Supplementary Information

An ultrasensitive polarity-specific two-photon probe for revealing of autophagy in living cells during scrap leather-induced neuroinflammation process

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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. PC12 cells were obtained from Procell Life Science & Technology Co., Ltd. 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 MΩ·cm (purified by Milli-Q system, Millipore). Column chromatography was performed using silica gel 60 (230 ± 400 mesh, 0.040 ±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).

Synthetic route to AMN.

Compound 1 and **AMN** were synthesized through the previously reported route with some modifications.[1] In general synthetic process, ethanol is used as solvent, and the substitution reaction of 4-bromo-1,8-naphthalic anhydride and 4-(2-aminoethyl)-morpholine can generate product **1**; Subsequently, the condensation reaction between compound **1** and 4-ethynylaniline under CuI and with $Pd(PPh_3)_2Cl_2$ as catalytic agent can synthesize product **AMN**. HRMS and NMR data of **AMN** as follows: ^{1}HNMR (400 MHz, DMSO-d_6) δ 8.66 (d, J = 8.3 Hz, 1H), 8.47 (d, J = 7.1 Hz, 1H), 8.34 (d, J = 7.6 Hz, 1H), 8.01 – 7.81 (m, 2H), 7.43 (d, J = 8.2 Hz, 2H), 6.63 (d, J = 8.3 Hz, 2H), 5.86 (s, 2H), 4.14 (t, J = 6.5 Hz, 2H), 3.53 (s, 4H), 2.59 – 2.51 (m, 2H), 2.45 (s, 4H). ^{13}CNMR (101 MHz, DMSO) δ 163.62, 163.31, 151.19, 133.97, 132.57, 131.59, 130.95, 130.65, 129.98, 128.29, 128.24, 127.85, 122.84, 120.86, 114.11, 107.27, 102.84, 84.93, 66.69,

55.98, 53.87, 37.32. HRMS m/z calc. for $C_{26}H_{23}N_3O_3$: 426.18122; found: 426.18127 $[M + H]^+$.

Spectroscopic measurements.

For the selectivity assay, superoxide anion ($O_2^{\bullet-}$) was prepared by dissolving KO_2 in DMSO solution. •OH was generated by Fenton reaction between Fe^{2+} (EDTA) and H_2O_2 quantitatively, and Fe^{2+} (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). H_2O_2 was determined at 240 nm ($\varepsilon_{240\,nm} = 43.6\ M^{-1}cm^{-1}$). NO_2^- was generated from $NaNO_2$.

Quantum yield measurements.

The measurement of the fluorescence quantum yield was measured by using quinine sulfate ($\Phi = 0.55$ in 0.1 M H_2SO_4 solution) as the reference, and using the following equation.

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

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. **AMN** was dissolved in 10 mM toluene, 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. δr 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 **AMN** (0-30 μM). 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 μL 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 **AMN** labelled group and the control group, respectively).

Cell Culture and Imaging.

PC12 cells were cultured with DMEM supplemented with 10% (v/v) fetal bovine serum (Gibco), 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified atmosphere with 5/95 (v/v) of CO_2/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×air objective.

Two-photon fluorescence imaging in tissues.

All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Shaanxi University of Science & Technology and experiments were approved by the Animal Ethics Committee of College of Biology (Shaanxi University of Science & Technology). Wild-type C57BL/6J mice (n = 300; 25–30 g) were purchased from Hubei Experimental Animal Research Center. (Hubei, China; No. 43004700018817, 43004700020932). Animals were housed in a room with controlled humidity (65 ± 5%) and temperature (25 ± 1 °C), under a 12/12-hour light/dark cycle with free access to food and water for at least 1 week before

the experiments. After the model was successfully established, 100 μL, 200 μM of the probe **AMN** was injected through the tail vein. After 1 h, the mice were anesthetized and dissected to remove the mouse brain tissue, and a 300 μm section was prepared with a microtome. Two-photon excited tissue fluorescence images were obtained by Zeiss LSM 710 multiphoton laser scanning confocal microscope.

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.

Statistical Analysis.

Statistical Product and Service Solutions (SPSS) software 19.0 was used for the statistical analysis. The error bars shown in the figures represented the mean ± s.d. Differences were determined with a one-way analysis of variance (ANOVA) followed by LSD test. Statistical significance was assigned at $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$. Sample size was chosen empirically based on our previous experiences and pre-test results. No statistical method was used to predetermine sample size and no data were excluded. The numbers of animals or samples in every group were described in the corresponding figure legends. The distributions of the data were normal. All experiments were done with at least three biological replicates. Experimental groups were balanced in terms of animal age, sex and weight. Animals were all caged together and treated in the same way. Appropriate tests were chosen according to the data distribution. Variance was comparable between groups in experiments described throughout the manuscript.

2. Structural Identifications of the Compounds.

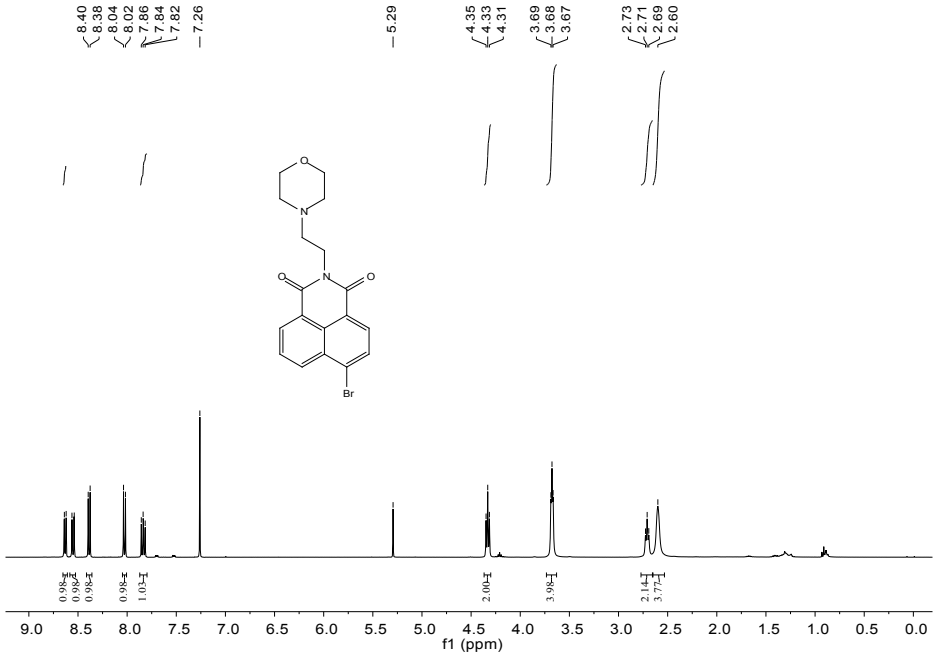

Fig. S1. ^{1}H NMR spectrum ($CDCl_3$) of compound **1**.

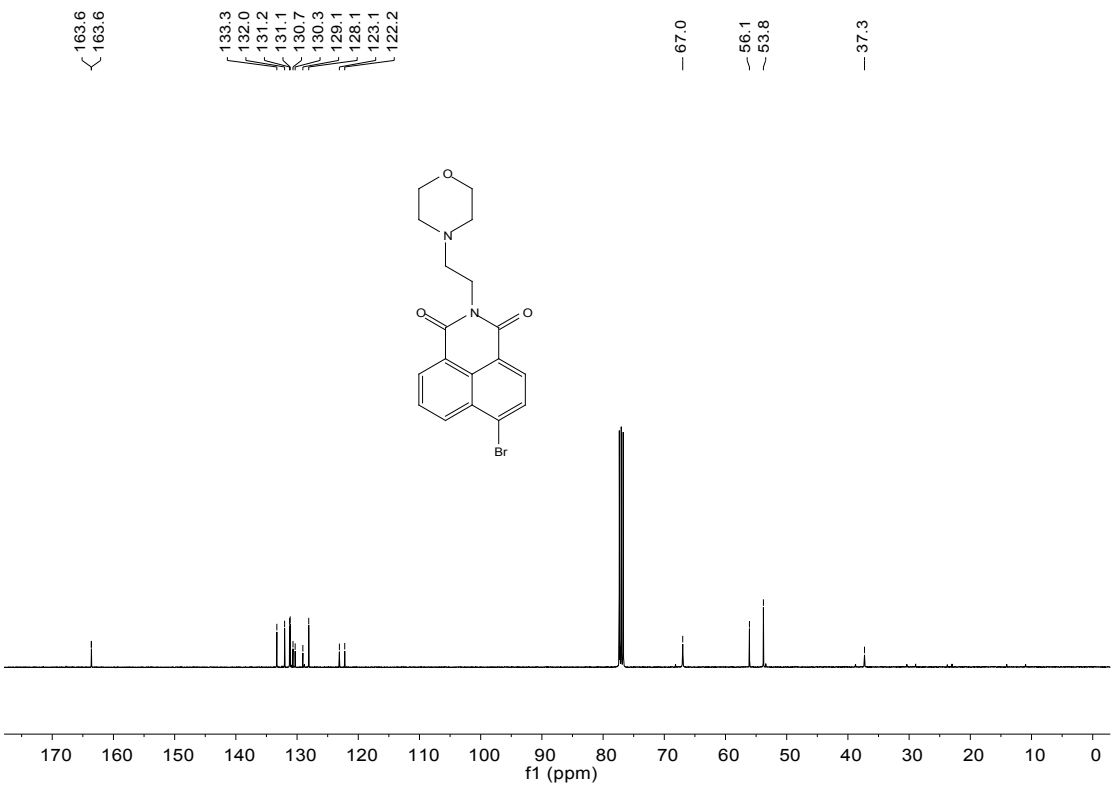

Fig. S2. ^{13}C NMR spectrum ($CDCl_3$) of compound **1**.

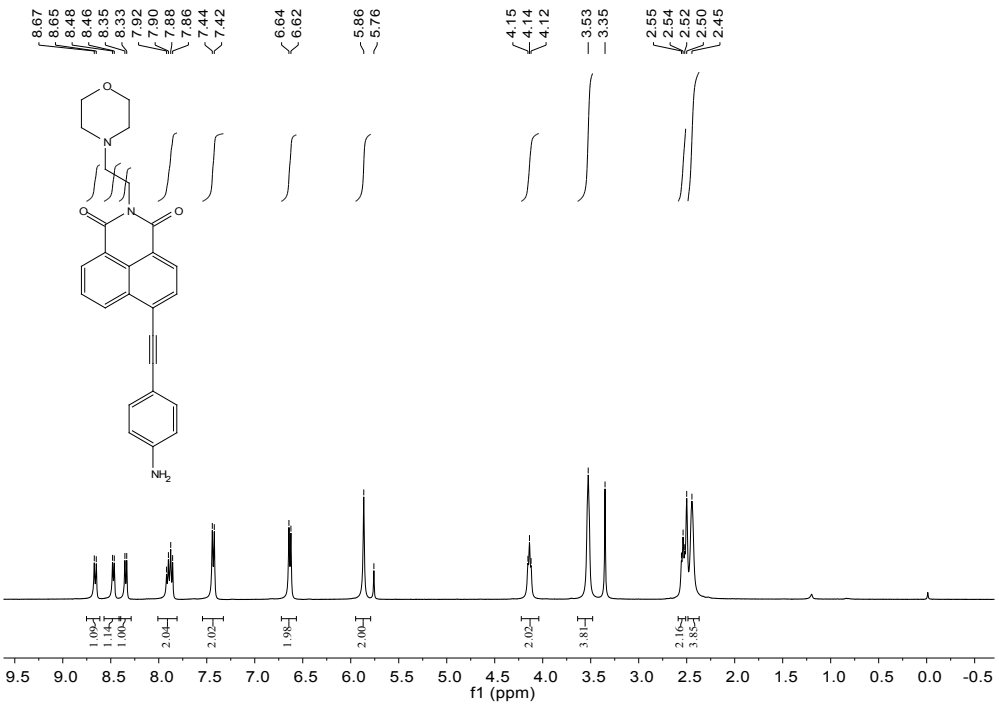

Fig. S3. [1] H NMR spectrum (DMSO-d_6) of compound **AMN**.

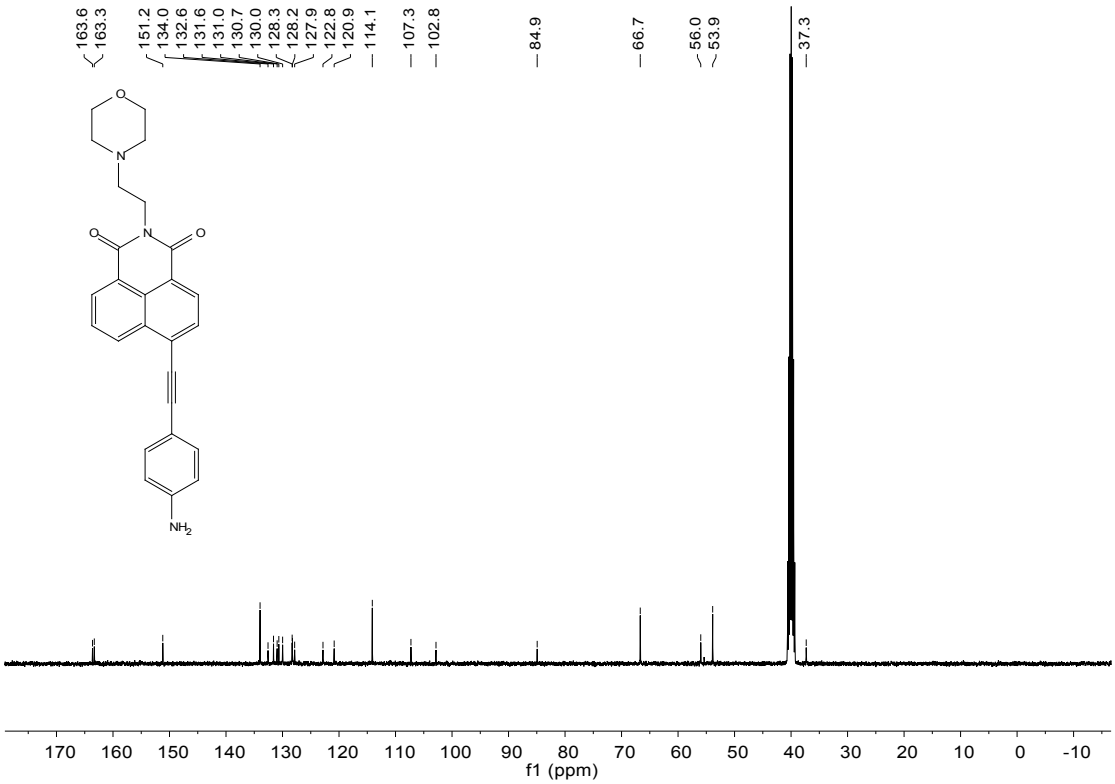

Fig. S4 ^{13} C NMR spectrum (DMSO-d_6) of compound **AMN**.

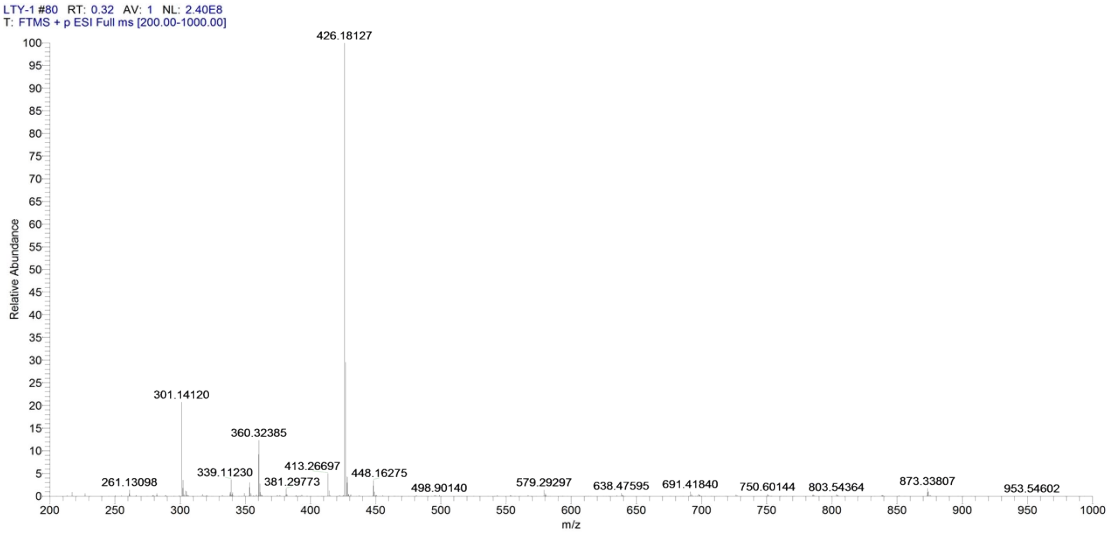

Fig. S5. HRMS spectrum of compound **AMN**.

3. Supporting Tables and Figures

Table S1 Photophysical properties of **AMN** in various solvents

Solvent	Dielectric Constant	λ_{abs} (nm)	λ_{em} (nm)	ε ($M^{-1}\cdot cm^{-1}$)	φ
Toluene	2.24	415	534	$3.36*10^4$	0.213
Dimethylformamide	36.71	429	603	$2.49*10^4$	<0.001
Chloroform	4.9	421	573	$2.34*10^4$	0.189
Tetrahydrofuran	7.58	453	558	$2.76*10^4$	0.0027
Ethanol	25.7	444	662	$3.17*10^4$	<0.001
Dichloromethane	9.1	415	587	$1.99*10^4$	0.161
Dimethyl sulfoxide	48.9	433	607	$3.27*10^4$	<0.001

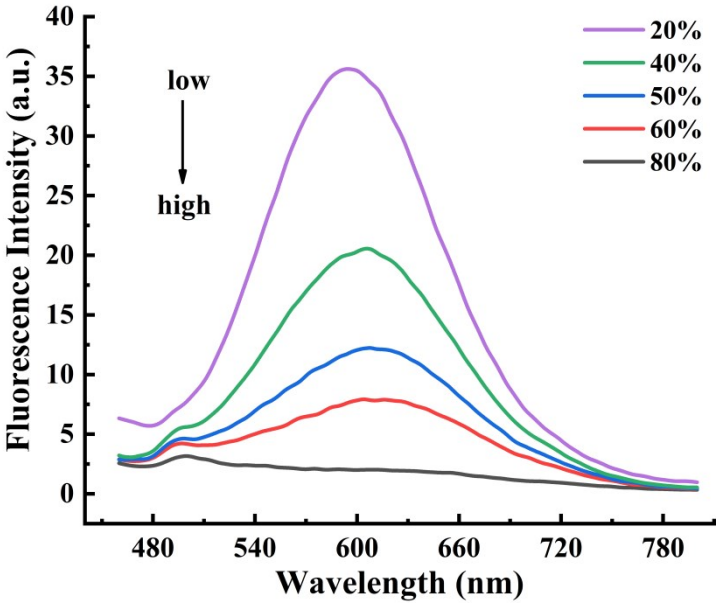

Fig. S6. Fluorescence emission spectra of **AMN** (10 μM) in different PBS/ dioxane mixtures (water from 20 % to 80 %, pH=7.4). λ_{ex} = 430 nm.

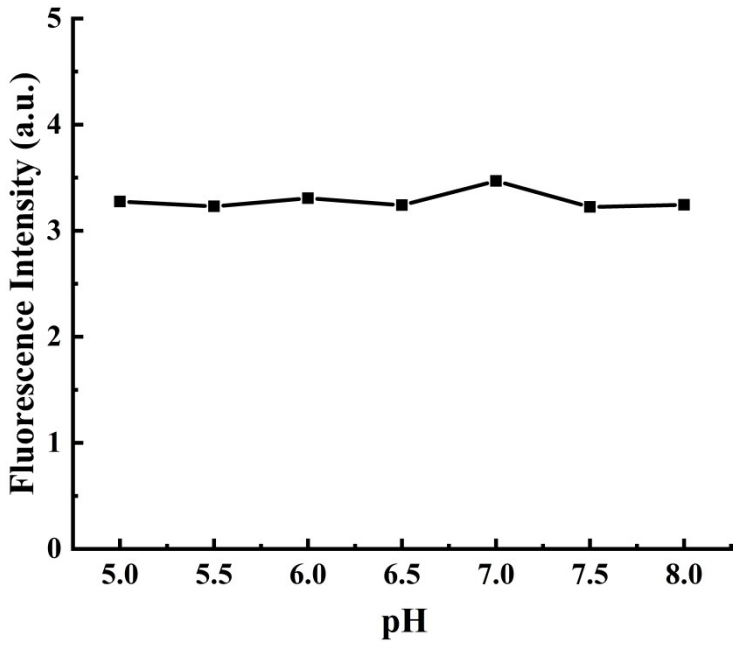

Fig. S7. Fluorescence emission spectra of **AMN** (10 μM) in PBS buffer (10 mM, containing 50% dioxane). λ_{ex} = 430 nm.

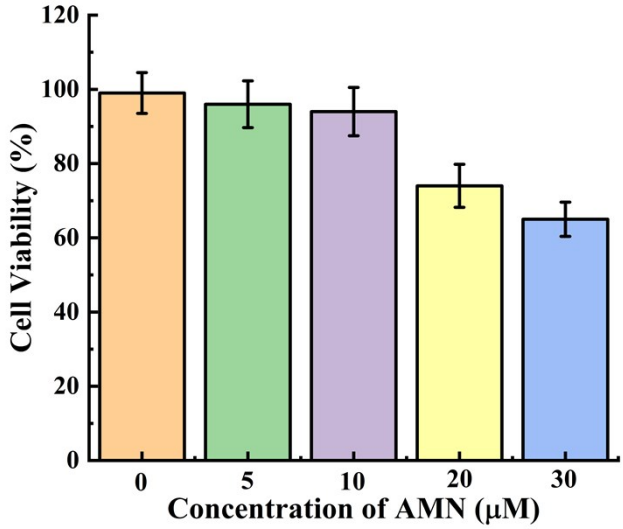

Fig. S8. MTT assay of PC12 cells treated with different concentration of **AMN** (0, 5, 10, 20, 30 μM).

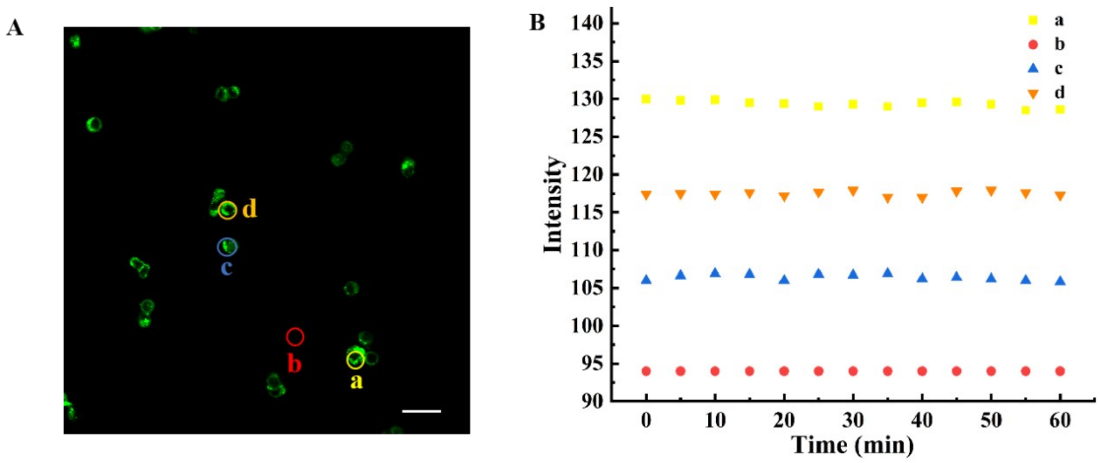

Fig. S9. (A) Two-photon fluorescence imaging of PC12 cells labeled with **AMN** (10 μM) for 1 h. (B) Fluorescence intensity from circle a-d as a function of time. The fluorescence intensity was collected with 5 min intervals for the duration of 1 h. λ_{ex} = 800 nm, λ_{em} = 500-710 nm. Scale bars: 20 μm.

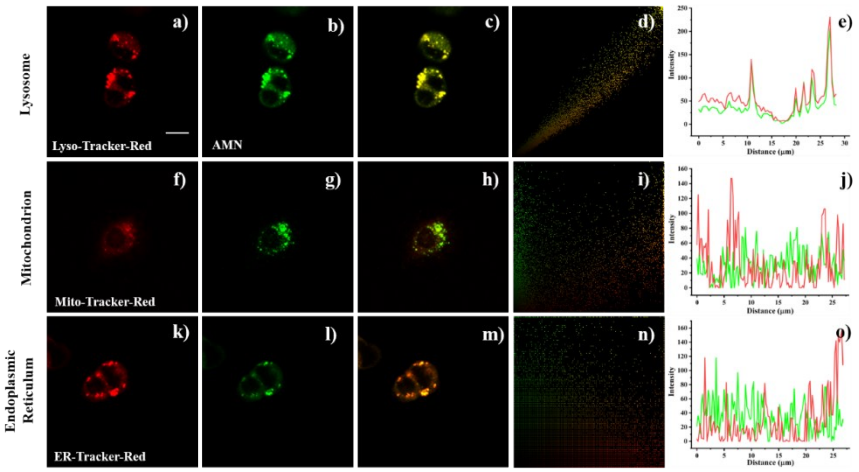

Fig. S10. Two-photon fluorescence co-localization cell imaging of **AMN** and commercial dyes including Lyso-Tracker Red (a-c), Mito-Tracker Red (f-h), ER-Tracker Red (k-m) in PC12 cell. Red channel (595–650 nm, λ_{ex} = 594 nm) for Lyso-Tracker Red, Mito-Tracker Red and ER-Tracker Red; Green channel (500-710 nm, λ_{ex} = 800 nm) for **AMN**. Fluorescence intensity correlation plot of **AMN** and commercial dyes (d, i, n). Scale bar: 50 μm. Fluorescence intensity profile of the region of interest across cells in the red and green channels (e, j, o).

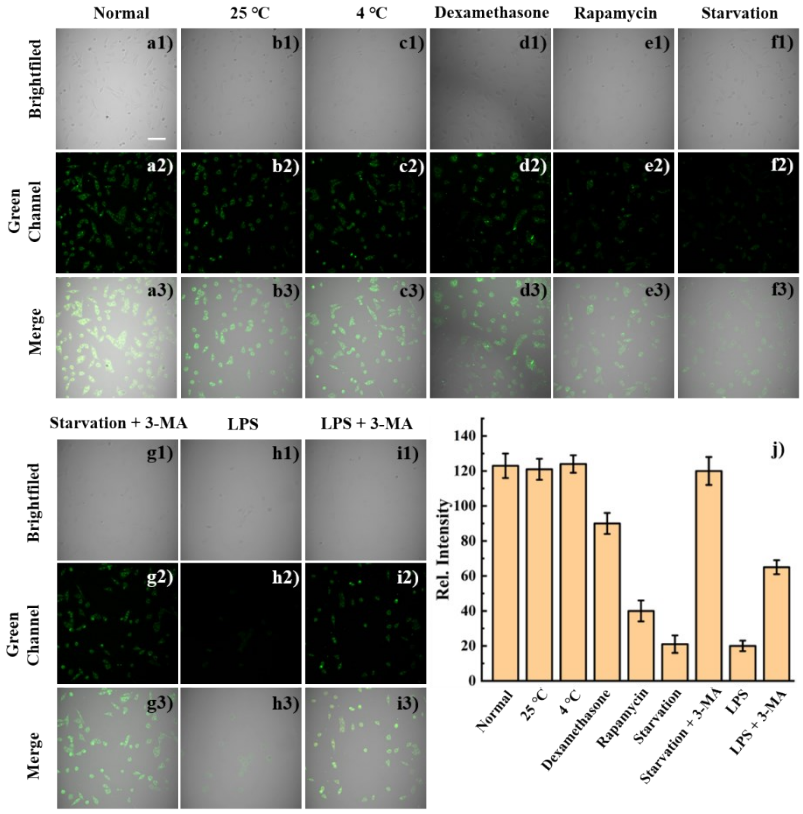

Fig. S11. Two-photon fluorescence imaging of PC12 cells incubated with **AMN** (10 μM) in (a 1-3) Normal group (control), (b1-3) 25°C (viscosity), (c1-3) 4°C (viscosity), (d1-3) dexamethasone (viscosity), (e1-3) rapamycin (autophagy), (f1-3) starvation (autophagy), (g1-3) starvation+3-MA (autophagy inhibited), (h1-3) LPS (inflammation) and (i1-3) LPS+3-MA (autophagy inhibited), respectively. (j) Histograms of average fluorescence intensity of (a-i). The data were shown as mean (± s.d.) (n = 7). λ_{ex} = 800 nm, λ_{em} = 500-710 nm. Scale bars: 20 μm.

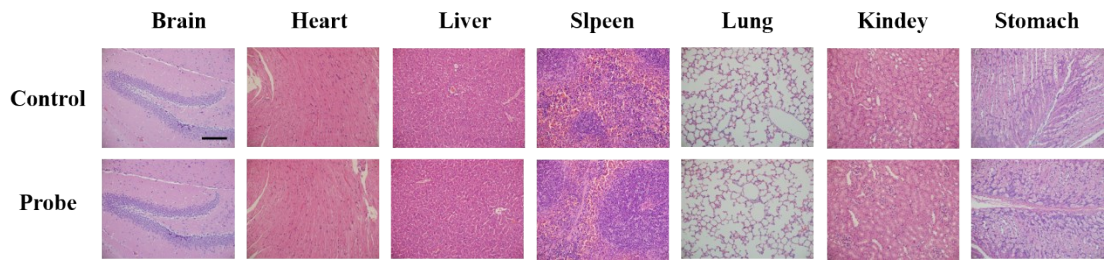

Fig. S12. H&E staining results of different organs collected from the control group and **AMN** (200 μL, 200 μM) treated group. Scale bar: 100 mm.

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.