Electronic Supplementary Information (ESI)

Visualization of nitroxyl (HNO) in vivo via a lysosome-targetable near-infrared fluorescent probe

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1. Experimental Section

**Instruments:** Mice imaging was performed on in vivo FX PRO (Bruker) system. Fluorescence spectra were obtained by Shimadzu RF-5301PC Spectrofluorometer with a Xenon lamp and 1.0-cm quartz cells. Absorption spectra were measured on TU-1810DSPC UV-visible spectrophotometer (Beijing Persee). Mass spectra were taken on LCQ Fleet LC-MS System (Thermo Fisher Scientific). \textsuperscript{1}H NMR, \textsuperscript{13}C, \textsuperscript{31}P NMR spectra
were recorded on a Bruker spectrometer. MTT Assay was carried out by a microplate reader (Tecan, Austria). The fluorescence images of cells were taken using a confocal laser scanning microscope (Japan Olympus Co., Ltd) with an objective lens (×40).

**Materials:** The purity of Lyso-JN was separated on a Shimadzu LC-20AT HPLC system equipped with fluorescence and UV-vis absorption detectors. When it was used for imaging, the purity of Lyso-JN was greater than 99.89%. HEPES was obtained from Aladdin and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma-Aldrich. Raw 264.7 Murine Macrophages (RAW264.7 cells) were obtained from the Committee on Type Culture Collection of the Chinese Academy of Sciences. Lyso-JN (1 mM) was prepared in DMSO and stored at 4°C in darkness. Angeli’s salt (AS) was prepared as reported by King and Nagasawa and stored dry at -20°C in a refrigerator.¹ S-nitrosoglutathione (GSNO) was synthesized from GSH, according to the published procedure.² Peroxynitrite (ONOO⁻) solution was synthesized as reported.³ NO was generated in the form of 3-(Aminopropyl)-1-hydroxy-3-isopropyl-2-oxo-1-triazene (NOC-5, 100 µM/ml).⁴ NO₂⁻ was generated from NaNO₂. O₂⁻ was created by the enzymatic reaction of xanthine/xanthineoxidase (XA/XO; 6.0 µM/3 mU) at 25 °C for 5 min.⁵ Methyl linoleate (MeLH) and 2,2’-azobis-(2,4-dimethyl) valeronitrile (AMVN) were used to produce MeLOOH.⁶ ClO⁻ was generated from NaClO. All other reagents and chemicals were all from commercial sources and used without further purification. Water used in all experiments was doubly distilled and purified by a Milli-Q system (Millipore, Bedford, MA, USA).

**Absorption Analysis:** Absorption spectra were obtained with 1.0-cm glass cells. The probe Lyso-JN (DMSO, 20 µL, 1 mM) was added to a 10.0-mL color comparison tube, and dilution to 2 µM with HEPES buffer (10 mM, 0.5% DMSO, 0.5% TW 80, pH 5.0). Lyso-1 and phosphine oxide were also performed as above.

**Fluorescence Analysis:** Fluorescence spectra were obtained with a Xenon lamp and 1.0-cm quartz cells. The probe (DMSO, 10 µL, 1 mM) was added to a 5.0-mL color comparison tube. After dilution to 2 µM with HEPES buffer (10 mM, 0.5% DMSO, 0.5% TW 80, pH 5.0), different concentrations of AS were added. The mixture was incubated
for 20 min before measurement. Then the fluorescence spectrum (670–760 nm) was measured with excitation at 690 nm.

**Cell Cultures:** RAW264.7 cells were obtained from the Committee on Type Culture Collection of the Chinese Academy of Sciences. RAW264.7 cells were cultured in DMEM (Dulbecco’s Modified Eagle Medium) supplemented with 10% FBS (fetal bovine serum) in an atmosphere of 5% CO₂ and 95% air at 37°C.

**Cell Staining Procedures and Colocalization-imaging Experiments.** The cells were plated on 6-well plates and allowed to adhere at 37 °C, 5% CO₂, 24 h before imaging. The culture medium was then removed, and the cells were washed once with 1 mL of phosphate-buffered saline (PBS). RAW264.7 cells were placed in 1 mL of PBS and loaded with 2 μM Hoechst 33342 for 10 min before staining with 10 μM Lyso-JN for 5 min. After washing the cells three times with PBS to remove the excess probe, the cells were placed in 1 mL of PBS and treated with 200 μM AS for 20 min. Finally, 2 μM NR was added and the cells were incubated for another 5 min at 37°C. Finally, the cells were rinsed with PBS three times and mounted on the microscope. Fluorescent images were acquired on an Olympus Fluo View FV1000 confocal laser-scanning microscope (Japan) with an objective lens (× 40). The spectrally separated images acquired from the three dyes were estimated using Image-Pro Plus software.

**Mice imaging:** The BALB/c mice were obtained from Binzhou Medical University. BALB/c mice (20–25 g) were given intraperitoneal (i.p.) injections of Lyso-JN (50 μM, 50 μL in 1:9 DMSO/ saline v/v), then mice 1 was intraperitoneally injected with AS (1 mM, 50 μL in saline) for 30 min. Finally, two mice were anesthetized by i.p. injections of 4% chloral hydrate (0.25 ml). Then two mice were imaged by using a FX PRO in vivo imaging system, with an excitation filter of 660 nm and an emission filter of 670 nm. The results were the mean standard deviation of five separate measurements.

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2. Synthesis and Characterization of Compounds

Synthesis of the Probe:

![Chemical Structure](image)

Aza-BODIPY fluorophore (Compound 1) was synthesized starting from 4’-hydroxychalcone following a previously reported protocol.⁷

**Synthesis of 2**: Compound 1 (52.9 mg, 0.1 mmol) was dissolved in dry acetone (25 mL), then treated with N-(3-chloropropyl) morpholine (15 µL, 0.1 mmol) and anhydrous potassium carbonate (27.5 mg, 0.2 mmol). The mixture was stirred under a dry argon atmosphere for 24 h and tracked with TLC. After the reaction, the mixture was cooled to room temperature, evaporated under reduced pressure and partitioned between CH₂Cl₂ and saturated NaBr solution. Finally, the organic layer was separated. Purification by column chromatography on silica eluting with EtOAc/CH₃OH (4:1) gave the product 2 as dark green crystals (16.40 mg, 25%).¹¹H NMR (500 MHz, CDCl₃-D₄) δ
(ppm): 9.53 (1H, s), 8.03-8.02 (6H, m), 7.72-7.69 (2H, t), 7.54-7.37 (7H, m), 7.01-6.94 (3H, t), 6.87-6.86 (2H, d), 4.32-4.29 (4H, t), 4.09-4.05 (2H, t), 2.57-2.51 (6H, m), 2.02-2.01 (2H, m). \(^{13}\)C NMR (125 MHz, CDCl\(_3\)-D): 167.78, 161.25, 159.10, 132.51, 132.46, 132.32, 131.97, 131.59, 130.94, 129.27, 129.16, 128.86, 128.53, 124.15, 123.70, 118.69, 118.53, 115.97, 114.70, 66.76, 66.09, 65.61, 55.48, 53.67, 30.58. ES-MS: \(m/z\) C\(_{39}\)H\(_{35}\)BF\(_2\)N\(_4\)O\(_3\), calcd 656.2770, found [M + H]\(^+\) 657.2841, [M - H\(^-\)] 655.2696.

**Synthesis of Lyso-JN:** Compound 2 (65.6 mg, 0.1 mmol), 2-(diphenylphosphino) benzoic acid (30.6 mg, 0.1 mmol), 4-(dimethylamino) pyridine (12.2 mg, 0.1 mmol), and 1-ethyl 3-(3-dimethylaminopropyl) carbodiimide hydrochloride (9.6 mg, 0.05 mmol) were dissolved in dry CH\(_2\)Cl\(_2\). The reaction mixture was stirred for 24 h at room temperature under a dry argon atmosphere, tracked with TLC. And then the mixture was poured into 1 N HBr and extracted with CH\(_2\)Cl\(_2\). The organic layer was separated, washed with NaBr in saturated aqueous solution, and dried over Na\(_2\)SO\(_4\). The solvent was evaporated, and the crude product of Lyso-JN was purified by column chromatography over silica gel with EtOAc/CH\(_3\)OH (9:1) gave Lyso-JN as dark green crystals (28.32 mg, 30%). \(^1\)H NMR (500 MHz, CDCl\(_3\)-D) \(\delta\) (ppm): 8.28-8.27 (1H, m), 8.11-7.99 (6H, m), 7.53 (1H, d), 7.51-7.29 (18H, m), 7.09-6.94 (6H, m), 5.46 (1H, s), 5.10 (1H, s), 4.13-4.10 (2H, t), 3.74-3.72 (4H, t), 2.56-2.49 (6H, m), 2.04-1.99 (2H, m). \(^{13}\)C NMR (125 MHz, CDCl\(_3\)) \(\delta\) (ppm): 165.21, 162.35, 159.42, 155.82, 152.42, 144.55, 142.49, 141.70, 141.47, 137.64, 137.55, 134.49, 134.15, 133.98, 133.38, 133.24, 132.65, 132.62, 132.34, 132.16, 132.06, 131.41, 130.92, 130.76, 129.61, 129.40, 129.26, 129.14, 128.83, 128.63, 128.62, 128.58, 128.38, 123.51, 121.78, 119.43, 118.29, 114.89, 66.92, 66.29, 65.58, 55.41, 53.73, 53.45, 30.59, 26.26. \(^{31}\)P NMR (200 MHz, CDCl\(_3\)-D) \(\delta\) (ppm): -4.075. ES-MS: \(m/z\) C\(_{58}\)H\(_{48}\)BF\(_2\)N\(_4\)O\(_4\)P, calcd 944.3474, found [M + H]\(^+\) 945.3549.


3. Effect of Tween 80
The effect of Tween 80 on the fluorescence intensity ($\lambda_{\text{ex}}=690 \text{ nm}, \lambda_{\text{em}}=700 \text{ nm}$) of Lyso-1 (2 μM) in HEPES (10 mM, 0.5% DMSO, pH 5.0). We evaluated the effect of Tween 80 (0.1%, 0.3%, 0.5%, 0.7%, 0.9%, 1.1%, and 1.3%) on the fluorescence intensity of Lyso-JN in HEPES (10 mM, 0.5% DMSO, pH 5.0). As shown in Figure S1, in the range from 0.1% to 1.3%, the fluorescent intensity undergoes unobvious optical changes.

4. Effect of pH Values

The absorption maximum of Lyso-1 and phosphine oxide were observed at 702 nm ($\varepsilon=3.80\times10^4 \text{ M}^{-1}\text{cm}^{-1}$) and 688 nm ($\varepsilon=3.75\times10^4 \text{ M}^{-1}\text{cm}^{-1}$), respectively.
Fig. S3 Absorption spectra of 2 μM Lyso-JN (black), and two products: 2 μM Lyso-1 (red), 2 μM phosphine oxide (blue). Spectra were acquired in HEPES buffer solution (10 mM, 0.5% DMSO, 0.5% TW 80, pH 5.0).


Angeli’s salt (AS) has been the most commonly used in most chemical and biological studies, including those studies regarding HNO detection with phosphine and Cu<sup>2+</sup>-based fluorophores in biological systems. When pH values less than 4, AS converts to an NO donor certainly. However, at pHs above 4, AS spontaneously decomposes to generate 1 equivalent each of HNO and NO<sub>2</sub>.<sup>8-10</sup> In living cells, different organelles have different organelle pHs. As the acidic organelles, lysosomes mainly functions during the pH range from 4.5 to 5.5. Therefore, AS mainly generate HNO in lysosomes.

Figure S4. Plot of the rate constants of decomposition of AS versus pH from Hughes and Wimbledon.<sup>11</sup> The ◆ show the rate of the disappearance of AS to yield nitric oxide (NO), and ■ show the rate of the disappearance of AS to give nitroxyl (HNO).


**7. Determination of Quantum Yields**

The fluorescence quantum yields of Lyso-1 and Lyso-JN were determined according to the following expression $^{12}$:

$$
\varphi_u = \frac{(\varphi_s)(FA_u)(A_s)(\lambda_exs)(\eta^2_u)}{(FA_s)(A_u)(\lambda_exu)(\eta^2_s)}
$$

Where $\varphi$ is fluorescence quantum yield; the subscripts $u$ and $s$ refer to the unknown and the standard, respectively; $F$ is integrated fluorescence intensity under the corrected emission spectra; $A$ is the absorbance at the excitation wavelength; $\eta$ is the refractive index of the solution. We chose Mg-tetra-tert-butyllphthalocyanine as standard, which has a fluorescence quantum yield of 0.84 according to the literature $^{13}$.


**8. MTT Assay**

The cytotoxicity of probe Lyso-JN was assessed by the MTT assay. RAW264.7 cells were seeded into 96-well cell culture plate at a final density of $8 \times 10^3$ cells/well. And then different concentrations of Lyso-JN (0.1 µM, 1 µM, 10 µM, 100 µM) were added to the wells. The cells were then incubated for 24 h at 37 °C under 5% CO$_2$. Subsequently, MTT was added to each well (final concentration 5 mg/mL) for an additional 4 h at 37 °C under 5% CO$_2$, then formazan crystals which were dissolved in 150 µL DMSO formed. The amount of MTT formazan was qualified by the absorbance (OD) at 570 nm using a microplate reader (Tecan, Austria). Calculation of IC50 values were done
according to Huber and Koella. The results are the mean standard deviation of six separate measurements.

![Cell Viability Graph](image)

**Fig. S5** Effects of Lyso-JN at varied concentrations on the viability of RAW264.7 cells.

**9. Figures for Supporting Data**

![Fluorescence Images](image)

**Fig. S6** Fluorescence image of RAW264.7 cells incubated with Lyso-JN in the absence of HNO. (a) DIC images; (b) 2 μM NR showing lysosomes (green channel: $\lambda_{ex} = 559$ nm, $\lambda_{em} = 570–650$ nm); (c) 2 μM Hoechst showing nuclei (blue channel: $\lambda_{ex} = 405$nm, $\lambda_{em} =425 – 525$nm); (d) Overlay of red channel, green channel, and blue channel. Scale bars represent 20 μm.

![DIC Images](image)

**Fig. S7** DIC images of RAW264.7 cells incubated with Lyso-JN in the presence of HNO.
Fig. S8 X-ray imaging in peritoneal cavity of the BALB/c mice.