

Supplementary Information

Observation of macrophage autophagy in the healing of diabetic ulcers via lysosome-targeting polarity-specific two-photon probe

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Table of Contents

- 1. General Information on Materials and Methods.**
- 2. Structural Identifications of the Compounds.**
- 3. Supporting Tables and Figures**
- 4. References.**

1. General Information on Materials and Methods.

Instruments and materials.

Unless otherwise stated, all solvents and reagents were purchased from commercial suppliers and were used as received without further purification. All the reagents were obtained from Aladdin Ind. Corp. (Shanghai, China). All other chemicals were from commercial sources and of analytical reagent grade, unless indicated otherwise. The reactions were performed in standard glassware. All aqueous solutions were prepared in ultrapure water with a resistivity of 18.25 M Ω ·cm (purified by Milli-Q system, Millipore). Column chromatography was performed using silica gel 60 (230 \pm 400 mesh, 0.040 \pm 0.063 mm) from Dynamic Adsorbents. NMR spectra were recorded on a Bruker-400 spectrometer, using TMS as an internal standard. High-resolution mass spectra (HRMS) were collected on a Thermo Scientific Q Exactive Plus Orbitrap spectrometer operating on ESI. Absorption spectra were recorded with a UV-vis spectrophotometer (Shimadzu UV-2550, Japan), and one-photon fluorescence spectra were obtained with a fluorimeter (Shimadzu RF-6000, Japan). Two-photon fluorescence spectra were excited by a mode-locked Ti: sapphire femto-second pulsed laser (Chameleon Ultra I, Coherent, America) and recorded with a DCS200PC photon counting with Omno-5008 monochromator (Zolix, China). Two photon microscopy was performed on a Zeiss LSM 710 multiphoton laser scanning confocal microscope (Carl Zeiss, Germany).

Synthesis of Compound 1.

6-iodoquinoline-2-carbaldehyde (684 mg, 2 mmol) was added Pd(PPh₃)₂Cl₂ (142 mg, 0.2 mmol), CuI (38 mg, 0.2 mmol), PPh₃ (107 mg, 0.4 mmol) and 4-(4-ethynylphenyl) morpholine (748 mg, 4 mmol). NEt₃ (2 mL) and THF (10 mL) were then added and the reaction mixture was refluxed for 12 h under an inert Ar atmosphere. The solvent was then removed and the crude residue was purified by column chromatography (1:6 v/v ethyl acetate/petroleum ether) to give the product as yellow solid. Yield: 351 mg (51.3%). ¹HNMR (400 MHz, DMSO-*d*₆) δ 10.12 (s, 1H), 8.59 (d, *J* = 8.5 Hz, 1H), 8.31 (d, *J* = 1.9 Hz, 1H), 8.22 (d, *J* = 8.7 Hz, 1H), 8.03 (d, *J* = 8.4 Hz, 1H), 7.95 (dd, *J* = 8.7, 1.9 Hz, 1H), 7.49 (d, *J* = 8.8 Hz, 2H), 7.01 (d, *J* = 8.9 Hz, 2H), 3.74 (q, *J* = 4.4 Hz, 5H), 3.26 – 3.15 (m, 5H).

Synthesis of XZTU-VIS.

Compound 1 (140 mg, 0.41 mmol), 2-(3,5,5-trimethylcyclohex-2-en-1-ylidene) malononitrile (148.8 mg, 0.8 mmol) and piperidine (catalyst, 10 drops) were dissolved in absolute ethanol (20

mL). The mixture was refluxed for 9 h under an inert Ar atmosphere. After cooling to room temperature, the red solid was filtered. Then, the crude solid was purified by column chromatography (1:100 v/v methanol/dichloromethane) to give the product as red solid. Yield: 69.4 mg (43.4%). ¹H NMR (400 MHz, Chloroform-*d*) δ 8.29 (s, 1H), 8.14 – 8.10 (m, 2H), 7.82 – 7.76 (m, 2H), 7.39 – 7.33 (m, 2H), 7.21 – 6.93 (m, 2H), 6.84 – 6.79 (m, 2H), 6.47 (d, *J* = 1.2 Hz, 1H), 3.75 (dt, *J* = 6.1, 3.6 Hz, 4H), 3.19 (dd, *J* = 6.1, 3.4 Hz, 4H), 2.46 – 2.26 (m, 4H), 1.04 (s, 6H). ¹³C NMR (101 MHz, Chloroform-*d*) δ 167.52, 154.62, 151.14, 145.97, 136.60, 132.92, 132.73, 130.25, 129.73, 127.80, 123.07, 121.11, 114.81, 113.19, 91.90, 87.64, 66.72, 48.38, 43.42, 29.69, 26.54, 25.61, 24.60. HRMS (MALDI-DHB): calcd. for C₃₄H₃₀N₄O [M+H]⁺ 511.24196 found 511.28525.

Spectroscopic measurements.

For the selectivity assay, superoxide anion (O₂^{•-}) was prepared by dissolving KO₂ in DMSO solution.¹ •OH was generated by Fenton reaction between Fe²⁺ (EDTA) and H₂O₂ quantitatively, and Fe²⁺ (EDTA) concentrations represented •OH concentrations.² The ONOO⁻ source was the donor 3-morpholinosydnonimine hydrochloride (sin-1, 200 mM).³ NO was generated in form of 3-(aminopropyl)-1-hydroxy-3-isopropyl-2-oxo-1-triazene (NOC-5, 200 μM). H₂O₂ was determined at 240 nm ($\epsilon_{240\text{ nm}} = 43.6\text{ M}^{-1}\text{cm}^{-1}$). NO₂⁻ was generated from NaNO₂.

Quantum yield measurements.

The measurement of the fluorescence quantum yield was measured by using quinine sulfate ($\Phi = 0.55$ in 0.1 M H₂SO₄ solution) as the reference, and using the following equation.

$$\Phi_s = \frac{A_r \cdot F_s \cdot n_s^2}{A_s \cdot F_r \cdot n_r^2} \Phi_r \quad (A \leq 0.05)$$

Where s and r represent the sample to be tested and the reference dye, respectively. A represents the absorbance at the maximum absorption wavelength, F represents the fluorescence spectrum integral at the maximum absorption wavelength excitation, and n represents the refractive index of the sample to be tested or the reference dye solvent.

Measurement of Two-photon Cross Section.

The two-photon absorption cross section (δ) was determined by using femtosecond (fs) fluorescence measurement technique as described. XZTU-VIS was dissolved in Dioxane or PBS with little dioxane, and the two-photon induced fluorescence intensity was measured at 750-900 nm

by using rhodamine B as the reference, whose two-photon property has been well characterized in the literature. The intensities of the two-photon induced fluorescence spectra of the reference and sample at the same excitation wavelength were determined. The TP absorption cross section was calculated by using the following equation.

$$\delta_s = (S_s \Phi_r n_s^2 c_r) / (S_r \Phi_s n_r^2 c_s) \delta_r$$

where the subscripts s and r stand for the sample and reference molecules, respectively. The intensity of the two-photon excited fluorescence was denoted as S. Φ is the fluorescence quantum yield, and Φ is the overall fluorescence collection efficiency of the experimental apparatus. The number density of the molecules in solution was denoted as c. δ_r is the two-photon absorption cross section of the reference molecule.

Cytotoxicity assay.

The cytotoxicity was evaluated by MTT assay. Briefly, PC12 cells were cultured in DMEM in 96-well microplates in incubator for 24 h. The medium was next replaced by fresh DMEM containing various concentrations of XZTU-VIS (0-30 μ M). Each concentration was tested in five replicates. Cells were rinsed twice with phosphate buffer saline (PBS) 24 h later and incubated with 0.5 mg/mL MTT reagent for 4 h at 37 °C. The culture was removed and 150 μ L DMSO was added to dissolve for mazan. After shaking for 10 min, the absorbance at 490 nm was measured by microplate reader (Synergy 2. BioTek Instruments Inc.). Cell survival rate was calculated by $A/A_0 \times 100\%$ (A and A_0 are the absorbance of the XZTU-VIS labelled group and the control group, respectively).

Cell Culture and Imaging.

BV-2 cells were cultured with DMEM supplemented with 10% (v/v) fetal bovine serum (Gibco), 100 U/mL penicillin, and 100 μ g/mL streptomycin in a humidified atmosphere with 5/95 (v/v) of CO₂/air at 37 °C. One day before imaging, cells were detached with a treatment of 0.2% (w/v) trypsin-EDTA solution (Gibco) and suspended in culture media. The cell suspension was then transferred to confocal dishes to grow with adherence. For imaging, PC12 cells at 80% confluence were harvested by scraping and transferred to confocal dishes to grow with adherence. Two-photon excited fluorescence images were obtained by Zeiss LSM 710 multiphoton laser scanning confocal microscope with a 20 \times air objective.

Calculation of mean fluorescence intensity.

The mean fluorescence density was measured by Image-Pro Plus (v. 6.0) and calculated via the equation (mean density = $IOD_{sum}/area_{sum}$), where IOD and area were integral optical density and area of the fluorescent region.

Immunofluorescence staining.

wound healing-operated mice were euthanized and perfused with cold PBS, followed by fixation with 4% paraformaldehyde for 2 days. The ischemic brains were cut into 50- μ m sections and the free-floating slices were blocked with 0.1 M PBS containing 5% fetal bovine serum and 0.3% Triton X for 1 h at room temperature. After washing, the slices were incubated at 4 °C overnight with the following primary antibodies: anti-IL-6 (1:200; 12912, Abcam, Cambridge, England) and LC3B (1:200; ab104224, Abcam, Cambridge, England). The slices were then rinsed anti- and incubated with an Alexa 594-conjugated antibody (1:200; ANT030, Millipore, Billerica, MA) or an Alexa 488-conjugated antibody (1:200; ANT024, Millipore, Billerica, MA for 2 h at room temperature. After thorough rinsing, the nuclei were stained with DAPI (94010, Vector Laboratories, Burlingame, CA, USA). All slices were photographed using a confocal fluorescence microscope (BX63, Olympus Optical Ltd, Tokyo, Japan). The number of immunoreactive cells in predefined areas were quantified using ImageJ software (Media Cybernetics Inc., Rockville, MD, USA). Six different fields for each mouse and six mice for each group were counted. All counts were conducted by blinded observers.

General procedure for detection of H₂O₂, TNF- α and IL-1 β concentration.

BV-2 cells were cultured in 96-well plates and treated with NS-398、APO after bearing scrap leather. After treatment, cell culture supernatants were collected for detection of TNF- α concentration using TNF- α ELISA kit (Invitrogen, ERA56RB) and IL-1 β ELISA kit (Invitrogen, EHC002b) according to the manufacturer's instructions. Meanwhile, the cells were collected for measurement of cellular H₂O₂ concentration using the Amplex Red Hydrogen Peroxide Assay Kit (Invitrogen, A22188) according to recommended protocol as described earlier.

Histological Staining of the Tissue Slices.

After imaging, the mice were killed, and the brains and other tissues (heart, liver, spleen, lung, kidney, stomach) were collected for tissue analysis. Through a series of standard procedures, including fixation in 10% neutral buffered formalin, embedding into paraffin and sectioning at 3 μ m thickness, the tissues were stained with hematoxylin-eosin (H&E). Thereafter, the prepared

slices were examined by a digital microscope.

Two-photon fluorescence cell imaging.

All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of South-central University of Nationalities and experiments were approved by the Animal Ethics Committee of College of Biology (South-central University of Nationalities). Wild-type C57BL/6J mice (n = 300; 25–30 g) were purchased from Hubei Experimental Animal Research Center. (Hubei, China; No. 43004700018817, 43004700020932). All animal experimental protocols were approved by the Animal Experimentation Ethics Committee of South-central University of Nationalities (No. 2020-scuec-043) and were conducted according to the Animal Care and Use Committee guidelines of South-central University of Nationalities. All BALB/c mice (age 4 weeks, weight 17–20 g) were randomly divided into Sham (n = 3), wound-control model (n = 3) and NS-398 model (n = 3) groups. Diabetic mice were prepared through intraperitoneal injection of streptozotocin (STZ, a diabetes-inducing drug), and man-made wounds were created on their right feet. After the model of Wild-type C57BL/6J mice mentioned above was successfully established, XZTU-VIS (100 μ L, 200 μ M) was injected through the tail vein, and the mice were anesthetized with isoflurane and euthanasia before fluorescence imaging two-photon excited tissue fluorescence images were obtained by Zeiss LSM 710 multiphoton laser scanning confocal microscope.

2. Structural Identifications of the Compounds.

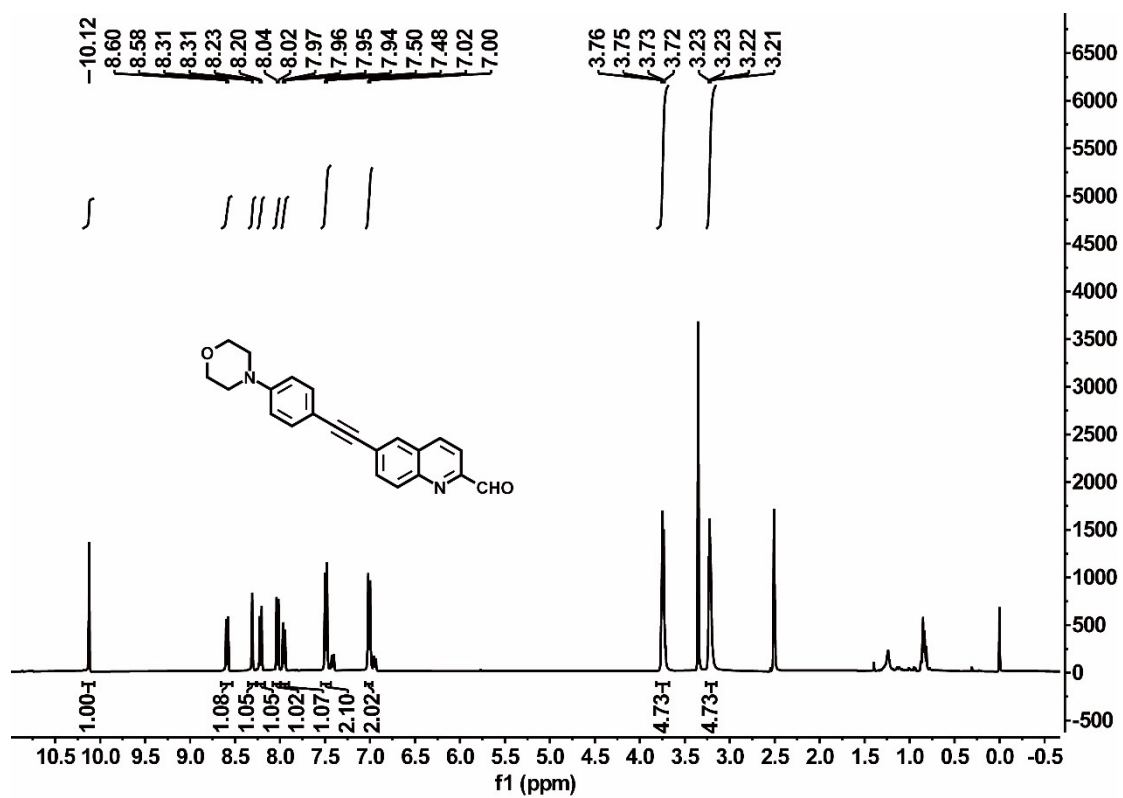


Figure S1. ¹H NMR spectrum (DMSO-*d*₆) of compound 1.

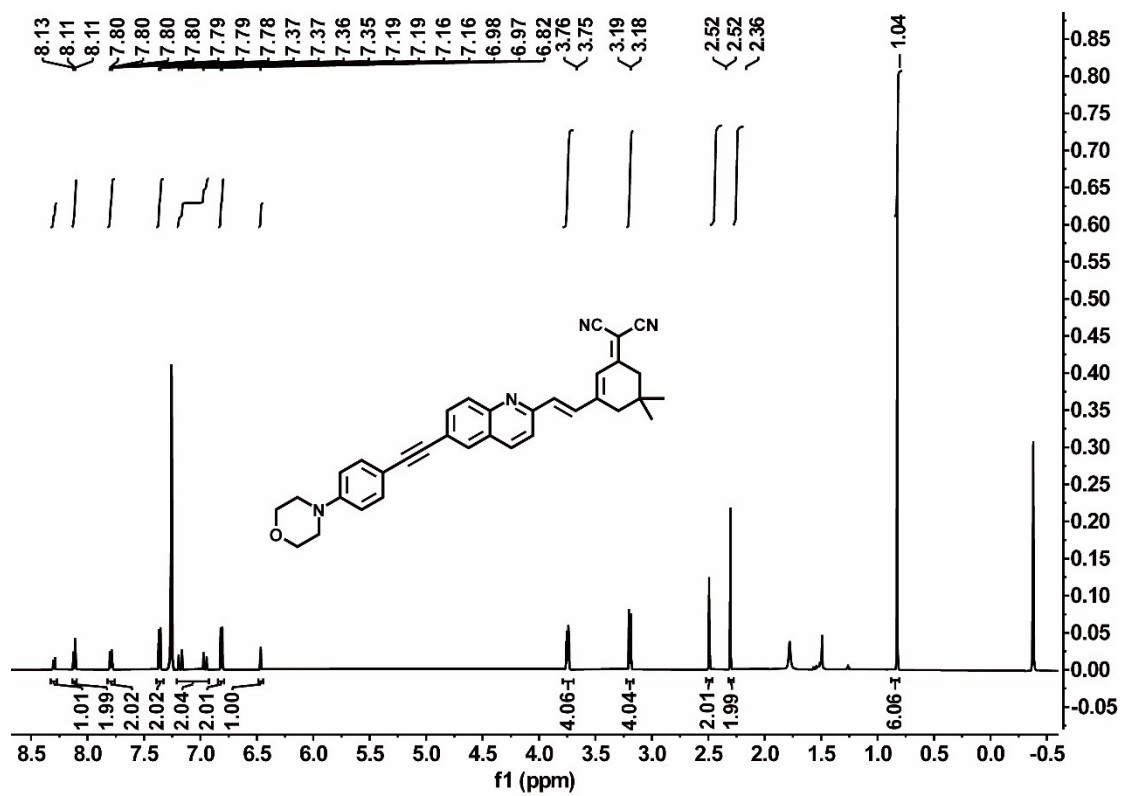


Figure S2. ¹H NMR spectrum (Chloroform-*d*) of XZTU-VIS.

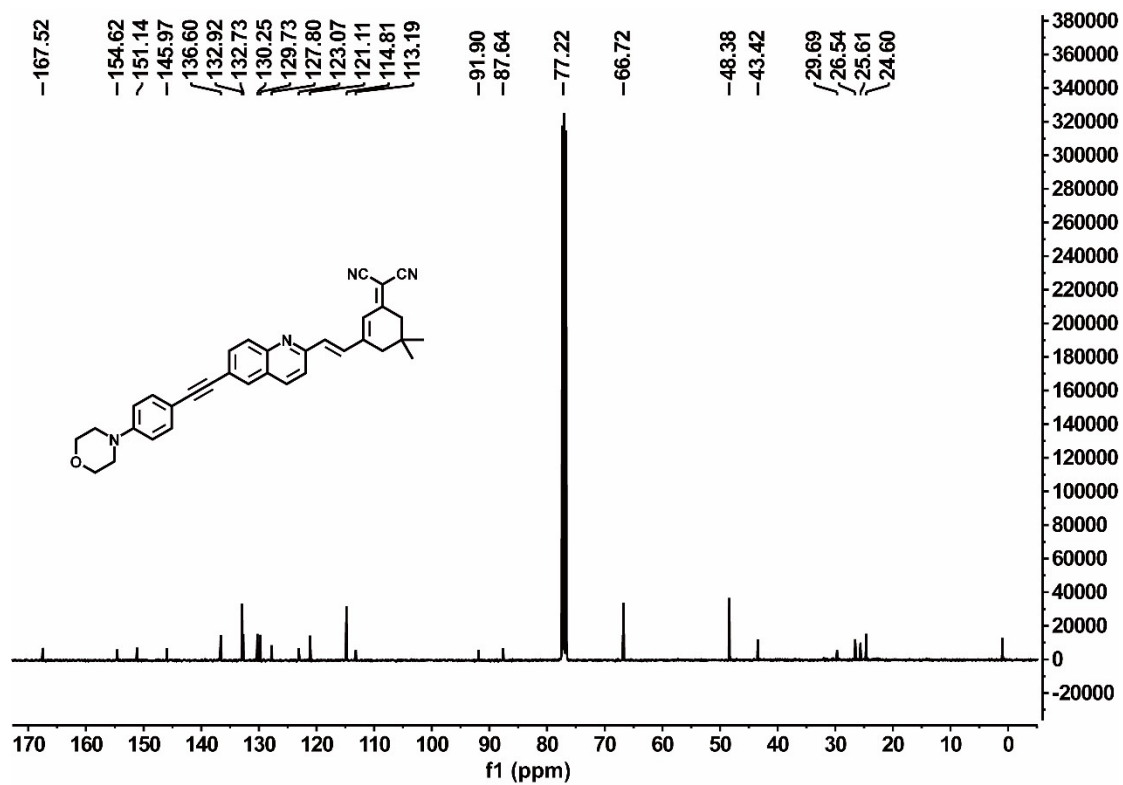


Figure S3. ¹³C NMR spectrum (Chloroform-*d*) of XZTU-VIS.

10 #78 RT: 0.31 AV: 1 NL: 3.60E7
T: FTMS + p ESI Full ms [150.00-800.00]

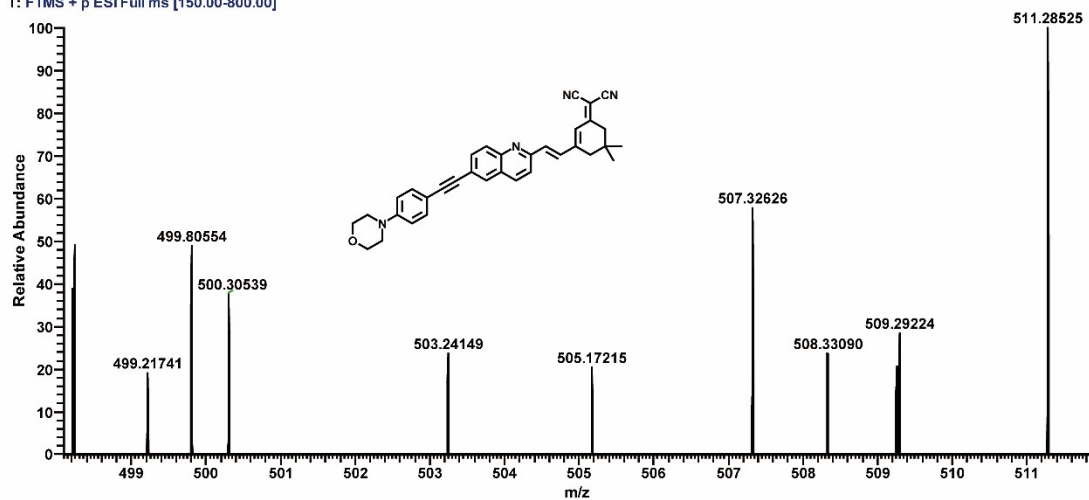


Figure S4. HRMS spectrum of compound XZTU-VIS.

3. Supporting Tables and Figures

Table S1 Photophysical properties of XZTU-VIS in various solvents.

Solvent	Dielectric Constant	λ_{abs} (nm)	λ_{em} (nm)	ϵ ($\text{M}^{-1} \text{cm}^{-1}$)	ϕ
Toluene	2.24	339	457	1.62×10^4	0.263
Dioxane	2.2	345	494	1.43×10^4	0.173
Dimethylformamide	36.71	340	588	1.35×10^4	0.008
Methanol	31.2	347	420	0.88×10^4	<0.001
Chloroform	4.9	332	501	1.42×10^4	0.154
Tetrahydrofuran	7.58	348	543	2.30×10^4	0.079
Acetonitrile	46.0	373	420	1.78×10^4	<0.001
Dichloromethane	9.1	336	543	1.29×10^4	0.065
Dimethyl sulfoxide	48.9	340	428	1.20×10^4	0.061
PBS	80.10	n.d.	454	n.d.	0.011
Ethanol	51.9	333	546	1.12×10^4	<0.001

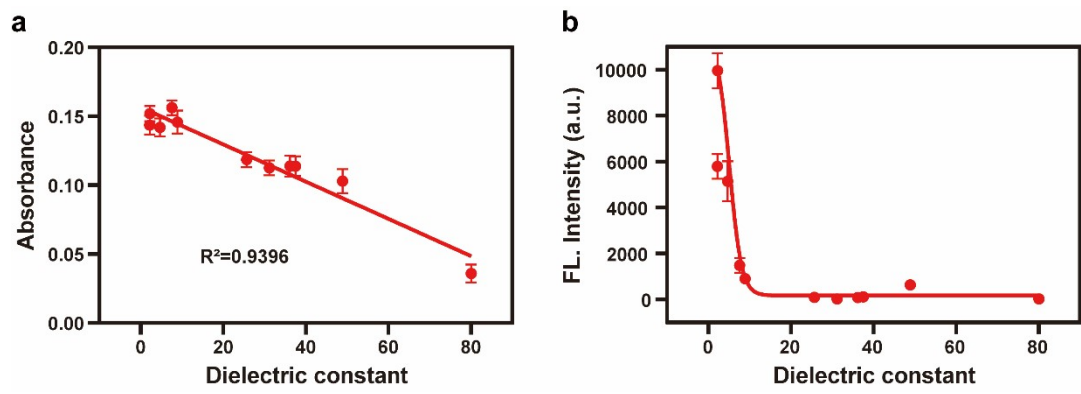


Figure S5. The absorption (a) and fluorescence intensity (b) with the dielectric constant.

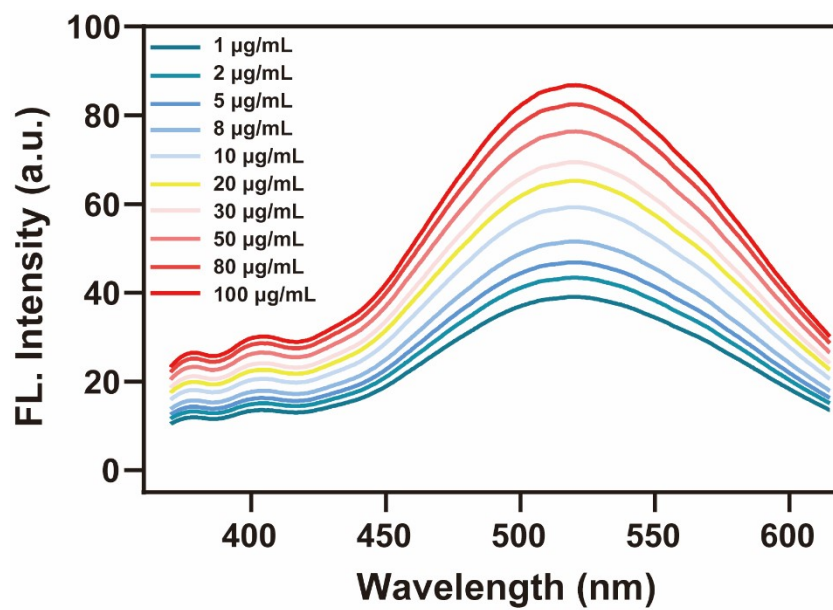


Figure S6. One-photon fluorescence spectra of XZTU-VIS in PBS with different concentrations of bovine serum albumin.

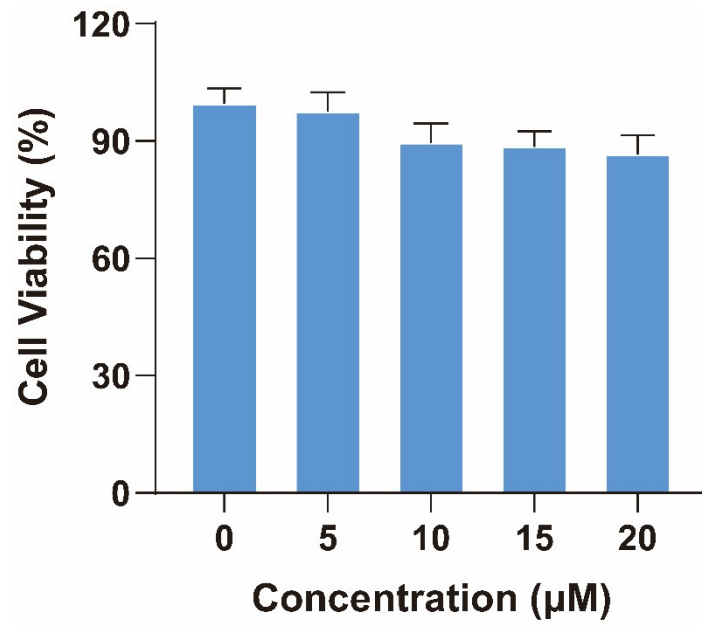


Figure S7. MTT assay of BV2 cells treated with different concentration of XZTU-VIS (0, 5, 10, 15, 20 µM).

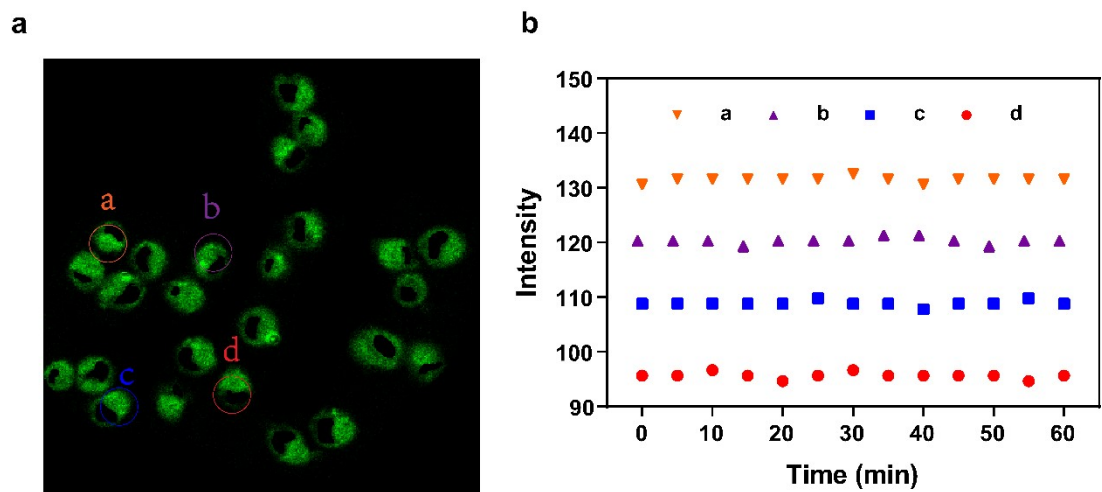


Figure S8. (a) Images of BV2 cells labeled with XZTU-VIS (10 μ M) for 1 h. (b) Fluorescence intensity from circle a-d as a function of time. The fluorescence intensity was collected with 5 min intervals for the duration of 60 min.

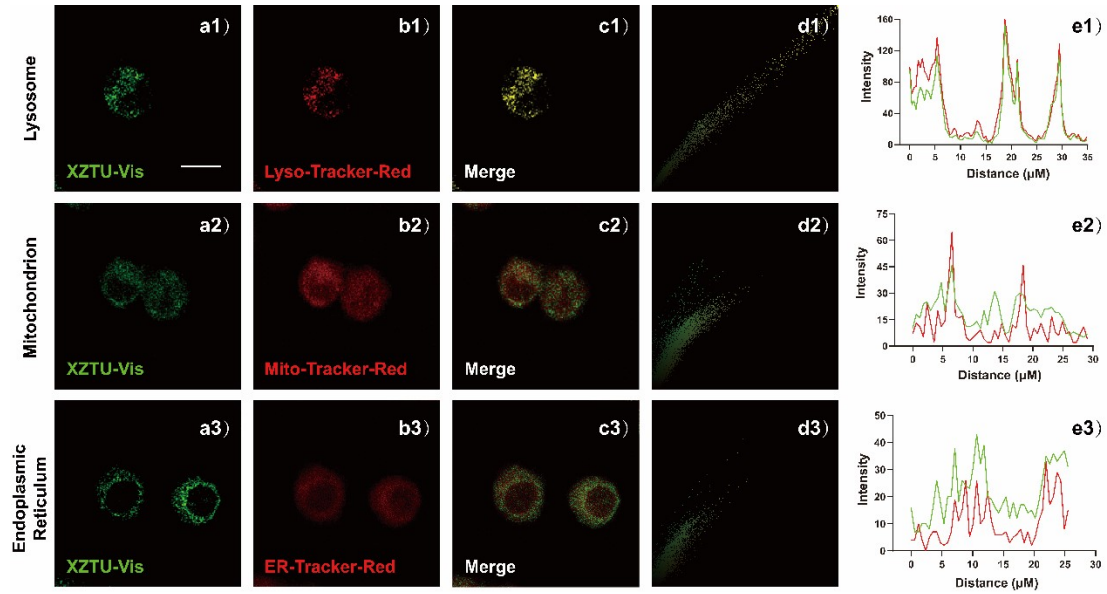


Figure S9. Two-photon fluorescence co-localization cell imaging of XZTU-VIS and commercial dyes including Lyso-Tracker Red (a1-c1), Mito-Tracker Red (a2-c2), ER-Tracker Red (a3-c3) in BV2 cell. Red channel (595–650 nm, $\lambda_{ex} = 594$ nm) for Lyso-Tracker Red, Mito-Tracker Red and ER-Tracker Red; Green channel (500-550 nm, $\lambda_{ex} = 810$ nm) for XZTU-VIS. Fluorescence intensity correlation plot of XZTU-VIS and commercial dyes (d1, d2, d3). Scale bar: 50 μm . Fluorescence intensity profile of the region of interest across cells in the red and green channels (e1, e2, e3).

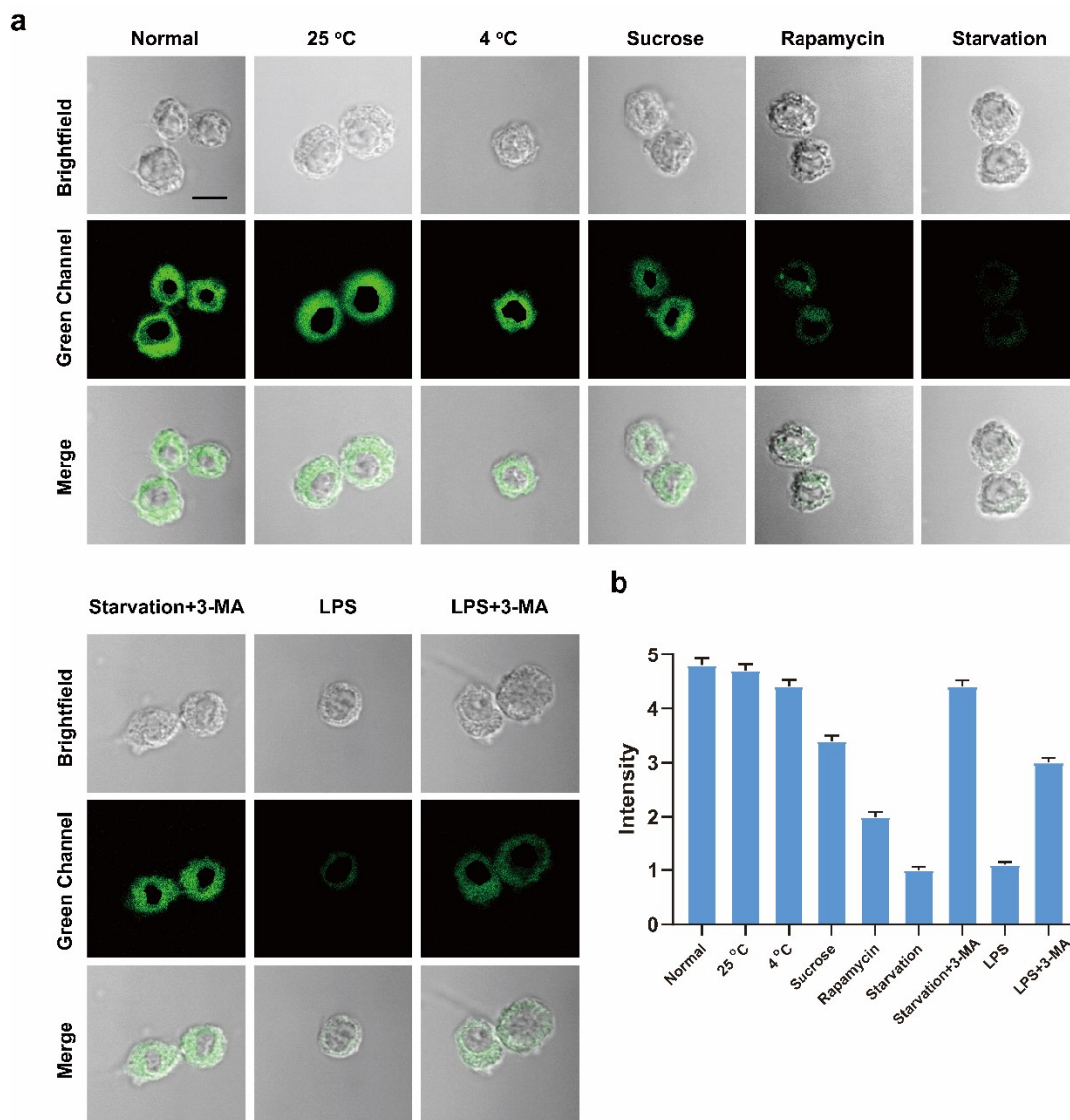


Figure S10. (a) Two-photon fluorescence imaging of BV-2 cells incubated with XZTU-VIS (10 μ M) in Normal group (control), 25 °C (viscosity), 4 °C (viscosity), Sucrose (polarity), Rapamycin (autophagy), starvation (autophagy), starvation+3-MA (autophagy inhibited), LPS (inflammation) and LPS+3-MA (autophagy inhibited), respectively. (b) Histograms of average fluorescence intensity of (a). Data are presented as the mean \pm SD (Normal group: n = 44 cells from three cultures; 25 °C: n = 27 cells from three cultures; 4 °C: n = 28 cells from three cultures; Sucrose: n = 40 cells from three cultures; Rapamycin: n = 37 cells from three cultures; starvation: n = 36 cells from three cultures; starvation+3-MA: n = 33 cells from three cultures; LPS: n = 46 cells from three cultures; LPS+3-MA: n = 51 cells from three cultures). λ_{ex} = 810 nm, λ_{em} = 500-550 nm. Scale bars: 20 μ m.

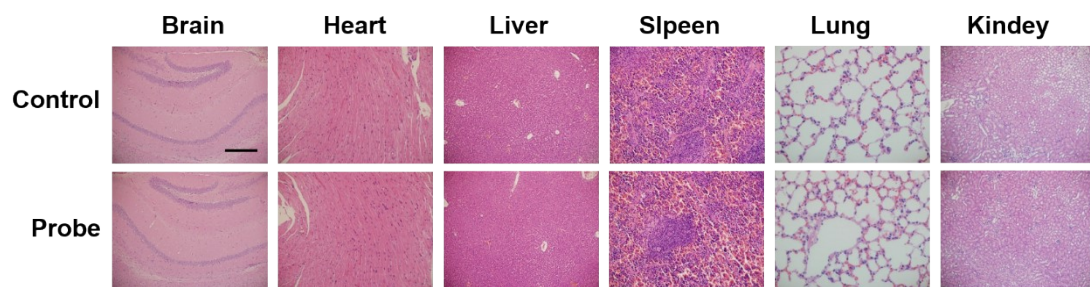


Figure S11. H&E staining results of different organs collected from the control group and XZTU-VIS (200 μ L, 100 μ M) treated group. Scale bar: 100 μ m.

4. Reference.

1. L. Dai, D. Wu, Q. Qiao, W. Yin, J. Yin and Z. Xu, *Chem. Comm*, 2016, **52**, 2095-2098.
2. L. Zeng, T. Xia, W. Hu, S. Chen, S. Chi, Y. Lei and Z. Liu, *Anal. Chem.*, 2018, **90**, 1317-1324.
3. R. M. Uppu, *Anal. Biochem.*, 2006, **354**, 165-168.