Electronic Supplementary Information

A near-infrared fluorescent probe for imaging peroxynitrite levels in paw edema mice and drug evaluation

Ling Hu,[a] Si-Si Chen,[a] Hui Zhang,[a] Jing-Jing Chao,[a] Zhen-Zhen Peng,[a] Guo-Jiang Mao,[c] Liufang Hu,[a] Juan Ouyang,[a] Anjie Min,[b,*] Chun-Yan Li[a,*]

[a] Key Laboratory for Green Organic Synthesis and Application of Hunan Province, Key Laboratory of Environmentally Friendly Chemistry and Applications of Ministry of Education, Hunan Provincial University Key Laboratory for Environmental and Ecological Health, College of Chemistry, Xiangtan University, Xiangtan, 411105, PR China.

[b] Department of Oral and Maxillofacial Surgery, Center of Stomatology, Research Center of Oral and Maxillofacail Tumor, Insititute of Oral Cancer and Precancerous Lesions, National Clinical Research Center for Geriatric Disorders, Xiangya Hospital, Central South University, Changsha, 410008, PR China.

[c] Henan Key Laboratory of Organic Functional Molecule and Drug Innovation, Collaborative Innovation Center of Henan Province for Green Manufacturing of Fine Chemicals, Key Laboratory of Green Chemical Media and Reactions, Ministry of Education, School of Chemistry and Chemical Engineering, Henan Normal University, Xinxiang, 453007, PR China.

*Corresponding Author. E-mail: lichunyan@xtu.edu.cn (Chun-Yan Li), william0732@csu.edu.cn (Anjie Min).

Table of contents

1. Experimental Section S3

2. Synthesis of probes S6

3. Spectral data.....S11

4. Biological assays.....S15

1. Experimental Section.

Reagents and instruments. Boron tribromide (BBr_3), 4-(bromomethyl) benzene boronic acid pinacol ester, dexamethasone, indometacin and ibuprofen were purchased from Aladdin (Shanghai, China). Lipopolysaccharide (LPS), interferon-γ (INF-γ), and minocycline were purchased from Sigma-Aldrich (St. Louis, USA). TNF-α Elisa kit was from Jingmei (Jiangsu, China). Unless otherwise stated, all chemicals were purchased from commercial suppliers and used without further purification.

Nuclear magnetic resonance (NMR) spectra were measured on a Bruker Avance II NMR spectrometer (Germany). Mass spectra (MS) was acquired on a Bruker Autoflex MALDI-TOF mass spectrometer (Germany). High-resolution mass spectra (HRMS) were obtained on an Agilent 1260II/6230 liquid chromatography mass spectrometer (USA). High-performance liquid chroma-tography (HPLC) experiments were conducted on Agilent 1260 LC system with a C18 column (USA). The fluorescence spectra were determined by using a Hitachi F-4600 spectrophotometer (Japan). The absorption spectra were determined by a PerkinElmer Lambda 25 UV/vis spectrophotometer (USA). The fluorescence images of cells were determined by using an Olympus FV3000 confocal scanning laser microscope (Japan). The fluorescence images of mice were operated on a PerkinElmer IVIS Lumina XR small animal optical in vivo imaging system (USA).

The process for fluorometric analysis of $ONOO^-$ in solution. The stock solution of TX-P (1.0×10^{-4} M) was prepared by dissolving TX-P in DMSO. The stock solution of $ONOO^-$ (1.0×10^{-2} M) was diluted to 3.0×10^{-4}-2.5×10^{-6} M stepwise. The solutions of TX-P with $ONOO^-$ were prepared with 1.0 mL of TX-P solution and 1.0 mL of $ONOO^-$ solution with a

series of concentrations and then were diluted to 10 mL with phosphate-buffered saline solution (PBS, 10 mM, pH 7.4). The final concentration TX-P were 1.0×10^{-5} M and $ONOO^-$ were 3.0×10^{-5} - 2.5×10^{-7} M. The excitation wavelength is 600 nm, and the emission wavelength is 670-900 nm. The excitation slit and emission slit were both set at 10 nm.

Fluorescence imaging of $ONOO^-$ in cells. The RAW 264.7 cells (mouse mononuclear macrophage), HeLa (human cervix cancer cells), and HepG2 (human hepatocellular carcinomas) were provided by Procell Life Science &Technology Co. Ltd. (Wuhan, China). The cells were cultured in DMEM (Dulbecco's modified Eagle's medium) containing 1% antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin) and 10 % FBS (fetal bovine serum) in an atmosphere of 37 °C and 5% CO_2.

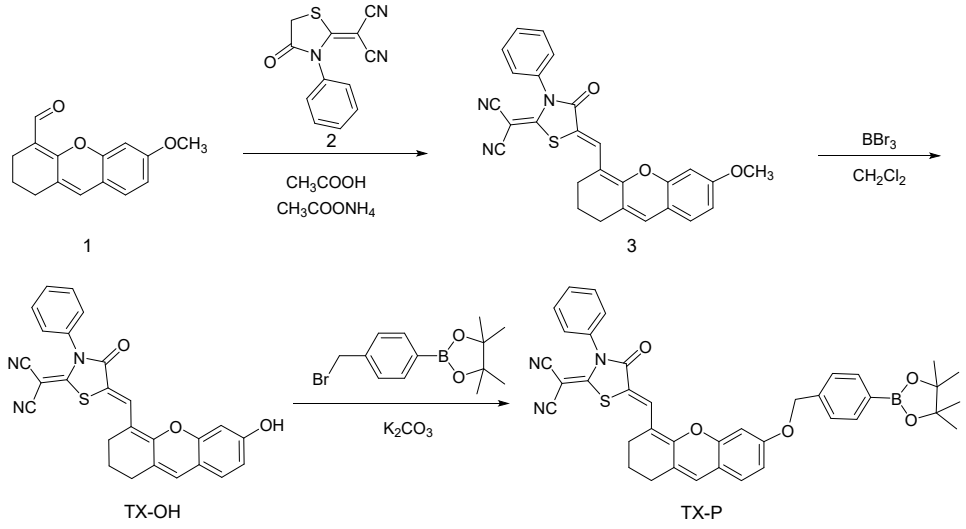
In the imaging of exogenous $ONOO^-$, RAW 264.7 cells were pretreated with different $ONOO^-$ concentration (0, 10, 20, 30 μM) for 30 min, then treated with TX-P (10 μM) at 37 °C for 30 min. In the imaging of endogenous $ONOO^-$, RAW 264.7 cells were divided into three groups. The first group were treated with TX-P (10 μM) at 37 °C for 30 min. The second group was stimulated with LPS (1 μg/mL) and IFN-γ (50 ng/mL) for 8 h and then treated with TX-P (10 μM) 37 °C for 30 min. The third group was treated with LPS (1 μg/mL), IFN-γ (50 ng/ml) and minocycline (200 μM) for 8 h, then treated with TX-P (10 μM) at 37 °C for 30 min. All the cells were washed by PBS buffer solution (10 mM, pH 7.4) three times and fluorescence image of cells were performed by a confocal scanning laser microscope.

Fluorescence imaging of acute paw edema inflammation in mice. The male Kunming mice of 25-30 g in weight were purchased from Hunan Slike Jingda Experimental Animal Co. Ltd. (Changsha, China), kindly kept in all the experimental process. All experiments were

performed in accordance with “Regulations of Hunan province on the administration of experimental animals” and approved by the ethics committee at Hunan Slike Jingda Experimental Animal Co. LTD. To image exogenous $ONOO^-$ in normal mice, TX-P (100 μM, 50 μL) was injected subcutaneously into the left and right paws of mice, respectively. Saline was then injected in the same area on the left side, and $ONOO^-$ (100 μM, 50 μL) was injected in the same region on the right side.

Endogenous $ONOO^-$ was imaged using TX-P in mice with paw edema. The first group was control group, in which a certain amount of saline was injected into the right hind paw of the mice. TX-P was then injected in situ, the imaging was performed at 0, 10, 20, 30, and 40 min. The second group was the paw edema group, in which the mice were injected with λ-carrageenan (20 mg kg^{-1}) in the right hind paw. TX-P was then injected in situ, recorded fluorescence imaging over time. The third to fifth groups were the treatment groups, in which the mice were injected with λ-carrageenan (20 mg kg^{-1}) to induce paw edema, then were injected separately with ibuprofen (IBU, 10 mg kg^{-1}), dexamethasone (DXM, 10 mg kg^{-1}), and indomethacin (INM, 10 mg kg^{-1}). TX-P was then injected in situ, recorded fluorescence imaging over time. One milliliter of blood was collected from the paw of mice, and the serum level of TNF-α was measured by an enzyme-linked immunosorbent assay kit. Histology analysis was performed by sacrificing the mice after the above treatments. The paws and the major organs from the mice were fixed in 4% paraformaldehyde solution, and then embedded in paraffin. The sliced tissue from the paw, heart, liver, spleen, lung and kidney were stained with hematoxylin and eosin (H&E) and then observed by an optical microscope.

2. Synthesis of TX-P.



Scheme S1. Synthetic route for TX-P.

Compound **1** was synthesized according to the reference (Sens. Actuators B Chem., 2020, 320, 128296). Compound **2** was synthesized according to the reference (ACS Appl. Mater. Interfaces 2018, 10, 17081-17088).

Compound 3. Compound 1 (0.25 g, 1.2 mmol) and Compound 2 (0.24 g, 1.0 mmol) were dissolved in CH_3COOH (20 mL). Next, CH_3COONH_4 (0.015 g) was added under stirring condition, the mixture was refluxed for 12 h. The obtained solution was concentrated and the crude product was purified by column chromatography (CH_2Cl_2:CH_3OH = 100:1) to afford a purple solid. Yield: 0.24 g (52%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.59 (t, J = 8.0 Hz, 1H), 7.39 (s, 1H), 7.34 (d, J = 4.0 Hz, 3H), 7.10 (d, J = 8.4 Hz, 1H), 6.81 (s, 1H), 6.63 (t, J = 8.5 Hz,3H), 4.03 (s, 3H), 2.77-2.62 (m, 4H), 1.42-1.25 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 166.6, 160.0, 158.5, 154.0, 133.8, 133.2, 131.2, 129.7, 129.2, 128.5, 127.7, 125.4, 114.0, 112.8, 110.4, 107.9, 106.6, 101.9, 53.0, 49.2, 29.4, 26.2, 22.4. MS (TOF): calcd: 466.1, found: 466.1.

Compound TX-OH. Compound 3 (0.23 g, 0.5 mmol) was dissolved in dry CH_2Cl_2 (10 mL), and BBr_3 (2.53 g, 10.0 mmol) was slowly added to the above solution at 0 °C. Then the

solution was continued to stir at 25 °C for 16 h. Next, the saturated $NaHCO_3$ solution was added to quench the reaction at 0 °C and the solution was extracted with CH_2Cl_2/ CH_3OH (100:1, v/v) for multiple times. The collected organic layer dried by anhydrous Na_2SO_4 and then the solvent was removed. The obtained crude product was further purified by column chromatography (CH_2Cl_2:CH_3OH = 90:1) to afford a dark purple solid. Yield: 0.17 g (77%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.34 (s, 1H), 8.14 (s, 1H), 7.57-7.50 (m, 5H), 7.22 (d, J = 8.3 Hz, 1H), 6.99 (s, 1H), 6.60 (t, J = 6.6 Hz, 2H), 2.66 (d, J = 6.4 Hz, 2H), 2.56 (d, J = 6.4 Hz, 2H), 1.97 (q, J = 7.3 Hz, 2H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 167.2, 160.6, 157.2, 153.9, 140.6, 134.1, 129.9, 129.7, 128.8, 128.7, 128.4, 127.7, 127.5, 127.0, 125.8, 115.2, 114.1, 109.0, 107.9, 101.6, 52.2, 29.4, 26.7, 22.6; MS (TOF): calcd: 451.1, found: 451.1.

Compound TX-P. TX-OH (113 mg, 0.25 mmol), 4-(bromomethyl) benzeneboronic acid pinacol ester (88 mg, 0.3 mmol) and K_2CO_3 (68 mg, 0.5 mmol) were dissolved in DMF (5 mL) under the nitrogen atmosphere. The solution was stirred for 5 h at 60 °C. Then, CH_2Cl_2 was added into the mixture, followed by the extraction of the organic layer with deionized water for multiple times. The obtained organic layer dried by anhydrous Na_2SO_4 was filtered, and then the solvent was evaporated. The crude product was further purified by silica gel column chromatography (CH_2Cl_2:CH_3OH = 120:1) to afford a purple solid. Yield: 73 mg (44%). ^{1}H NMR (400 MHz, $CDCl_3$: CD_4O = 5:1): δ 7.75 (d, J = 8.0 Hz, 3H), 7.54 (d, J = 4.0 Hz, 4H), 7.35 (d, J = 8.0 Hz, 1H), 7.06 (d, J = 8.0 Hz, 1H), 6.78 (s, 1H), 6.70 (d, J = 8.0 Hz, 2H), 5.04 (s, 2H), 2.68 (d, J = 4.0 Hz, 2H), 2.55 (d, J = 4.0 Hz, 2H), 1.84 (s, 2H), 1.28 (s, 12H). ^{13}C NMR (100 MHz, $CDCl_3$: CD_4O = 5:1) δ 166.6, 160.8, 157.8, 154.1, 139.1, 134.8, 133.7, 133.3, 131.4, 131.0, 129.9, 128.8, 128.2, 127.6, 126.7, 126.5, 115.3, 114.1, 112.7, 110.5, 108.4, 108.0, 101.3,

84.0, 70.2, 29.6, 24.7, 20.8. HRMS m/z: calcd for $C_{39}H_{34}BN_3O_5S$ $[M+Na]^+$: 690.2312, found: 690.2223.

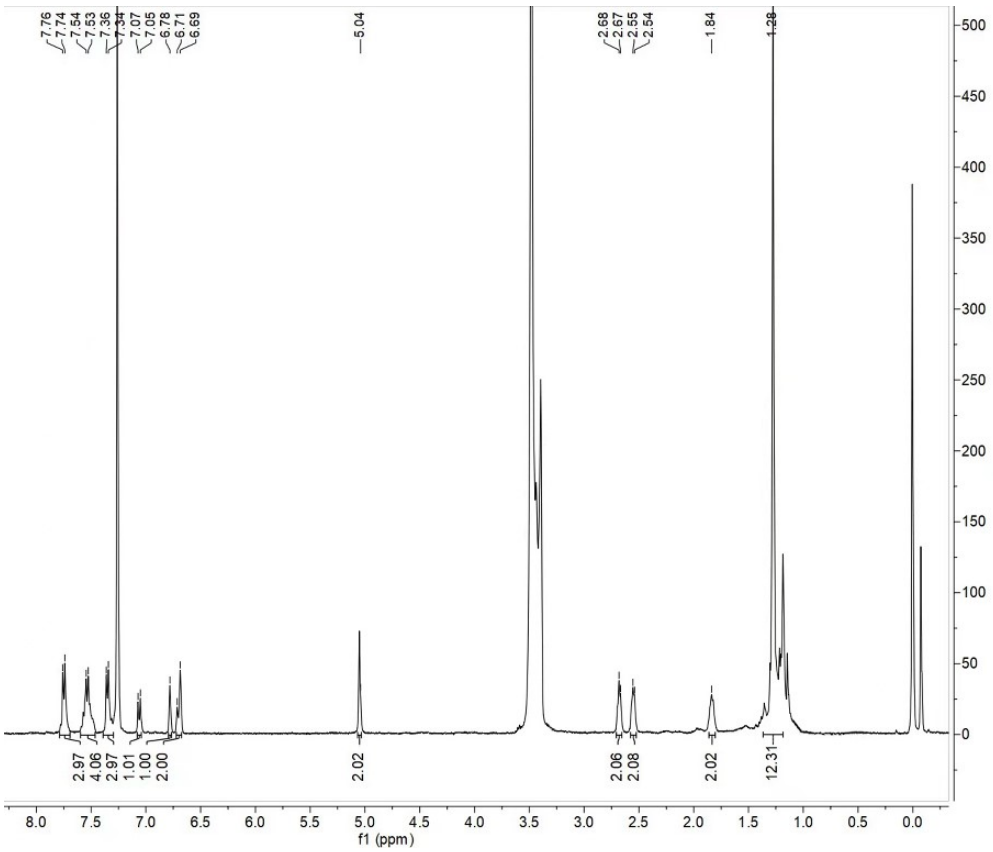


Fig. S1 ^{1}H NMR spectra of TX-P in $CDCl_3$: CD_4O = 5:1.

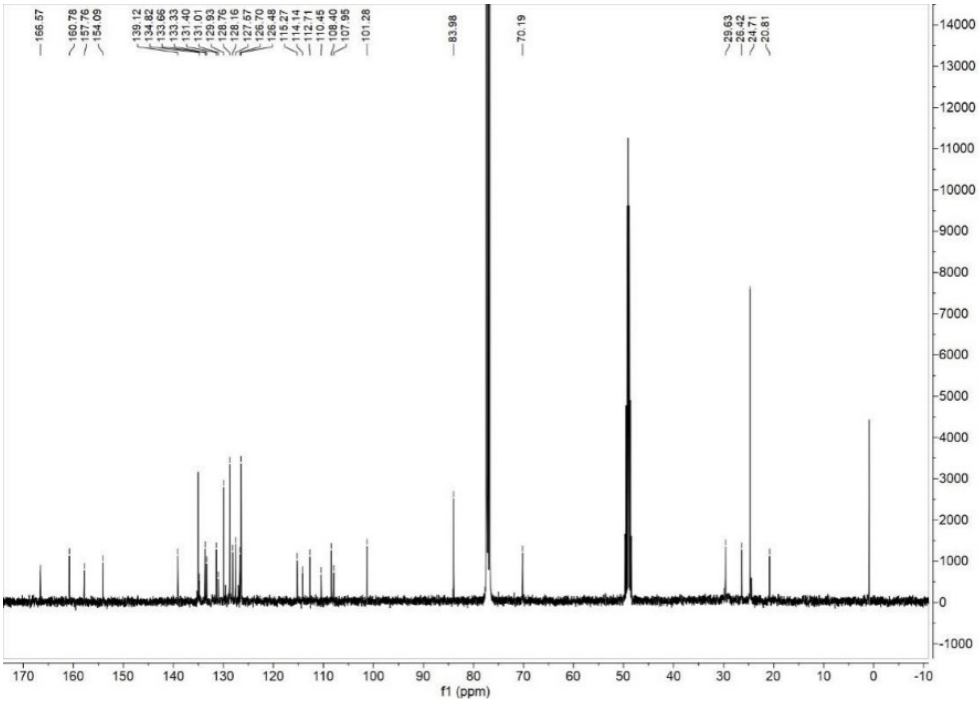


Fig. S2 ^{13}C NMR spectra of TX-P in $CDCl_3$: CD_4O = 5:1.

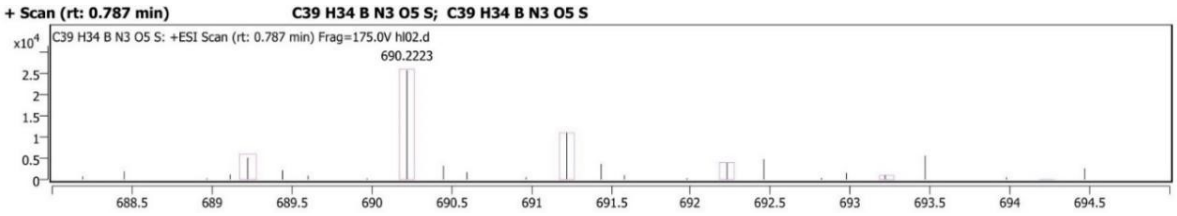
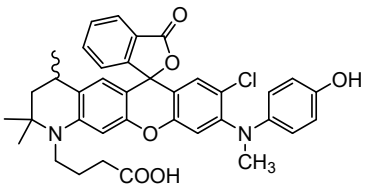
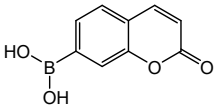
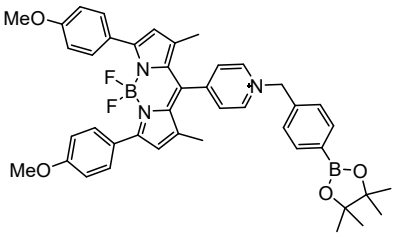
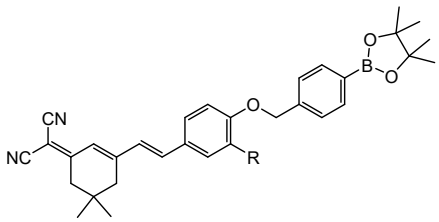
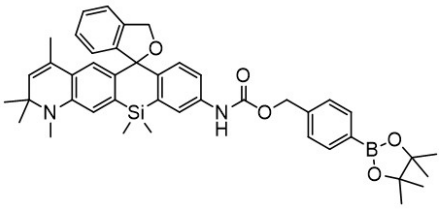
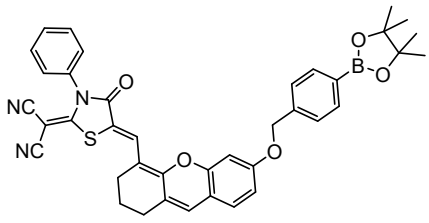


Fig. S3 HRMS spectra of TX-P.

3. Spectral data.

Table S1 Comparison of the performance of this probe with the reported probes for ONOO-

Probe	Excitation (nm)	Emission (nm)	Stoke shift (nm)	Applications	Ref.
	543	570	37	Cells & tissues	12
	332	475	143	Seawater	13
	463	613	148	Cells & organ	14
	455	590/676	135/221	Cells & inflammatory mice	15
	645	696	51	Cells & glioma mice	17
	600	725	125	Cells & paw edema mice	This work

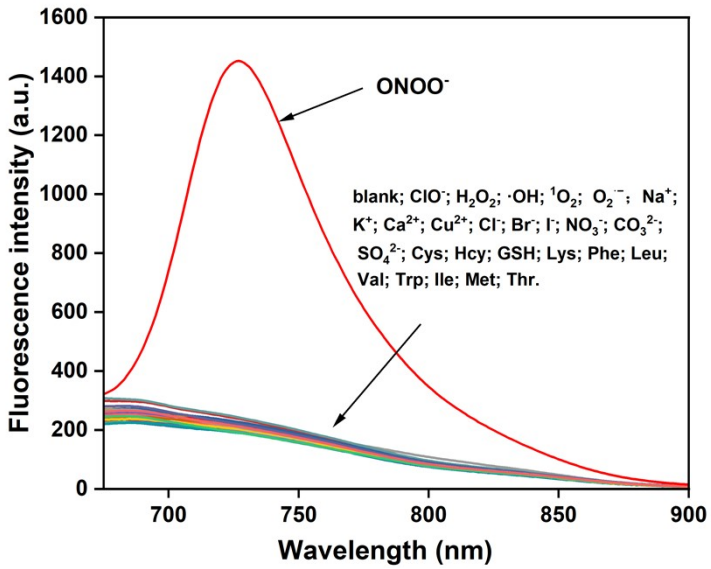


Fig. S4 Fluorescence spectra of TX-P (10 μM) toward $ONOO^-$ (30 μM) and other analytes (100 μM) in PBS buffer solution (10 mM, pH 7.4).

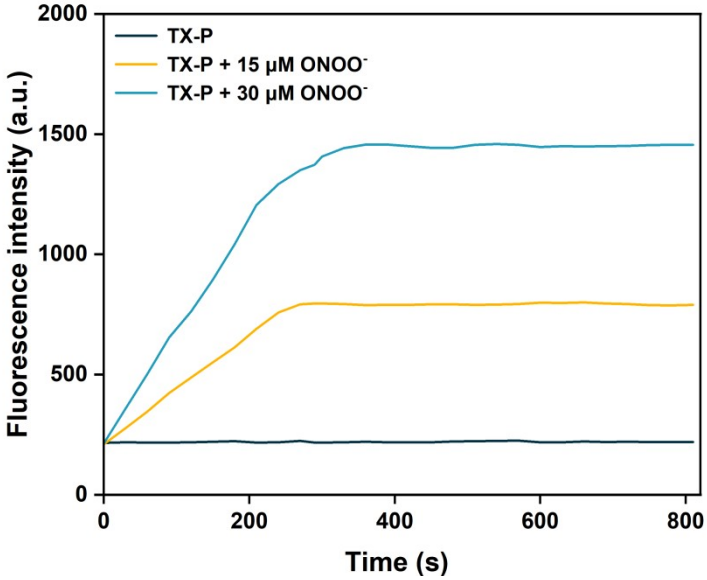


Fig. S5 Time-dependent fluorescence intensity of TX-P (10 μM) upon the addition of $ONOO^-$ (0, 15, 30 μM) in PBS buffer solution (10 mM, pH 7.4).

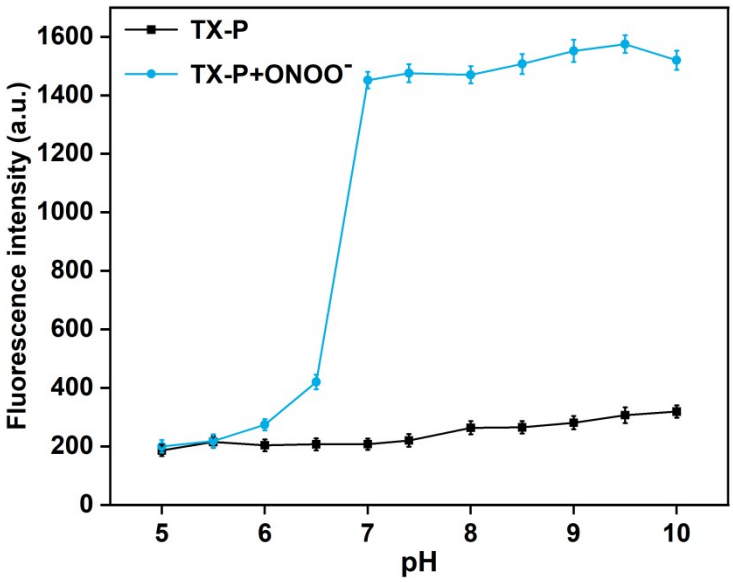


Fig. S6 Effect of pH on the fluorescence intensity of TX-P (10 μM) before and after reaction with $ONOO^-$ (30 μM).

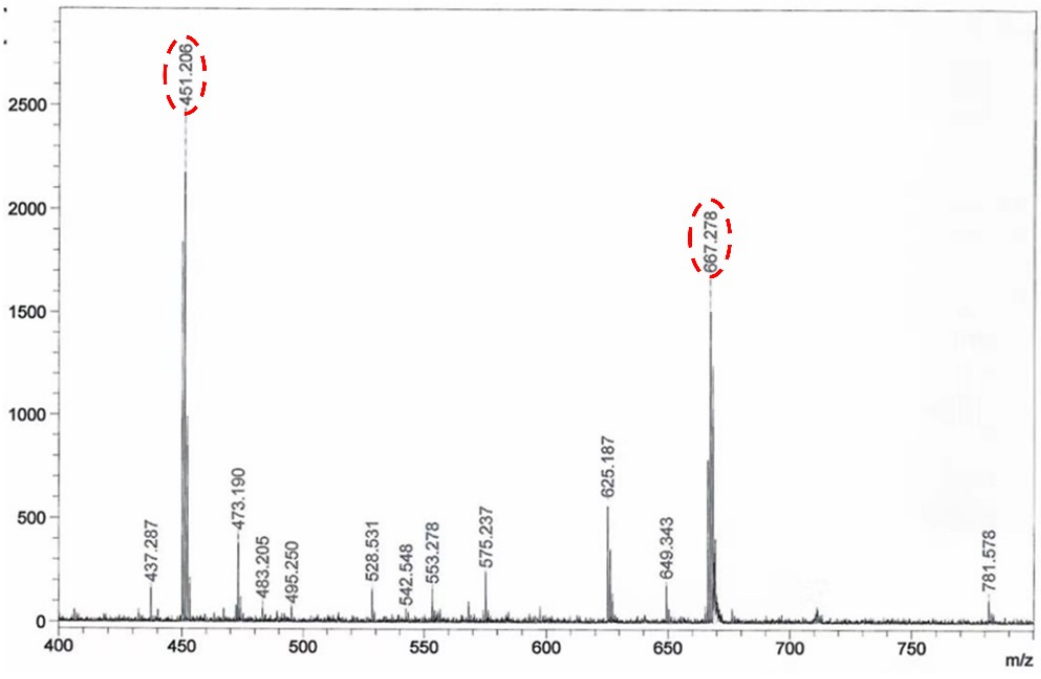


Fig. S7 Mass spectra of TX-P with $ONOO^-$.

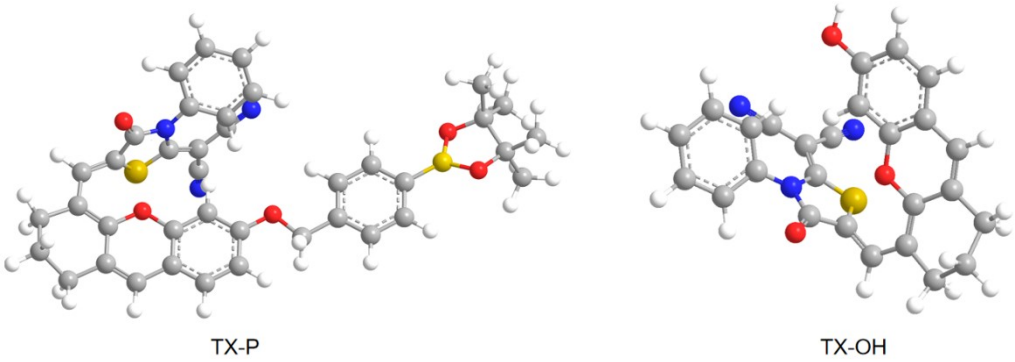


Fig. S8 The optimized structures of TX-P and TX-OH. In the ball-and-stick model, carbon, oxygen, sulfur and nitrogen atoms are colored in gray, red, yellow and blue, respectively.

4. Biological assays.

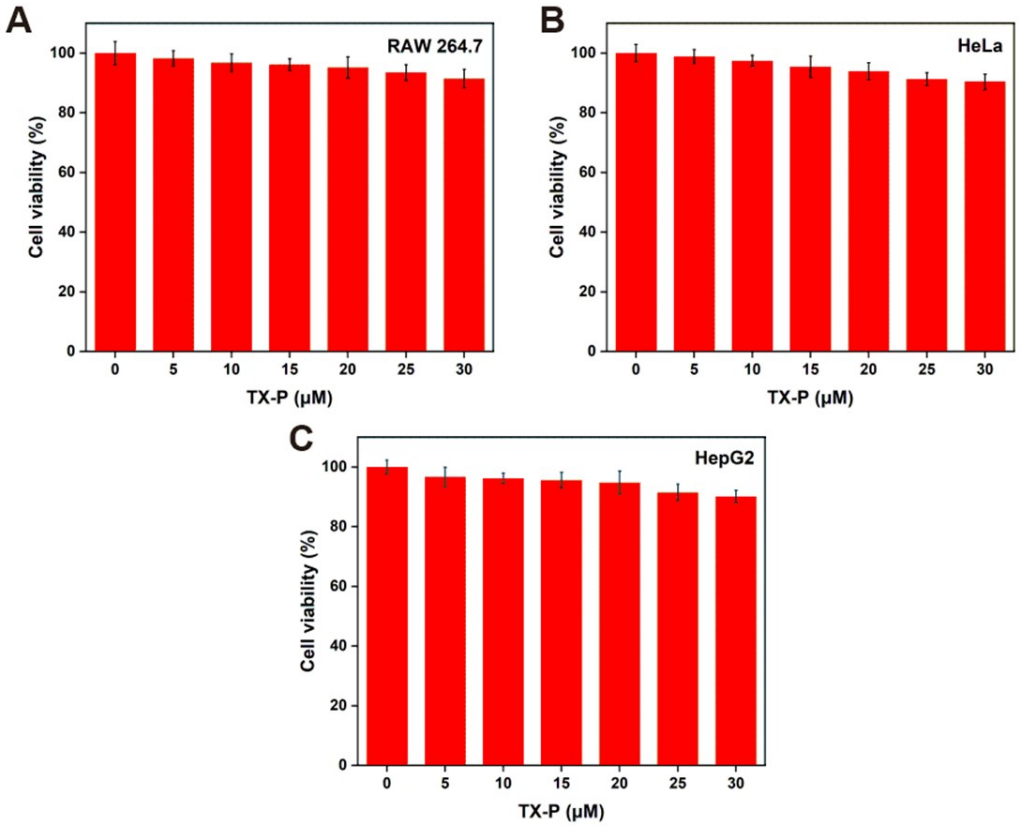


Fig. S9 CCK-8 assay for estimating cell viability (%) of (A) RAW 264.7 cells, (B) HeLa cells and (C) HepG2 cells treated with various concentrations of TX-P (0-30 μM) after 24 h incubation.

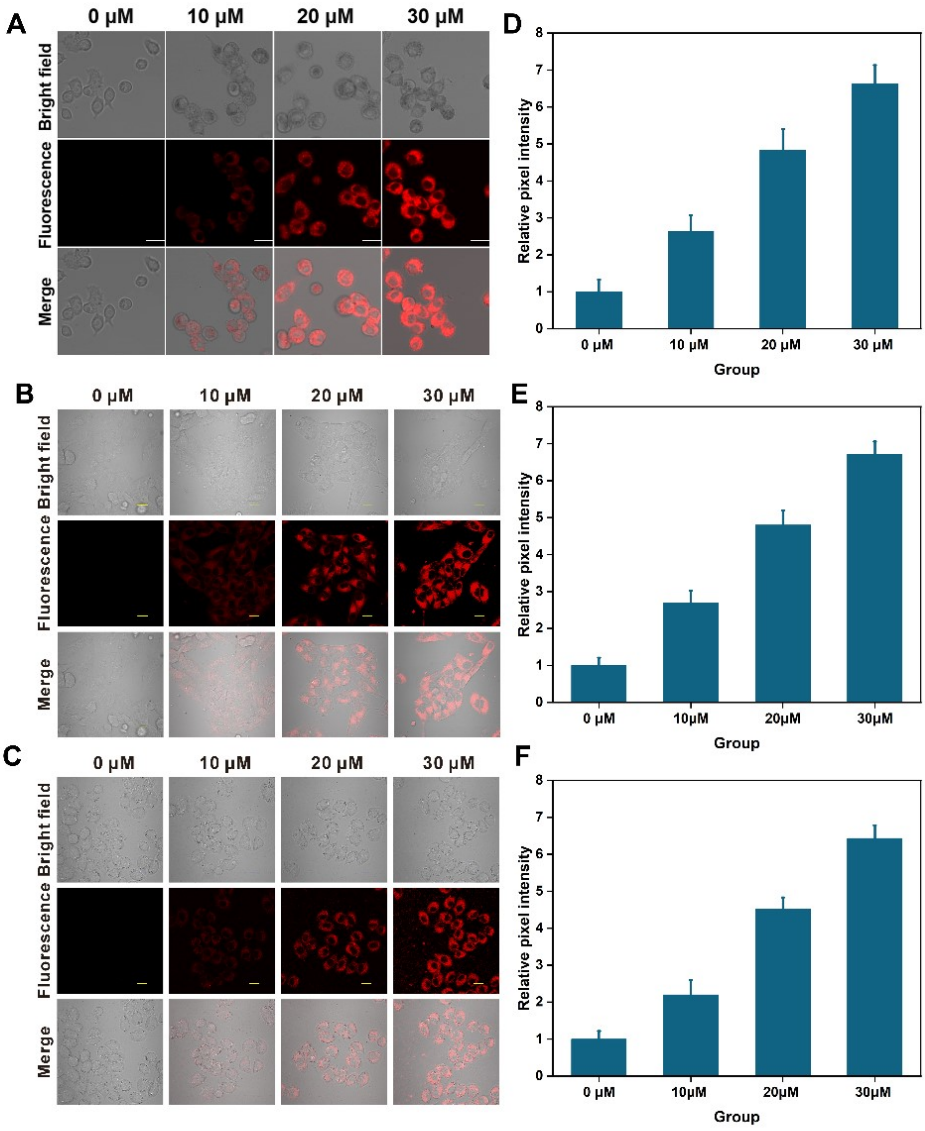


Fig. S10 Fluorescence imaging of exogenous $ONOO^-$ in (A) RAW 264.7, (B) HeLa and (C) HepG2 cells. The cells were incubated with different concentration of $ONOO^-$ (0, 10, 20, 30 μM), and then treated with TX-P (10 μM). (D), (E) and (F) Relative pixel intensity in (A), (B) and (C). λ_{ex} = 640 nm, λ_{em} = 700-775 nm; Scale bar: 20 μm.

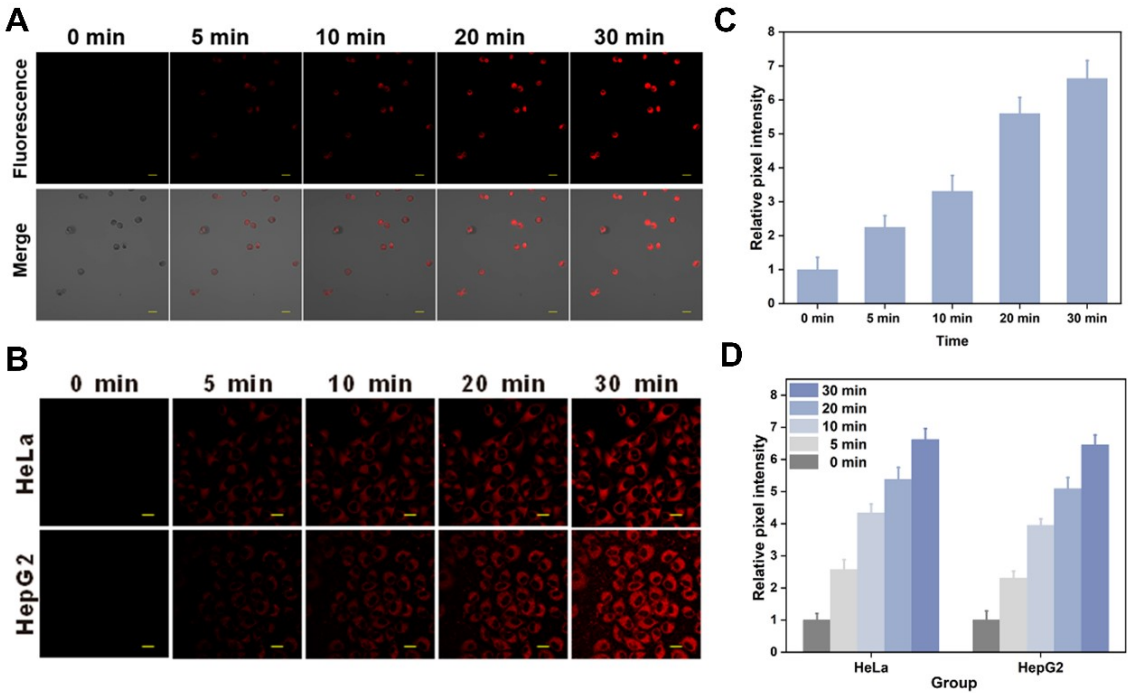


Fig. S11 Fluorescence imaging of exogenous $ONOO^-$ in (A) RAW 264.7, (B) HeLa and HepG2 cells. The cells were treated with $ONOO^-$ (30 μM) for 30 min, and then incubated with TX-P (10 μM) at different time points: 0, 5, 10, 20, 30 min. (C) and (D) Relative pixel intensity in (A) and (B). λ_{ex} = 640 nm, λ_{em} = 700-775 nm; Scale bar: 20 μm.

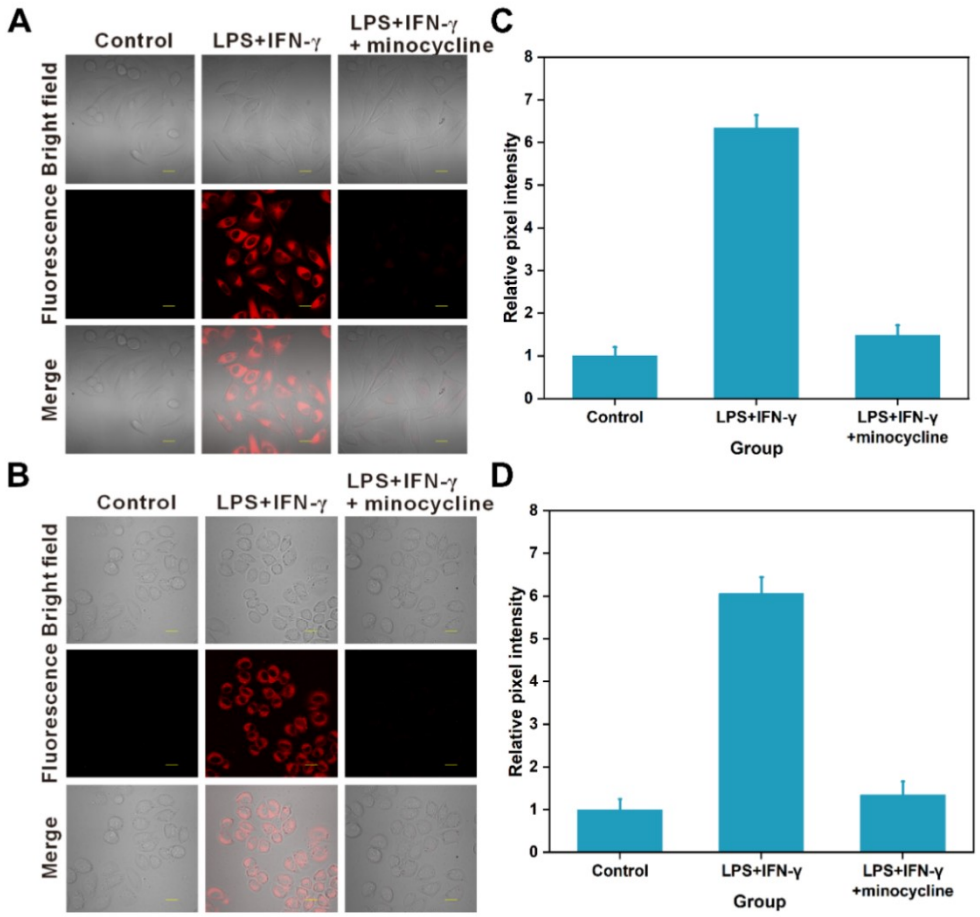


Fig. S12 Fluorescence imaging of endogenous $ONOO^-$ in (A) HeLa and (B) HepG2 cells. Control group: the cells were treated with TX-P (10 μM). LPS+IFN-γ group: the cells were treated with LPS (1 μg/mL) and IFN-γ (50 ng/mL), and then incubated with TX-P (10 μM). LPS+IFN-γ+minocycline group: the cells were treated with LPS (1 μg/mL), IFN-γ (50 ng/mL), minocycline (200 μM), and then incubated TX-P (10 μM). (C) and (D) Relative pixel intensity in (A) and (B). λ_{ex} = 640 nm, λ_{em} = 700-775 nm; Scale bar: 20 μm.

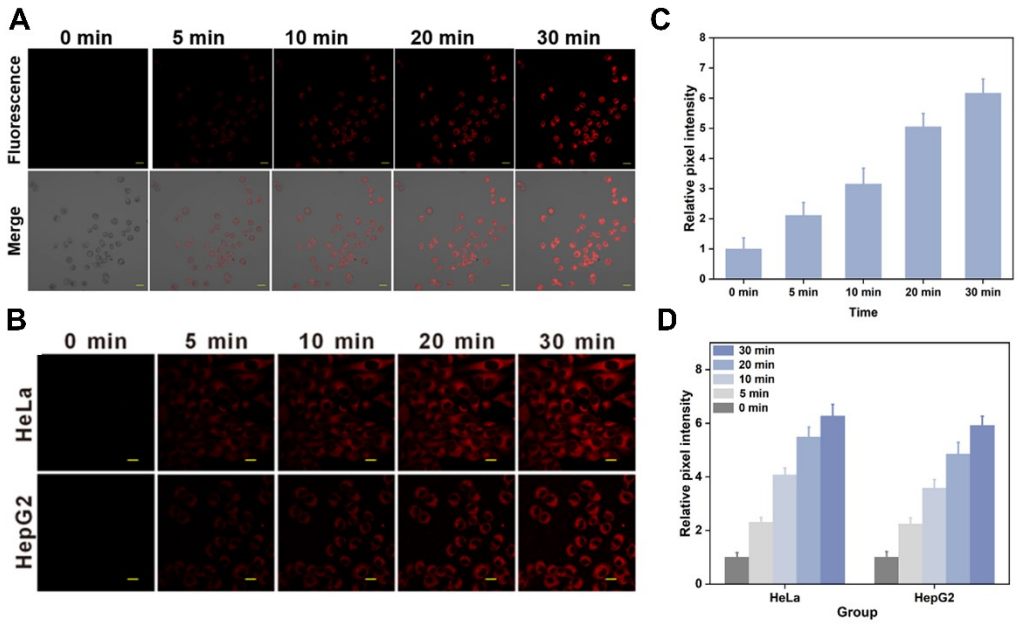


Fig. S13 Fluorescence imaging of endogenous $ONOO^-$ in (A) RAW 264.7, (B) HeLa and HepG2 cells. The cells were stimulated with LPS (1 μg/mL)/IFN-γ (50 ng/mL) for 8 h and then incubated with TX-P (10 μM) at different time points: 0, 5, 10, 20, 30 min. (C) and (D) Relative pixel intensity in (A) and (B). λ_{ex} = 640 nm, λ_{em} = 700-775 nm; Scale bar: 20 μm.

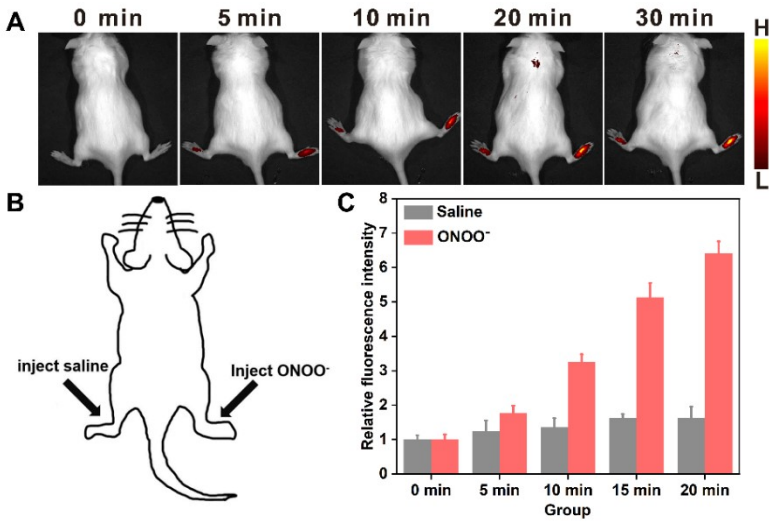


Fig. S 14 (A) Fluorescence images for exogenous $ONOO^-$ in mice. (B) Schematic diagram of the mice model. The mice were injected with saline in the left paw and $ONOO^-$ (1 mM) in the right paw, and then probe TX-P (100 μM) was injected subcutaneously at the same site. (C) Relative fluorescence intensity in (A). λ_{ex} = 640 nm, λ_{em} = 680-780 nm.

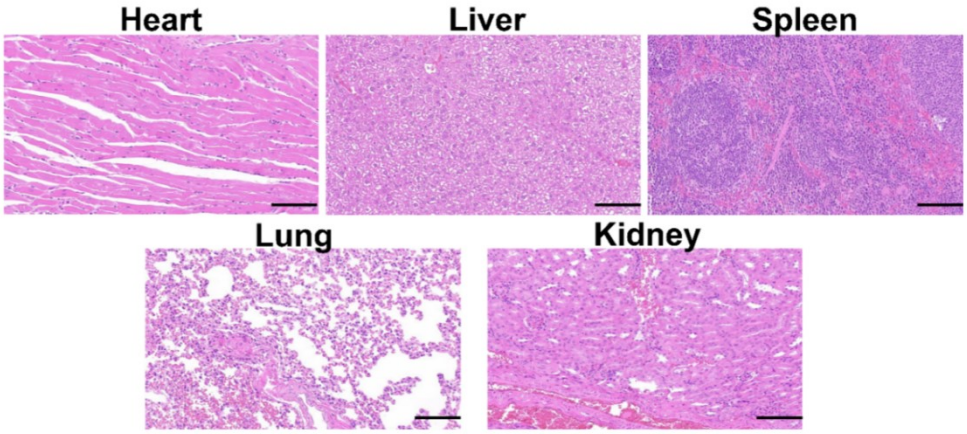


Fig. S15 H&E staining of isolated organs (heart, liver, spleen, lung, kidney) after the injection of TX-P in mice. Scale bar: 100 μm.