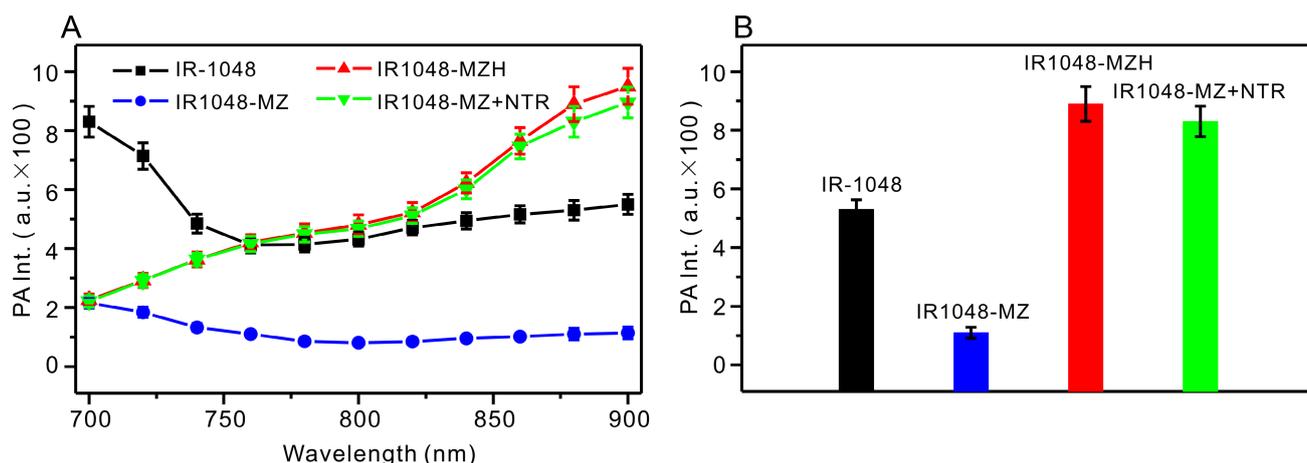
**Figure S8. The fluorescence intensity of IR-1048 and IR1048-MZH in different water/DMSO mixtures. (A)** Fluorescence spectra of 5  $\mu\text{g/mL}$  IR-1048 in different water/DMSO solutions ( $\lambda_{\text{ex}} = 980$  nm). **(B)** Fluorescence spectra of 5  $\mu\text{g/mL}$  IR1048-MZH in different water/DMSO solutions ( $\lambda_{\text{ex}} = 980$  nm). **(C)** The FI of IR-1048 ( $\lambda_{\text{em}} = 1086$  nm) and IR1048-MZH ( $\lambda_{\text{em}} = 1046$  nm) in different water/DMSO mixtures. **(D)** A plot of FI of IR-1048 vs. FI of IR1048-MZH in different water/DMSO mixtures.



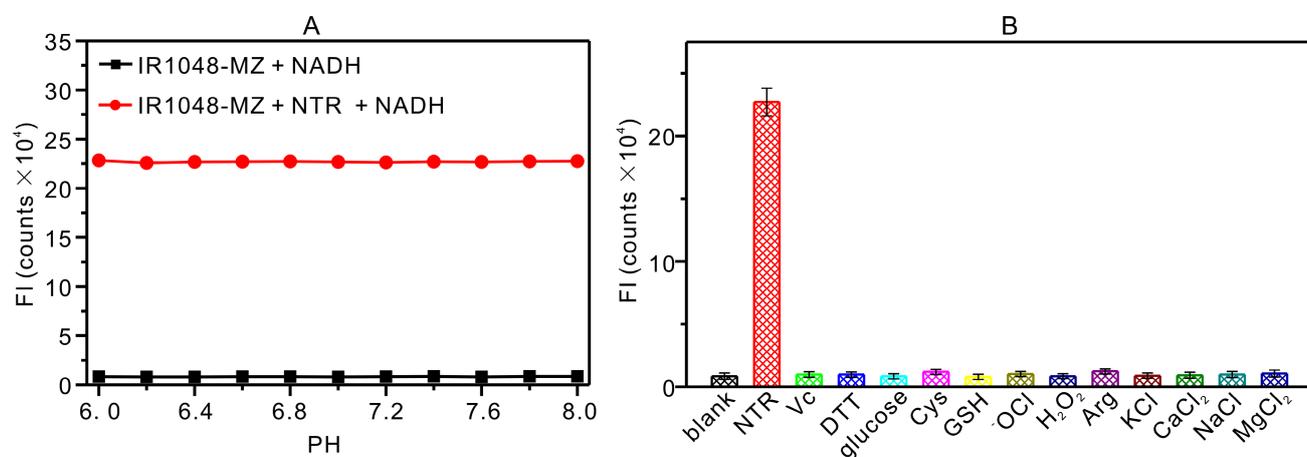
**Figure S9.** The absorption spectra of IR-1048 and IR1048-MZ in different water/DMSO mixtures. **(A)** Absorption spectra of 5  $\mu\text{g/mL}$  IR-1048 in different water/DMSO solutions. **(B)** Absorption spectra of 5  $\mu\text{g/mL}$  IR1048-MZ in different water/DMSO solutions.



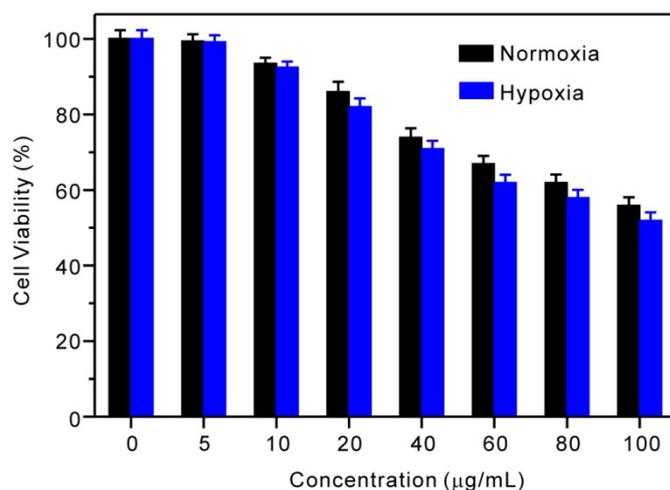
**Figure S10.** Time-dependent fluorescence intensity (FI) of IR1048-MZ (5  $\mu\text{g/mL}$ ) in the presence of NTR (0, 5 and 10  $\mu\text{g/mL}$ ) under hypoxic.



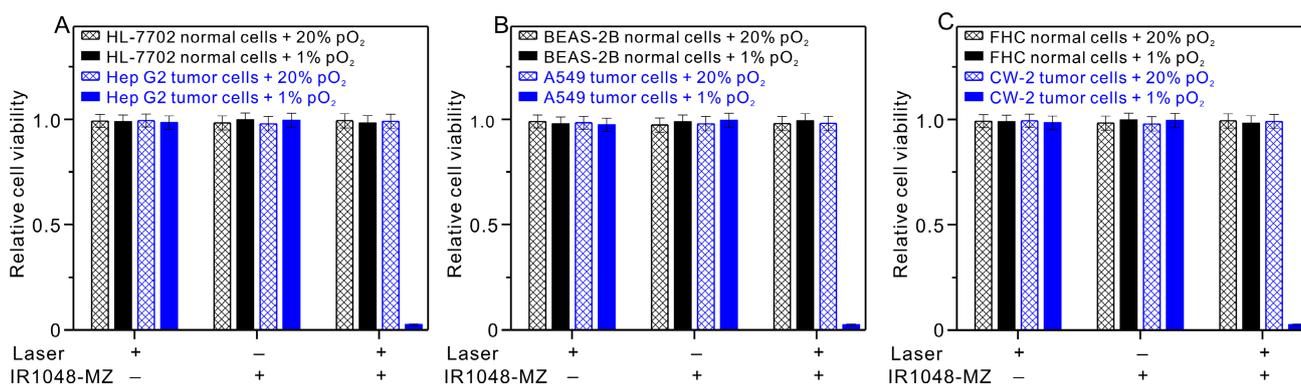
**Figure S11.** The PA signal of IR-1048, IR1048-MZ, IR1048-MZH and NTR-activated IR1048-MZ. (A) PA spectra of 5  $\mu\text{g/mL}$  IR-1048, IR1048-MZ, IR1048-MZH and NTR-activated IR1048-MZ (5  $\mu\text{g/mL}$ ). (B) The PA signal of IR-1048, IR1048-MZ, IR1048-MZH and NTR-activated IR1048-MZ (5  $\mu\text{g/mL}$ ) at 880 nm impulse excitation.



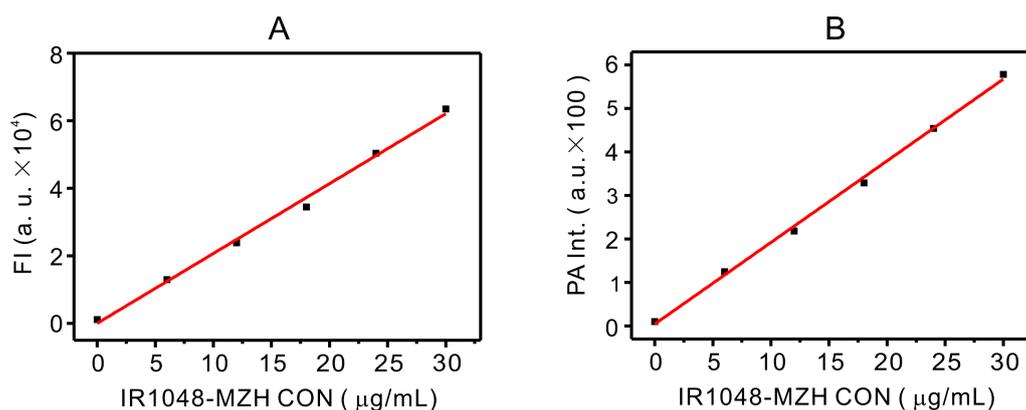
**Figure S12.** The reaction selectivity of IR1048-MZ to NTR. (A) Effects of pH on the reaction between IR1048-MZ (5  $\mu\text{g/mL}$ ) and NTR at 37  $^{\circ}\text{C}$ . (B) Fluorescence responses of IR1048-MZ (5  $\mu\text{g/mL}$ ) at 1046 nm to different kinds of species. All the data were acquired in PBS with pH 7.4 in the presence of 1% DMSO as a co-solvent at 37  $^{\circ}\text{C}$  under hypoxic ( $\lambda_{\text{ex}}/\lambda_{\text{em}} = 980/1046 \text{ nm}$ ).



**Figure S13. Effect of probe IR1048-MZ on the viability of A549 cells.** Cell viability was determined by MTT assay after treatment with different concentrations of IR1048-MZ probe for 24 h under the normoxic or hypoxic conditions. The results showed low toxicity of the probe in both normoxia and hypoxia condition.



**Figure S14. The evaluation of hypoxia-activated photothermal therapy of IR1048-MZ in tumor cells and normal cells under normoxic (20% pO<sub>2</sub>) or hypoxic condition (0% pO<sub>2</sub>).** The relative cell viability of (A) HL-7702 normal cells or HepG-2 tumor cells, (B) BEAS-2B normal cells or A549 tumor cells, (C) FHC normal cells or CW-2 tumor cells after different treatment.

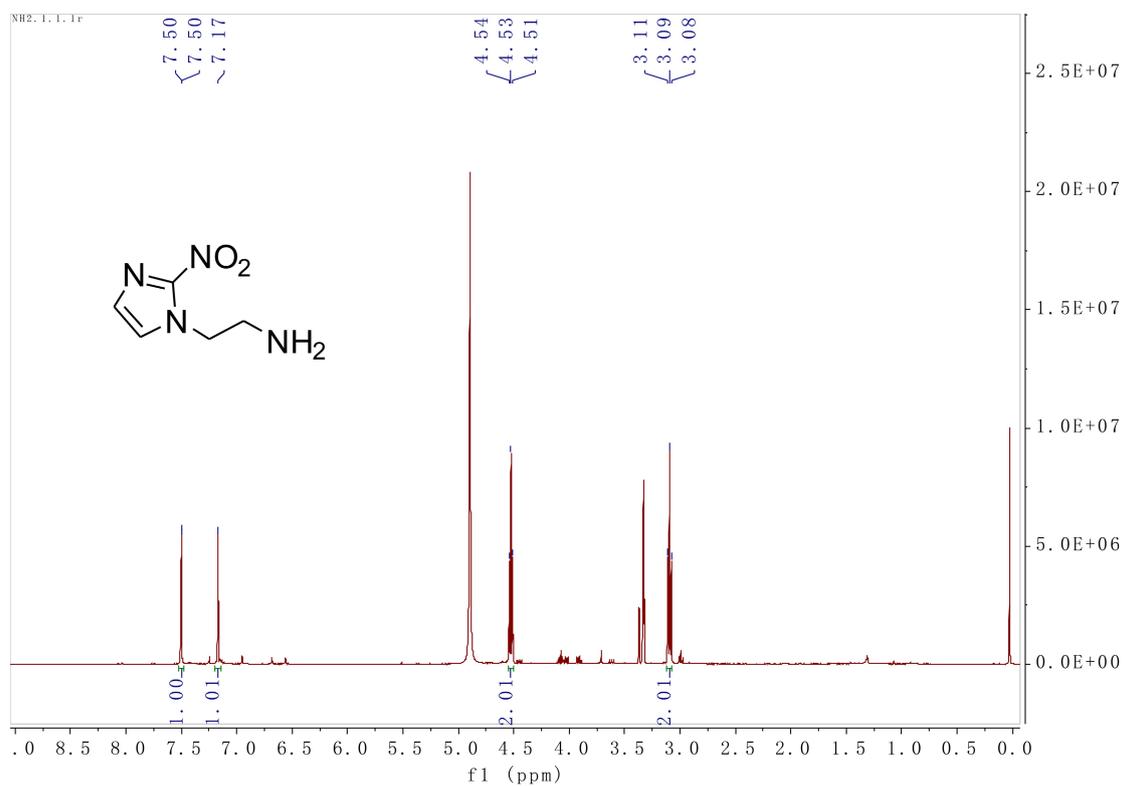
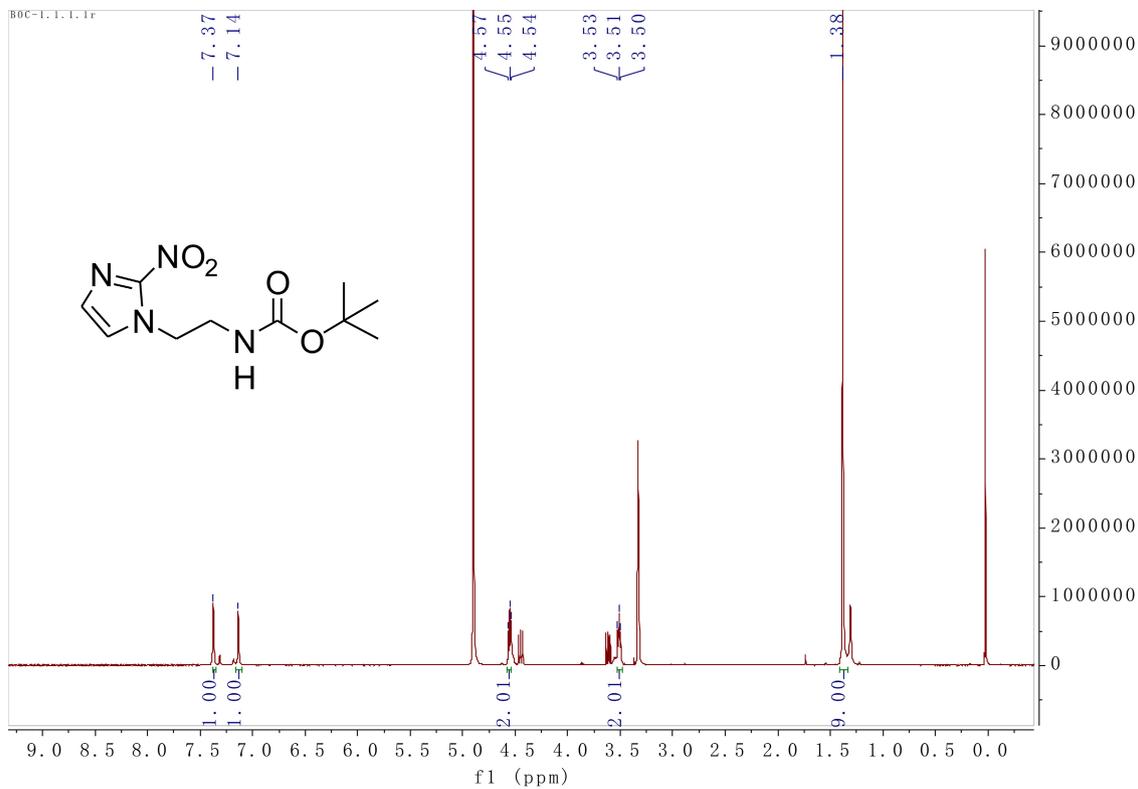


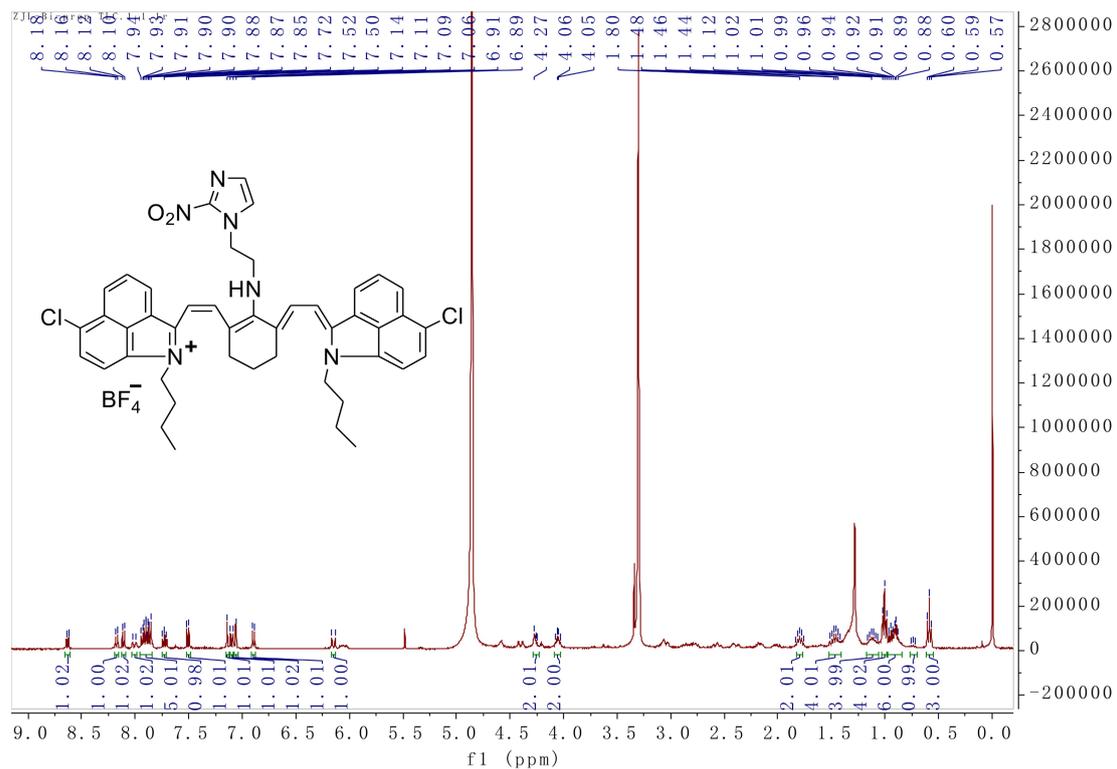
**Figure S15. NIR II fluorescence /PA intensity of IR1048-MZH with different concentration (CON).** (A) NIR II fluorescence intensity of IR1048-MZH with different concentration measured by Maestro<sup>TM</sup> *in vivo* NIR II fluorescence imaging system with a 980 nm excitation and a 1000 nm longpass emission filter. FI: fluorescence intensity. (B) PA intensity of IR1048-MZH with different concentration measured by photoacoustic tomography scanner. The impulse excitation wavelength was 880 nm. PA Int.: PA intensity.

**Table S1. Blood biochemistry and hematology data of Balb/c nude mice.**

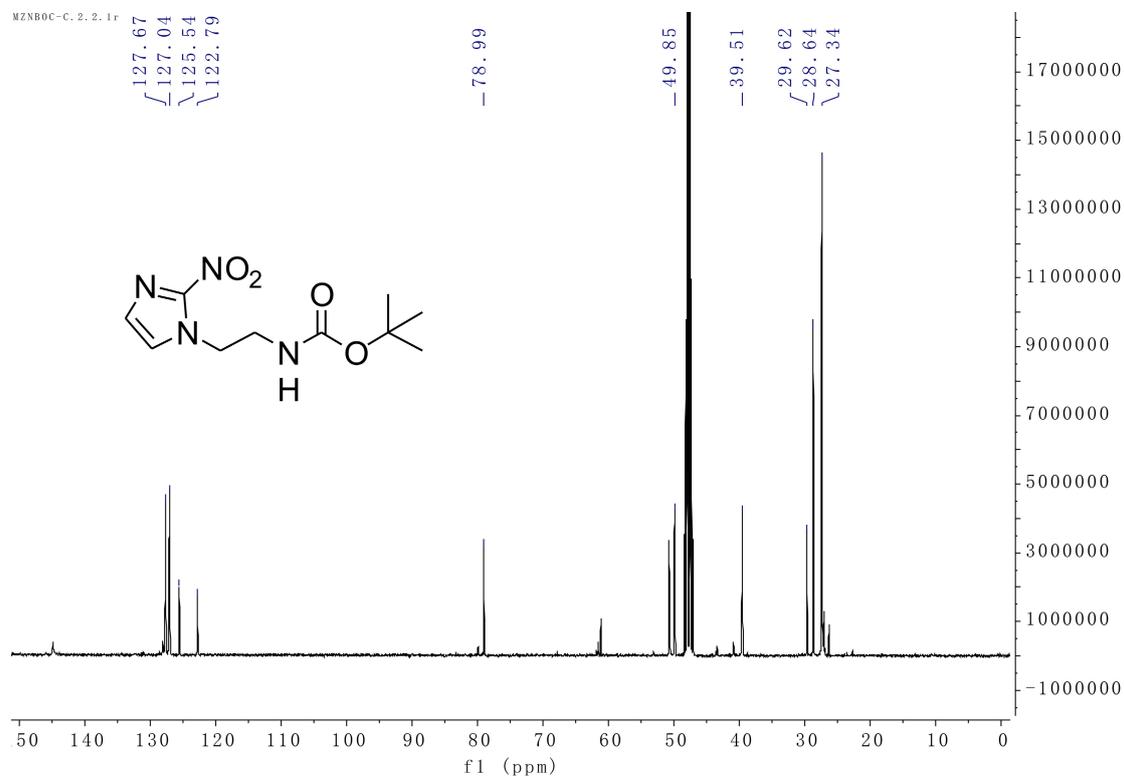
Project Name	Treatment Group	Control Group	units
	Mean ± SD	Mean ± SD	
ALT	43.32 ± 4.28	42.64 ± 4.86	U L <sup>-1</sup>
AST	152.42 ± 17.24	153.62 ± 16.62	U L <sup>-1</sup>
ALP	135.2 ± 16.9	140.3 ± 24.5	U L <sup>-1</sup>
A/G	0.4 ± 0.01	0.4 ± 0.02	
BUN	6.21 ± 0.67	6.22 ± 0.63	mmol/L
WBC	12.20 ± 1.62	12.19 ± 1.52	10 <sup>9</sup> L <sup>-1</sup>
RBC	9.73 ± 0.52	9.85 ± 0.63	10 <sup>12</sup> L <sup>-1</sup>
HGB	164.26 ± 2.42	165.04 ± 2.63	g L <sup>-1</sup>
PLT	838.25 ± 47.25	839.37 ± 48.74	10 <sup>9</sup> L <sup>-1</sup>
HCT	46.3 ± 2.1	46.4 ± 1.6	%
MCV	53.52 ± 5.53	53.61 ± 4.04	fL
MCH	16.73 ± 0.94	17.02 ± 0.65	pg

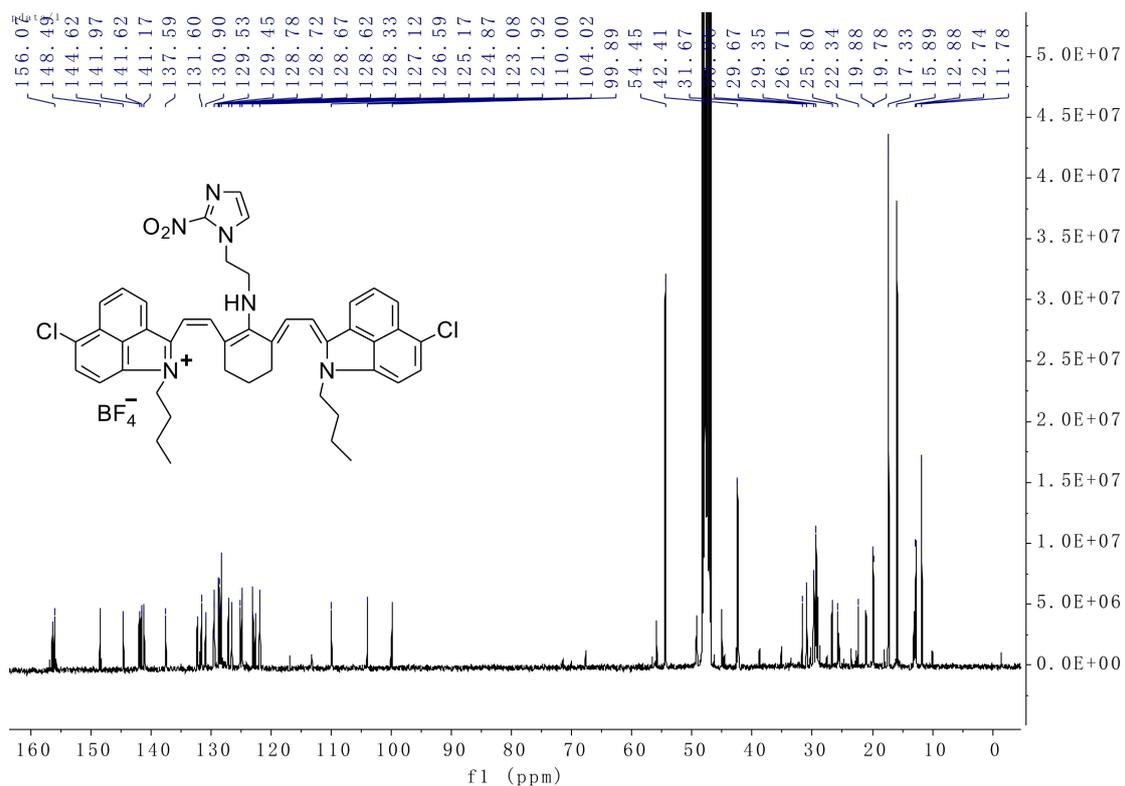
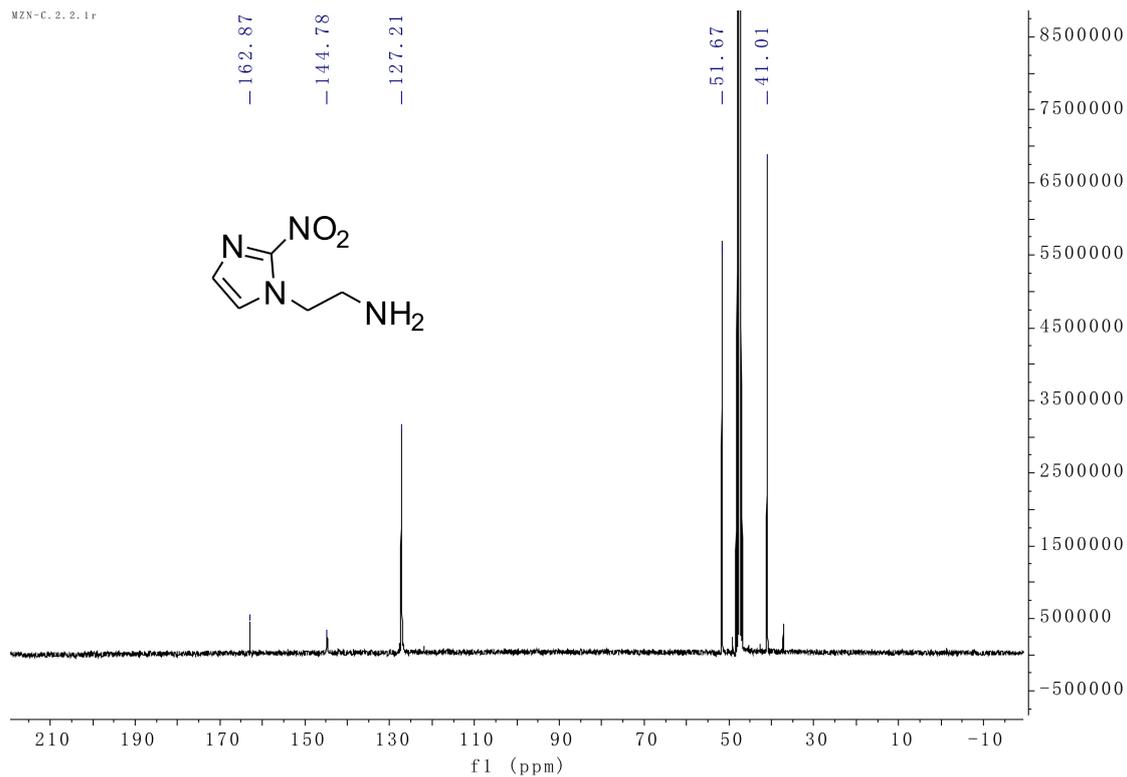
MCHC

 $314.62 \pm 4.07$  $314.95 \pm 4.14$  $\text{g L}^{-1}$  **$^1\text{H}$  NMR data:**



**<sup>13</sup>C NMR data:**





**HRMS data:**

