Electronic Supplementary Information for:

Mitochondria-targeted fluorescent probe for imaging viscosity in Hepatic Ischemia−Reperfusion Injury cell model

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7. Experimental

Materials and Reagents. 1,1,2-Trimethyl-1*H*-benzo[*e*]indole, iodoethane, glycerol, staurosporine and 4-carboxybenzaldehyde were purchased from Innochem. nystatin from Aladdin Co., Ltd. Staurosporine was obtained from J&K Scientific Ltd (Bejing, China). Monensin, 2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA) and nystatin were purchased from Sigma-Aldrich. A phosphate buffered saline (PBS, 10 mM) solution and (4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was bought from Sangon Biotech Co., Ltd (Shanghai, China). All other available chemicals and reagents used were of analytical grade and provided by local suppliers. The stock solution preparation of probe **X-V** (1 mM) was made by dissolving an appropriate amount of **X-V** in DMSO. All solutions were prepared with ultrapure water from a Heal Force water purification system (Shanghai, China).

Characterization Methods. ^{1}H and ^{13}C NMR spectra were performed on a Bruker Avance III 400 MHz spectrometer. Electrospray ionization mass spectrum (ESI-MS) was executed by a LC-MS 6410B instrument (triple quadrupole, Agilent Technologies, CA, USA). High resolution electrospray ionization mass spectra (HR-ESI-MS) were carried out at an APEX IV FTMS instrument (Bruker, Daltonics). Steady fluorescence intensity spectral measurements were taken on an Edinburgh FS5 Fluorescence Spectrophotometer. Confocal laser scanning microscopy images were obtained on Leica TCS SP8 confocal laser scanning microscope (Germany). The absorbance for MTT analysis was measured on a multifunctional microplate reader (Molecular Devices SpectraMax M5, America). Flow cytometry data were collected by BD Biosciences FACS AriaIII instrument.

Synthesis of Probe X-V. A synthetic route for **X-V** and model molecule was depicted in Scheme 1.

Compound **1**: 1,1,2-trimethyl-1*H*-benzo[*e*]indole (500 mg, 2.39 mmol) and iodoethane (745 mg, 4.78 mmol) were added in a 50 mL two-necked flask with 10 mL acetonitrile under nitrogen atmosphere. The mixture solution was refluxed and stirred at 75℃ for 16 h. The reaction was monitored by TLC. The solvent was removed by rotary evaporator. Then the residue was purified by silica gel chromatography, with eluting agent dichloromethane/methanol (v/v, 25:1), affording **1** (324 mg, yield: 56%). The chemical structure of Compound **1** was characterized by ^{1}H NMR and ^{13}C NMR spectra and HRMS, as provided in Figures S1, S2 and S3, respectively. ^{1}H NMR (400 MHz, CD3OD-d6): δ 8.32 (d, J = 16.4 Hz, 1H), 8.31 (d, J = 8.4 Hz, 1H), 8.30 (d, J = 8.4 Hz, 2H), 8.24 (d, J = 8.8 Hz, 1H), 8.21 (d, J = 8.8 Hz, 1H), 8.16 (d, J = 8.4 Hz, 1H), 7.82 (t, J = 7.6 Hz, 1H), 7.78 (d, J = 3.2 Hz, 1H), 7.68 (d, J = 5.2 Hz, 1H), 7.40 (d, J = 8.4 Hz, 2H), 4.83 (d, J = 7.2 Hz, 2H), 2.31 (s, 3H), 2.15 (s, 6H), 1.84 (t, J = 7.2 Hz, 3H). ^{13}C NMR (100 MHz, CD3OD-d6): δ 195.9, 139.5, 136.9, 133.9, 131.1, 132.1, 131.2, 129.8, 128.4, 127.2, 126.4, 123.1, 122.8, 112.3, 112.2, 79.2, 55.7, 42.8, 35.4, 20.9, 14.1. HRMS calcd for $C_{17}H_{20}N^{+}$ $[M]^{+}$: 238.1590, found 238.1584.

Probe **X-V**: compound **1** (300 mg, 1.25 mmol), 4-carboxybenzaldehyde (374 mg, 2.50 mmol) and sodium acetate (307 mg, 3.75 mmol) were dissolved in 5 mL of acetic anhydride. The mixture solution was stirred for 12 h at 50 ºC. Then the reaction mixture was extracted three times with CH_2Cl_2 and H_2O. The organic phase was separated, and dried with anhydrous $MgSO_4$. The solvent was removed by rotary evaporator. The crude product was purified by silica gel flash chromatography using dichloromethane/methanol (v/v, 25:1) as eluent, affording

X-V as a yellow solid (298 mg, 64% yield). The chemical structure of **X-V** was characterized by ^{1}H NMR and ^{13}C NMR spectra and HRMS, as provided in Figures S4, S5 and S6, respectively. ^{1}H NMR (400 MHz, DMSO-*d*$_6$, 298 K): δ 13.34 (d, *J* = 13.2 Hz, 1H), δ 8.35 (d, *J* = 15.4 Hz, 1H), 8.23 (d, *J* = 8.2 Hz, 1H), 8.04 (d, *J* = 8.2 Hz, 1H), 8.08 (d, *J* = 8.2 Hz, 1H), 7.37 (d, *J* = 9.0 Hz, 2H), 7.45 (d, *J* = 9.0 Hz, 1H), 7.21 (t, *J* = 7.5 Hz, 1H), 7.57 (t, *J* = 7.5 Hz, 1H), 7.23 (d, *J* = 15.6 Hz, 1H), 6.89 (d, *J* = 7.8 Hz, 2H), 5.34 (s, 2H), 4.87 (q, *J* = 6.9 Hz, 2H), 2.32 (s, *6*H), 1.53 (t, *J* = 6.9 Hz, 3H); ^{13}C NMR (100 MHz, DMSO-*d*$_6$, 298 K): δ 182.4, 158.4, 157.9, 138.2, 136.4, 133.1, 132.0, 126.2, 123.0, 122.5, 116.4, 112.1, 109.0, 101.1, 47.35, 47.36, 41.9, 26.0, 27.4, 12.5, 12.4. HRMS calcd for $C_{25}H_{24}NO_2^+$ $[M]^+$: 370.1802, found 370.1800.

Measurement of Viscosity. X-V solutions (10 μM) of different viscosity were obtained by water-glycerol mixture in different volume ratios. The solution were continuously shaken for 1 hour and then allowed to stand for 30 minutes to eliminate bubbles. The mixed solution was measured fluorescence spectra with $\lambda_{ex/em}$ = 490/615 nm and both excitation and emission slit widths of 8 nm.

Cytotoxicity Assay. In order to estimate the cytotoxicity of **X-V** on HepG2 cells, we did a routine MTT test, as described previously.[17] MTT assays were carried out to evaluate the toxicity of **X-V**. HepG2 cells (10^6 cells/mL) were cultured into 96-well microtiter plates with total volumes of 200 mL/well. After 24 h of incubation, various concentrations of **X-V** (0 μM, 5 μM, 10 μM, 25 μM, 50 μM, 75 μM) were added, and the HepG2 cells were cultured for another 24 h. Afterwards, 10 μL of MTT solution (10 mg/mL) was added to each well. After 4 h, the MTT solution was removed, and 150 μL of DMSO was added to each well. Finally, the absorbance at 490 nm was measured using a multifunctional microplate reader.

Intracellular ROS detection. Commercial dye 2',7'-dichlorofluorescin diacetate (DCFH-DA) was used to detect the production of intracellular reactive oxygen species (ROS) during HIRI. The final concentration was 10 μM and the incubation time was 20 min.

Fluorescence (FL) intensity Imaging of Viscosity in HepG2 Cells. HepG2 cells were incubated in RPMI (Roswell Park Memorial Institute) 1640 medium with 10% fetal bovine serum (FBS), and 1% (v/v) penicillin-streptomycin at 37 °C under a humidified atmosphere containing 5% CO_2. Then cells were seeded into cell culture dishes at a density of 2.0×10^4 in growth medium. For stimuli experiments, the cells were pretreated with different drugs (monensin, nystatin and staurosporine) for different time and then incubated with **X-V** (10 μM) for 30 min at 37 °C, respectively. Finally, the cells were washed twice with PBS and FL imaging was recorded by confocal luminescence microscope.

Hepatic ischemia−reperfusion injury models in cells. Hepatic ischemia−reperfusion injury (HIRI) cell models were established by oxygen-glucose-serum deprivation/reperfusion. HepG2 cells were cultured in DMEM (without glucose and serum) and deoxygenated sodium dithionite (0.5 mM) for 30 min. During this time, photos were taken every 10 minutes. Then these cells were incubated with high glucose and serum DMEM (standard DMEM) in a 5 % CO2 and 95 % O2 atmosphere for another 30 min. During this time, photos were taken every 10 minutes.

Table 1. The viscosity of the water-glycerol mixture in different proportions

Water (v%)	Glycerol (v%)	Viscosity (cP)
100	0	0.89
90	10	1.15
80	20	1.54
70	30	2.16
60	40	3.18
50	50	5.04
40	60	8.82
30	70	17.96
20	80	45.86
10	90	163.60
0	100	945.00

1. Synthesis of probe X-V

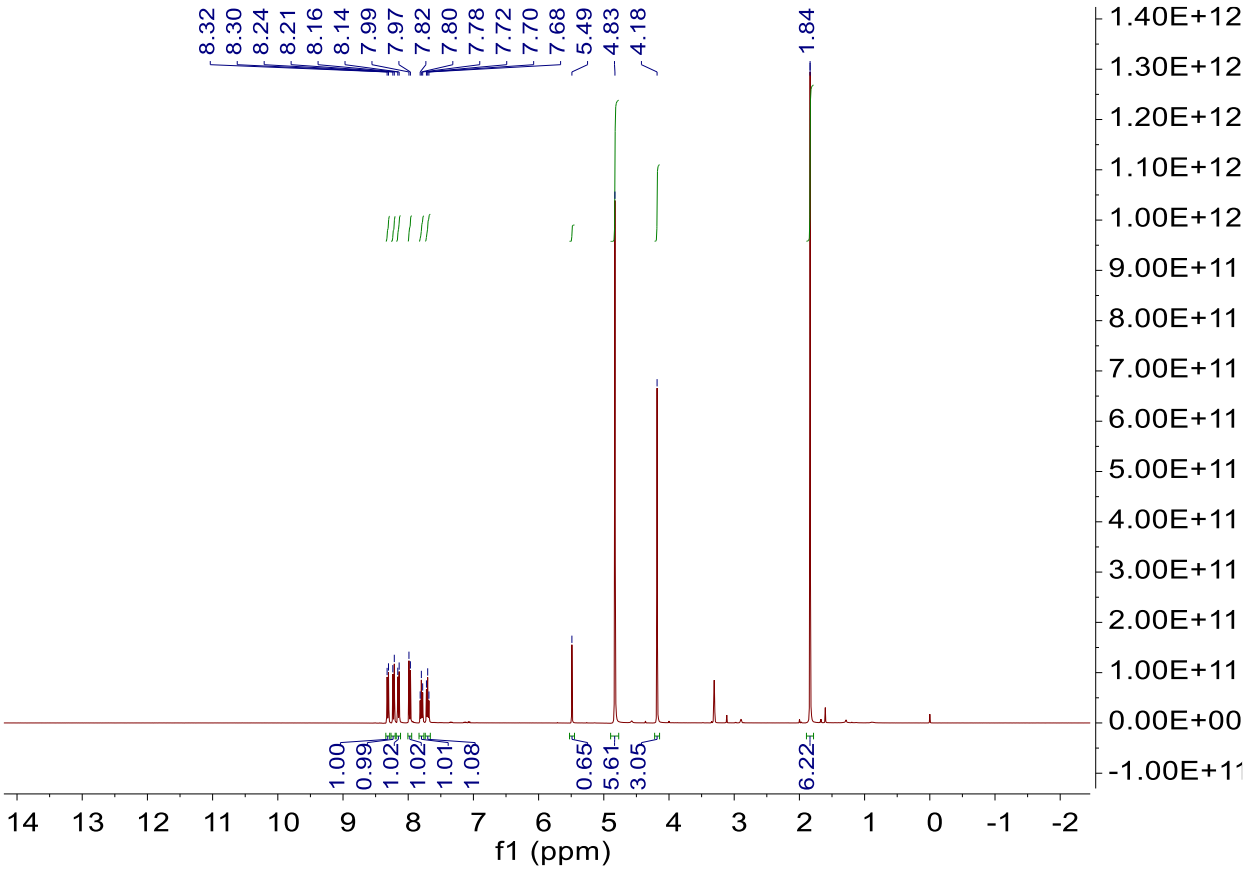

Figure S1. ^{1}H NMR spectrum of Compound **1** (400 MHz, CD3OD-d_6, 298 K).

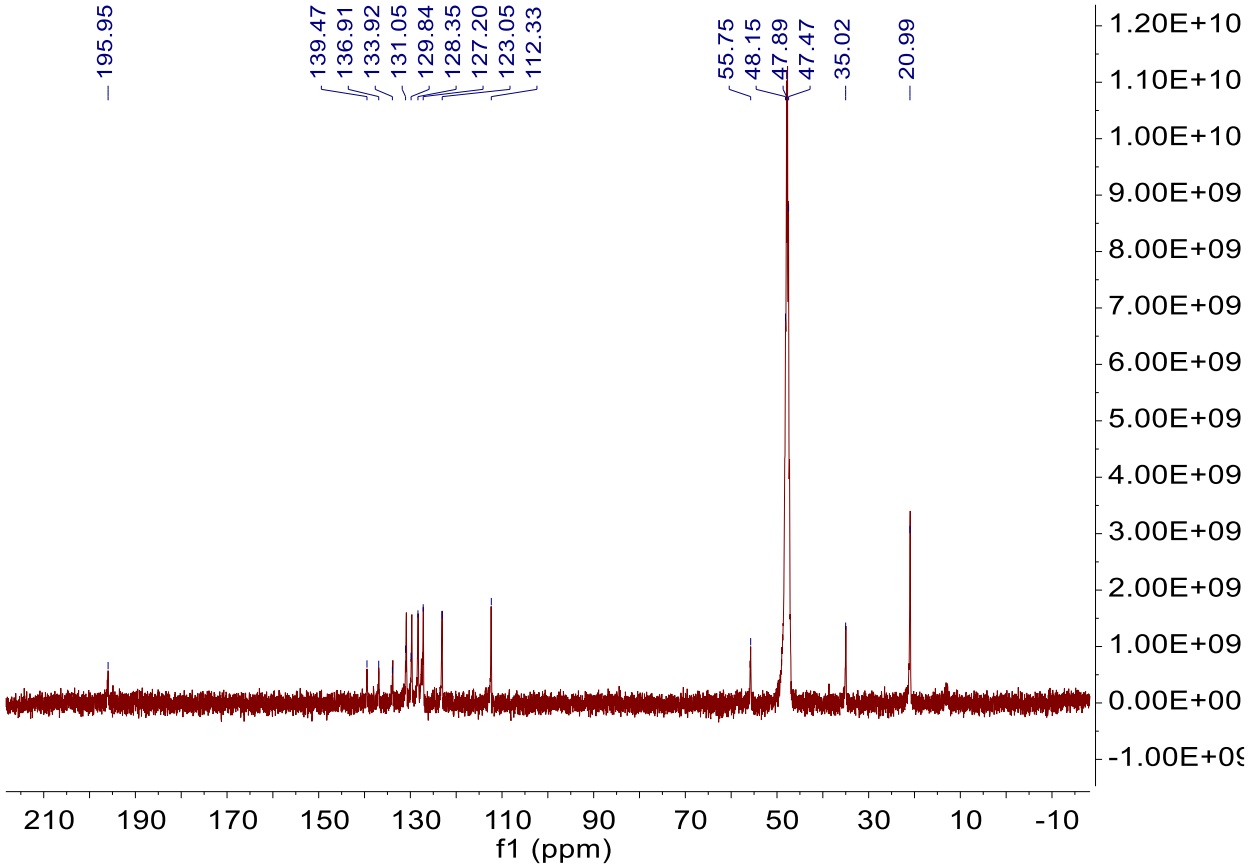

Figure S2. ^{13}C NMR spectrum of Compound **1** (100 MHz, CD3OD-d_6, 298 K).

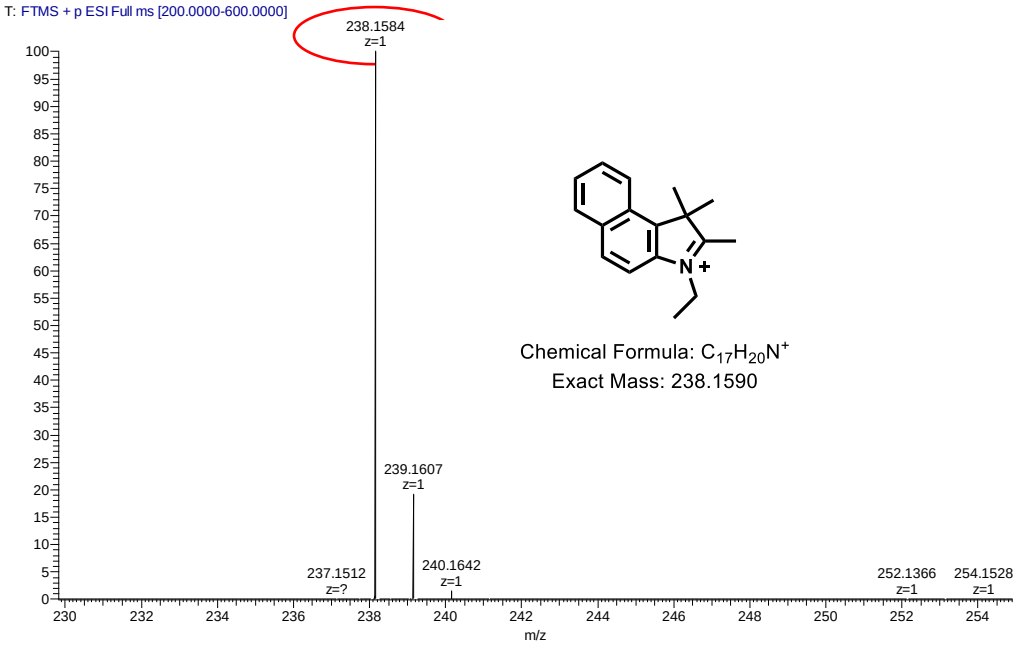

Figure S3. HRMS result of compound **1**.

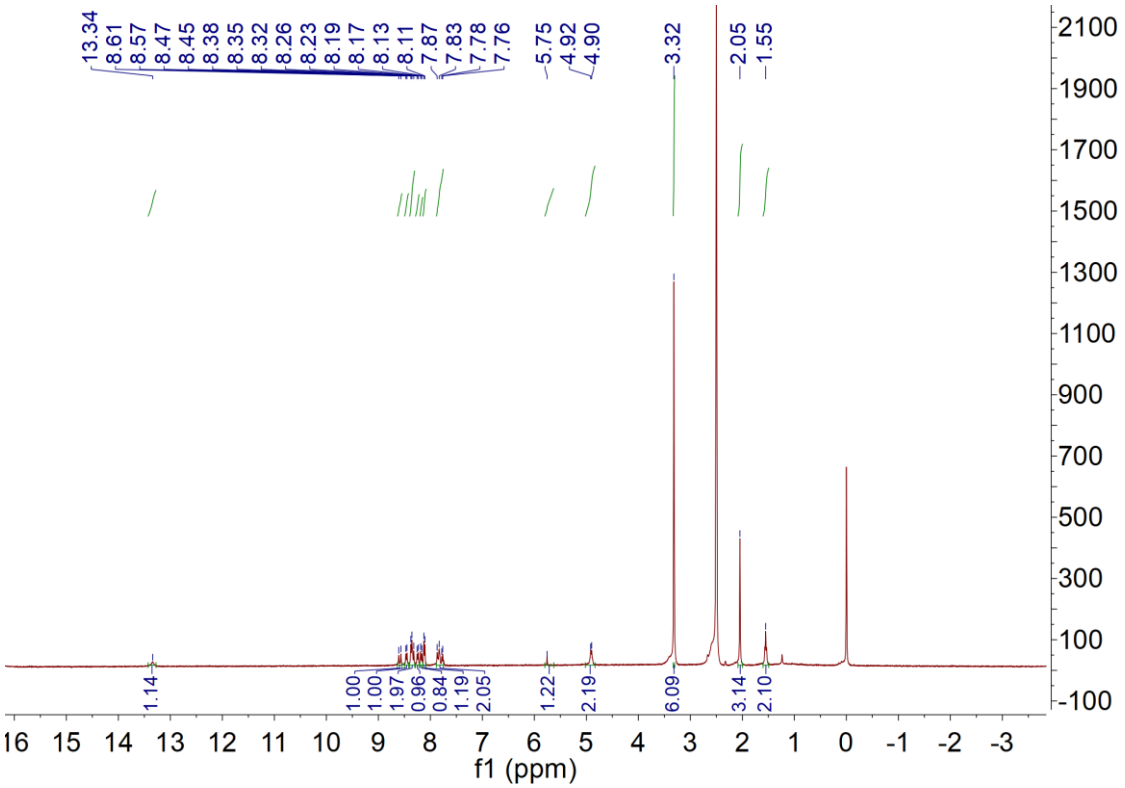

Figure S4. ^{1}H NMR spectrum of **X-V** (400 MHz, DMSO-d_6, 298 K).

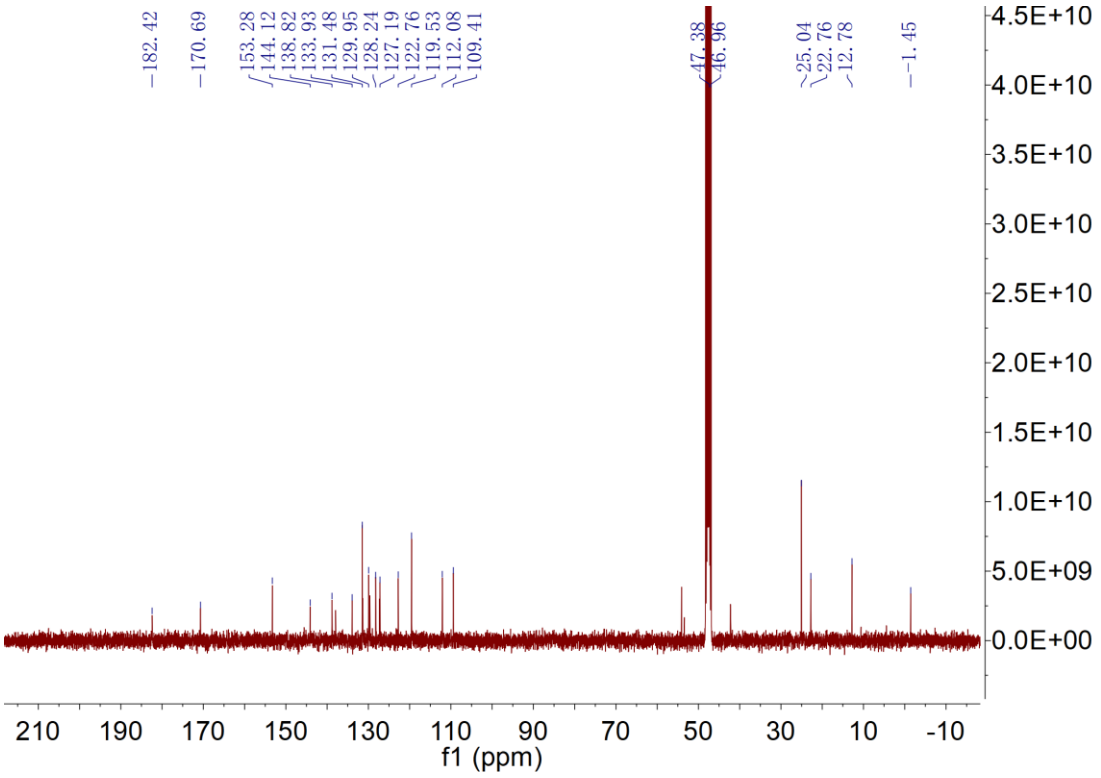

Figure S5. ^{13}C NMR spectrum of **X-V** (100 MHz, DMSO-d_6, 298 K).

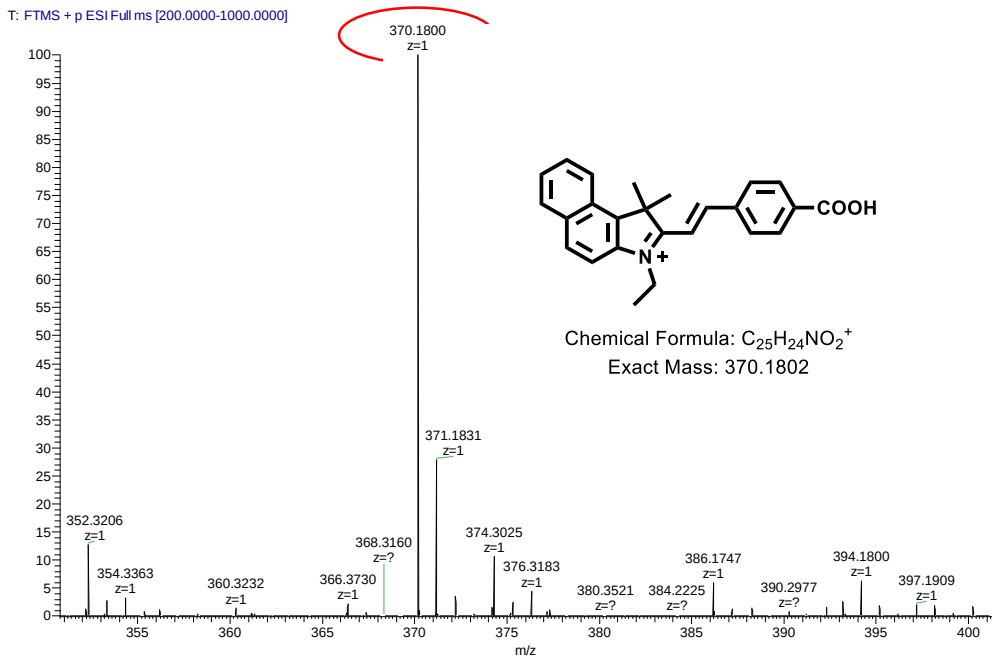

Figure S6. HRMS result of compound **X-V**.

2. Optimization of experimental conditions

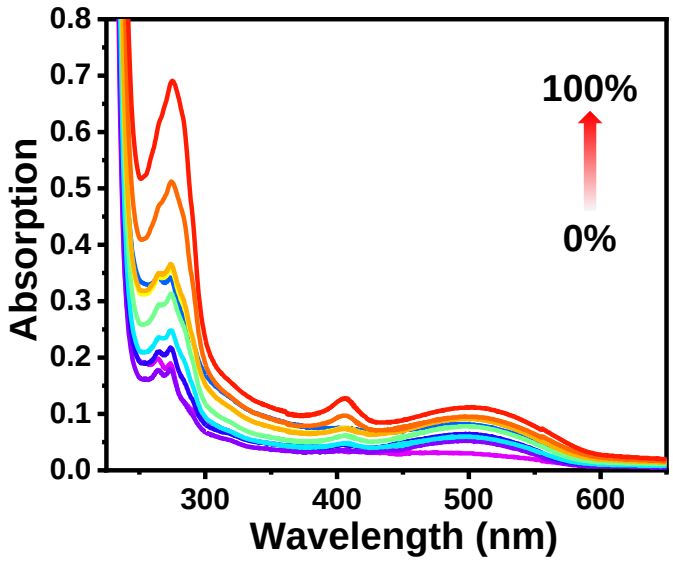

Figure S7. Absorption spectra of **X-V** (10 μM) in a water−glycerol system with different viscosities (the glycerol fractions of water-glycerol system are 0%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% and 100% respectively from the bottom to top).

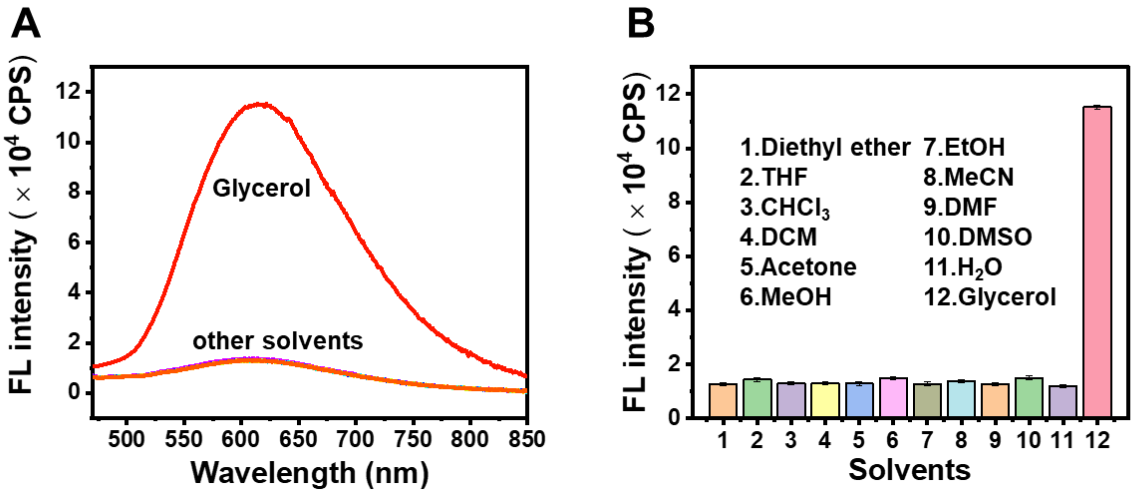

Figure S8. (A) FL spectra of 10 μM **X-V** in various solvents with different polarities. (B) Bar diagram of panel A. $\lambda_{ex/em}$ = 490/615 nm.

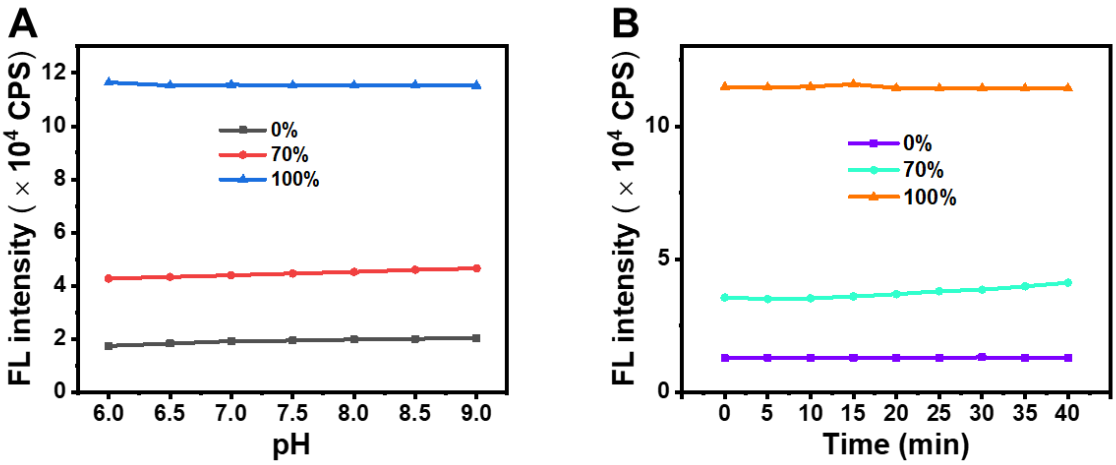

Figure S9. (A) FL intensity of **X-V** (10 μM) the reaction time in the presence of varied glycerol volumetric ratios. The effects of pH (B) on the fluorescence intensity of probe **X-V** (10 μM) in presence of varied glycerol volumetric ratios. $\lambda_{ex/em}$ = 490/615 nm.

3. Cytotoxicity assay

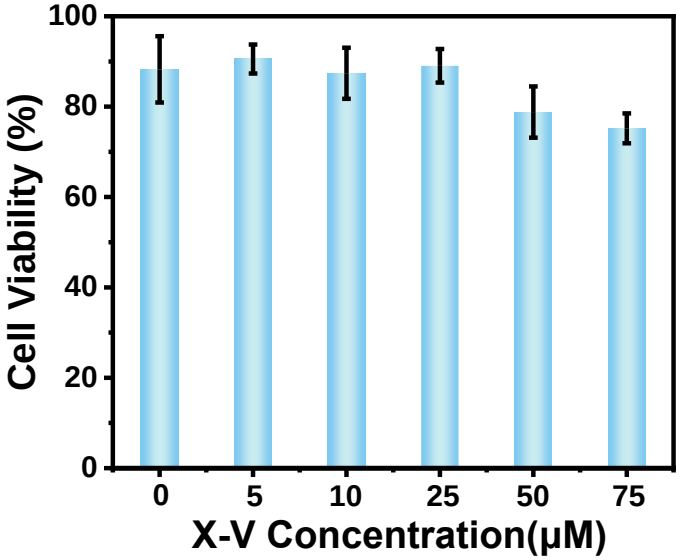

Figure S10. Effects of **X-V** with varied concentrations (0-75 μM) on the viability of HepG2 cells. The viability of the cells without **X-V** is defined as 100%. The results are the mean ± standard deviation of five separate measurements.

4. Optimal time and concentration for X-V to incubate HepG2 cells

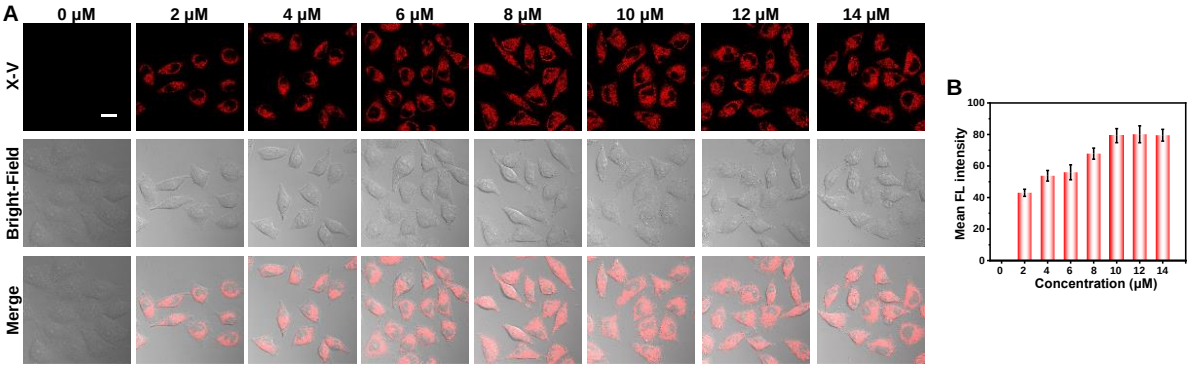

Figure S11. The confocal laser scanning microscopy (CLSM) images of HepG2 cells incubated with the **X-V** for 0 μM, 2 μM, 4 μM, 6 μM, 8 μM, 10 μM, 12 μM and 14 μM in cell culturing condition, then washed with PBS. Red fluorescence (**X-V**, Ex: 488 nm, Em: 600-650 nm). Scale bar: 20 μm.

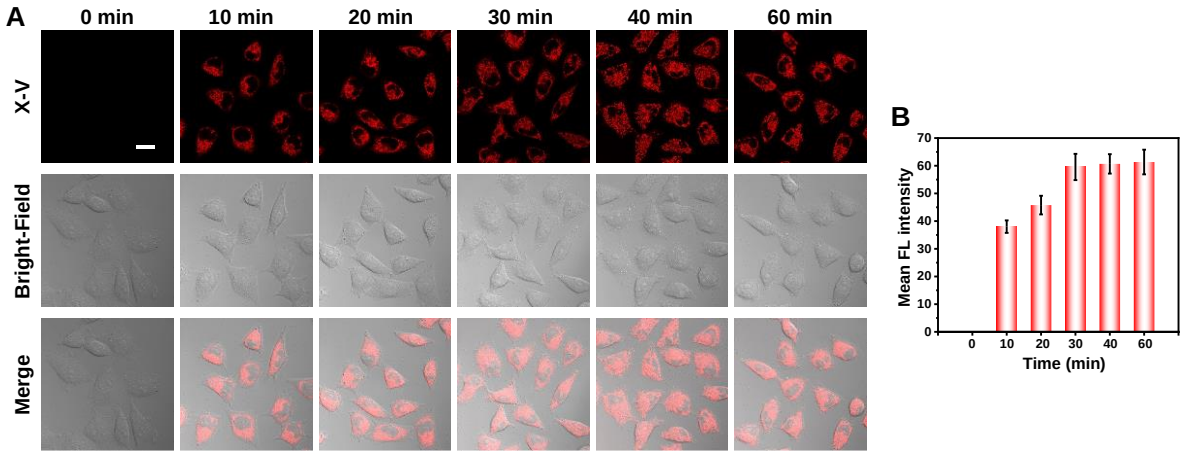

Figure S12. The CLSM images of HepG2 cells incubated with the **X-V** (10 μM) for 0 min, 10 min, 20 min, 30 min, 40 min, and 60 min in cell culturing condition, then washed with PBS. Red fluorescence (**X-V**, Ex: 488 nm, Em: 600-650 nm). Scale bar: 20 μm.

5. Intracellular photostability experiment

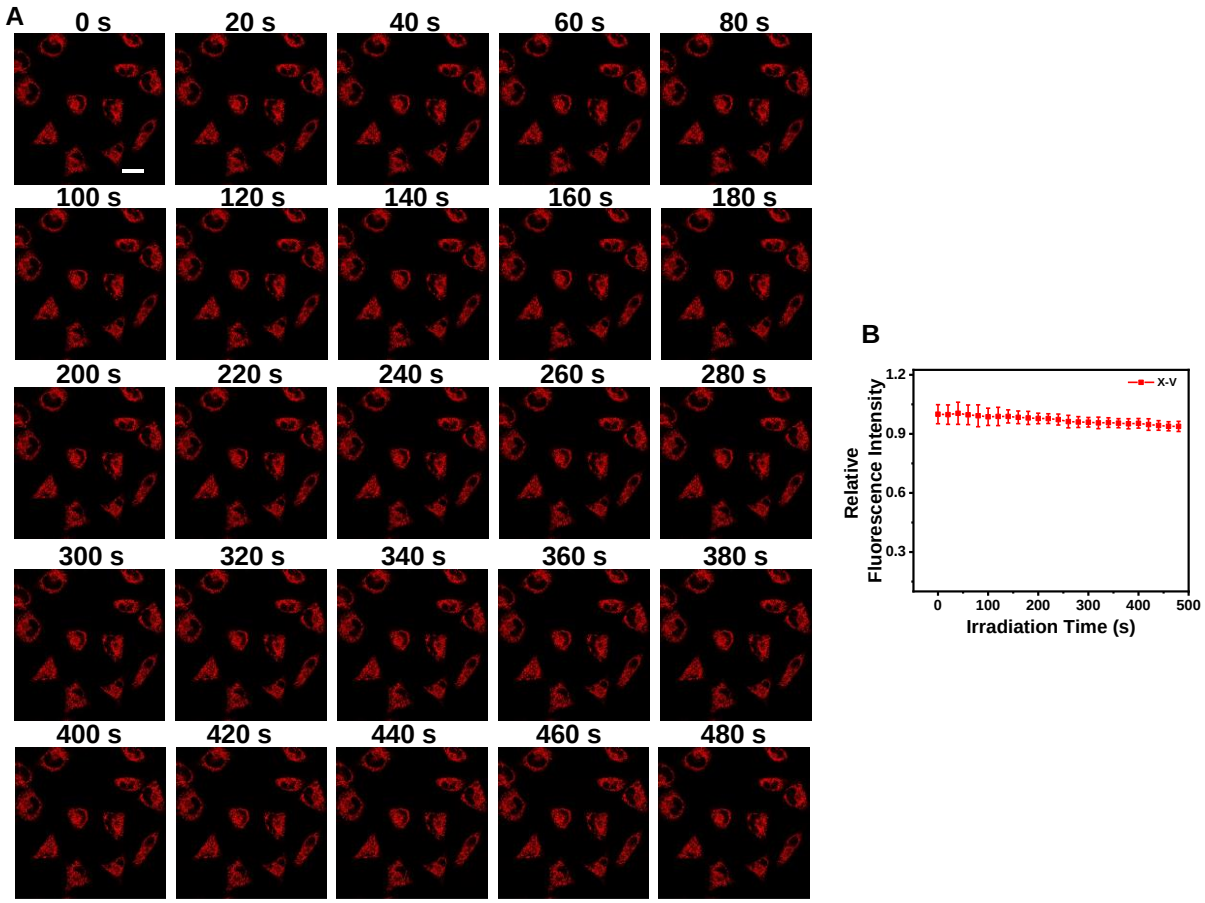

Figure S13. Intracellular photostability experiment of **X-V** under irradiation with a 488 nm laser (intensity 20 %) in HepG2 cells. A. Fluorescence images of HepG2 cells stained with 10 μM **X-V** were captured after every 20 s irradiation. B. The relative fluorescence intensity output of

A. Normalization was carried out against the initial fluorescence intensity. Ex = 488 nm, collected 600−650 nm. Scale bar: 20 μm.

6. Subcellular localization experiment

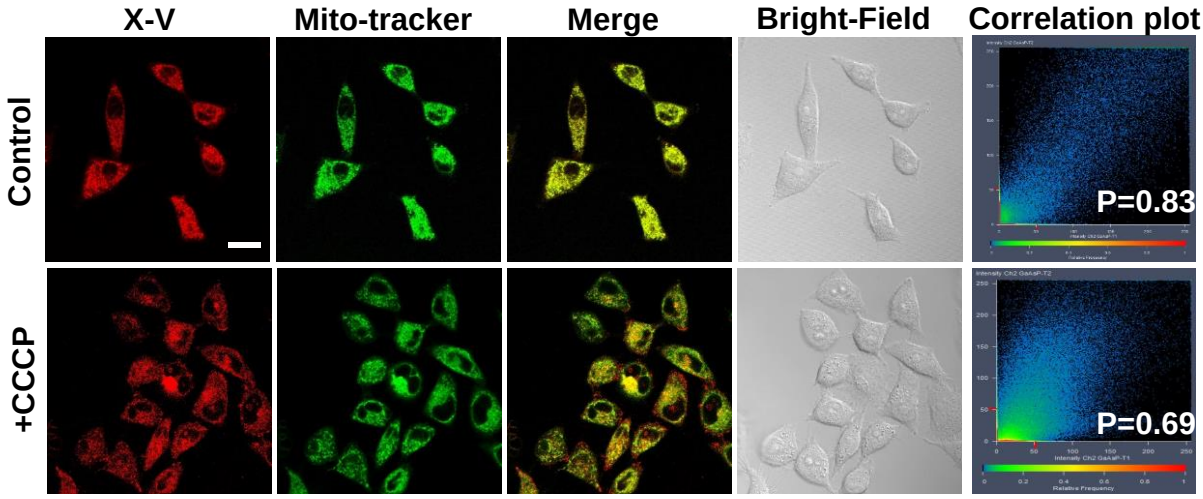

Figure S14. Subcellular localization in HepG2 cells by **X-V**. The fluorescence images for HepG2 cells after treatment with 10 μM **X-V** followed by further incubation for 1 h with PBS and CCCP (10 μM), respectively. Co-localization images of HepG2 cells incubated with **X-V** for the red channel (10 μM, Ex = 488 nm, collected from 590−630 nm) and the corresponding green channel (Mito-Tracker, 500 nM, Ex = 633 nm, collected from 680−726 nm). Scale bar: 20 μm.