Supplementary Information

Imaging of viscosity in ferroptosis-mediated acute alcoholic gastric injury and tumor models by an endoplasmic reticulum fluorescent probe

Hui Wang,[‡a] Ling Ma,[‡b] Sijie Li,[a] Rui Wang,[a] Shaomin Shuang,[a] Chuan Dong,[*a] and Li Fan[*a]

[a] *Institute of Environmental Science, School of Chemistry and Chemical Engineering, Shanxi University, Taiyuan, 030006, P. R. China.*
[b] *Department of Chemistry and Chemical Engineering, Jinzhong University, Jinzhong, 030619, P. R. China.*

[‡] These authors contributed equally to this work.

E-mail: fanli128@sxu.edu.cn; dc@sxu.edu.cn

Table of Contents

Experimental section

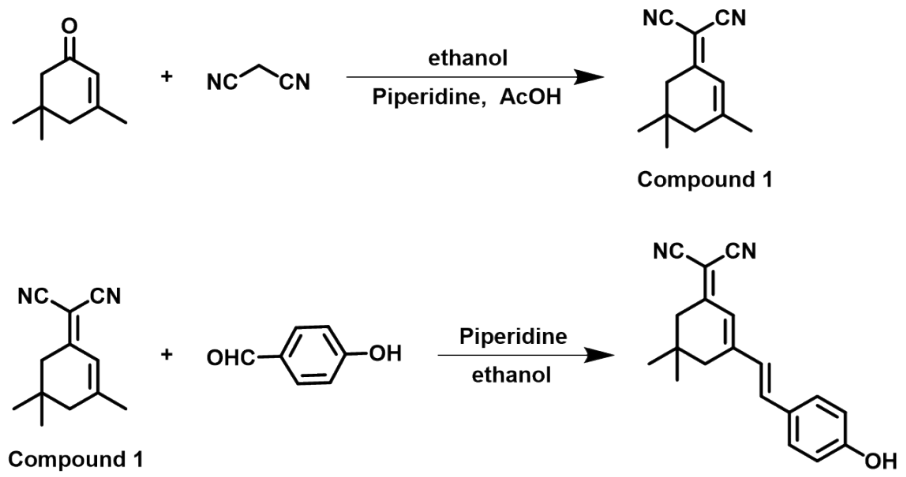
Materials and apparatus

All chemicals were commercially available and analytical grade. Isophorone, malononitrile, piperidine, glacial acetic acid, malononitrile, p-hydroxybenzaldehyde, bovine serum albumin (BSA), human serum albumin (HAS), esterase and other chemicals used in this work were purchased from Sigma-Aldrich Co., Ltd. ER Tracker Green (ER-TG) was purchased from Med Chem Express. Lipopolysaccharide (LPS), MitoTracker Green (MTG) and LysoTracker Green (LTG) were commercially available from Invitrogen. Tunicamycin (Tm) were purchased from Shanghai Macklin Biochemical Co., Ltd. Erastin, Ferrostatin-1 (Fer-1), 2′,7′-Dichlorodihydrofluorescein Diacetate (DCFH-DA), monensin (Mon) and cimetidine were purchased from Shanghai Aladdin Biochemical Technology Co., Ltd.

NMR spectra were acquired with a Bruker instrument (BrukerBioSpin AG Ltd., Beijing, China) with TMS as the internal standard in DMSO-d_6 of 600 MHz for ^{1}H NMR and 151 MHz for ^{13}C NMR, respectively. High resolution mass spectra were obtained on a Thermo Scientific Q Exactive mass spectrometer (Thermo Fisher Scientific). Deionized (DI) water from a Milli-Q water purification system (Millipore, Bedford, MA, USA) with a resistivity of 18.25 MΩ cm was used to prepare all aqueous solutions. pH values were obtained on a PHS-3G pH meter (INASE Scientific Instrument Co., Ltd, China). The UV-visible spectra were taken on a UV-26001 UV-vis spectrophotometer (Shimadzu Corporation, Japan). The fluorescence spectra were taken on a F-7100 fluorescence spectrophotometer (Hitachi High-Tech Corporation, Japan). Fluorescence lifetime were carried out on a FLS-920 Edinburgh Fluorescence Spectrophotometer (Edinburgh Co., Ltd., England) equipped with a xenon discharge lamp using 1 mL Fluor Micro Cell. The fluorescence images were taken on a confocal laser scanning microscope (Zeiss, LSM880, Germany). *In vivo* images were then taken by using a PerkinElmer IVIS Lumina LT III *vivo* imaging system.

Synthesis and characterizations

Synthesis routine of ER-V



Scheme S1 Synthetic routine of ER-V.

Synthesis of compound 1. Isophorone (3.46 g, 25 mmol), malononitrile (1.98 g, 30 mmol), 250 μL piperidine and 300 μL glacial acetic acid were dissolved in 50 mL anhydrous ethanol for refluxing overnight under argon atmosphere. After the solvent was removed, the residue was dissolved with 50 mL dichloromethane, washed with saturated saline, and dried over anhydrous Na_2SO_4. The solvent was evaporated under reduced pressure, and the crude product was purified by silica column chromatography (petroleum/dichloromethane = 1:1, v/v) to give a yellowish color solid (3.37 g, 72.5%). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 6.61 (h, J = 1.4 Hz, 1H), 2.51 (s, 2H), 2.18 (s, 2H), 2.03 (d, J = 1.2 Hz, 3H), 1.01 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ (ppm): 170.43, 159.85, 120.58, 113.21, 112.42, 78.21, 45.67, 42.64, 32.37, 27.81, 25.31.

Synthesis of ER-V. Compound 1 (0.559 g, 3 mmol), p-hydroxybenzaldehyde (427.4 mg, 3.5 mmol), and 150 μL piperidine were dissolved in 15 mL anhydrous ethanol for refluxing 8 h. Then the solvent was removed under reduced pressure, and the resulting residue was dissolved in 100 mL dichloromethane, washed with saturated saline, and dried over anhydrous Na_2SO_4. After the removal of the solvent, the crude product was purified by silica

column chromatography (dichloromethane) to obtain the ER-V as an orange red scaly solid (636.9 mg, 73.1%). ^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm): 9.98 (s, 1H), 7.55 (d, J = 8.4 Hz, 2H), 7.22 (d, J = 16.2 Hz, 1H), 7.18(d, J = 16.2 Hz, 1H), 6.80 (d, J = 8.4 Hz, 2H), 6.79 (s, 1H), 2.59 (s, 2H), 2.52 (s, 2H), 1.01 (s, 6H). ^{13}C NMR (151 MHz, DMSO-d_6) δ (ppm): 170.7, 159.8, 157.2, 138.8, 130.3, 127.6, 126.7, 121.9, 116.4, 114.6, 113.8, 75.3, 42.8, 38.7, 32.1, 27.9. HR-MS m/z: $[M+H]^+$ calculated for $C_{19}H_{18}N_2O$, 290.1419; measured, 289.1282.

UV-vis and fluorescence measurements

ER-V was dissolved in DMSO to prepare a stock solution (2.0 mM). The viscosity response measurement of ER-V (10 μM) was tested in water/glycerol mixtures with different glycerol fractions (f_G), in which 10 μL of the ER-V stock solution was added in 1990 μL of a mixture of different proportions of water/glycerol and ultrasonic for 5 min. λ_{ex} = 435 nm, slit width: d_{ex} = d_{em} = 5.0 nm.

Preparation of ROS/RNS

Peroxynitrite solution ($ONOO^-$) was synthesized as reported (*Anal. Biochem.*, 1996, **236**, 242-249). The concentration of $ONOO^-$ was determined by UV analysis with the extinction coefficient of 1670 M^{-1} cm^{-1} at 302 nm. H_2O_2 was generated by dilution of a 30% H_2O_2 solution in deionized water. ClO^- was generated by dilution of a NaClO (contains 6-14% active chlorine) solution in deionized water. Hydroxyl radicals (•OH) was generated by the Fenton reaction. For this purpose, hydrogen peroxide was added to a solution of Ferrous sulfate in deionized water. Singlet oxygen ($^1O_2$) was obtained by the reaction of ClO^- and H_2O_2. Superoxide ($O_2^{\bullet -}$) was produced from KO_2 in dry DMSO by an ultrasonic method.

Calculation of the Quantum Yield

The relative quantum yield of ER-V was determined according to the following equation:

$$\Phi_x = \Phi_{st}\left(\frac{D_x}{D_{st}}\right)\left(\frac{A_{st}}{A_x}\right)\left(\frac{\eta_x{}^2}{\eta_{st}{}^2}\right)$$

Where Φ_{st}, D and A refer to the reported quantum yield of the standard, the area under

the emission spectra and the absorbance at the excitation wavelength. η stands for the refractive index of the solvent used. Subscripts x and st represents sample and standard, respectively. Rhodamine 6G (Φ = 0.94 in EtOH) was used as the reference standard.

Cell culture and cell cytotoxicity assay

For Living Cells Culture. HeLa cells were kindly provided by Institutes of Biomedical Sciences (Shanxi University, Taiyuan, China), and cultured in DEME (Dulbecco's modified eagle medium) medium supplemented with 10% fetal bovine serum and 1% antibiotics at 37 ℃ in a 5 % CO_2 atmosphere.

Cell Cytotoxicity Assay. The cell cytotoxicity of ER-V to living HeLa cells was performed by a standard MTT assay. About 8×10^3 cells/well in 100 μL cell culture medium was seeded in 96-well microplate and then the medium was replaced with fresh medium that containing ER-V with various concentrations of 0 μM, 0.5 μM, 1 μM, 2 μM, 5 μM, 8 μM, 10 μM, 12 μM, 15 μM and 20 μM for 24 h, respectively. Six replicate wells were used for each control and test concentration. After washing the cells with fresh medium three times, 90 μL of fresh medium and 10 μL of MTT were added to each well for 4 hours. Then the medium containing MTT solution was discarded, 200 μL DMSO was added to each well, and each well was analyzed with an ELISA microplate reader and the absorbance was detected at 435 nm. The cell viability was expressed as relative to the control cells taken as 100 % metabolic activity.

$$\text{Cell viability (\%)} = (A_{\text{with probe}}–A_{\text{blank}}) / (A_{\text{control}}–A_{\text{blank}}) \times100\%.$$

Cell imaging experiment

For co-localization imaging. HeLa cells were co-incubated with ER-V (6 μM) and ER-TG (1 μM), MTG (1 μM) or LTG (1 μM) for 30 min, respectively. The fluorescence images were obtained on a confocal laser scanning microscope with a green channel (λ_{ex} = 488 nm, λ_{em} = 500 nm ~ 530 nm) for ER-TG, MTG or LTG; and a red channel (λ_{ex} = 405 nm, λ_{em} = 560 nm ~ 700 nm) for ER-V, respectively.

For imaging of viscosity in live cells. HeLa cells were incubated with ER-V (6 μM)

only for 30 min as control; pretreated with Mon (10 μM) for 30 min and then incubated with ER-V (6 μM) for another 30 min; pretreated with LPS (1 mg/mL) for 90 min and then incubated with ER-V (6 μM) for another 30 min; and pretreated with TM (30 μM) for 60 min and then incubated with ER-V (6 μM) for another 30 min, respectively. λ_{ex} = 405 nm, λ_{em} = 560 nm ~ 670 nm.

For imaging of viscosity in ferroptosis cells. HeLa cells were incubated with ER-V (6 μM) for 30 min as control; pretreated with erastin (10 μM) for 90 min and then incubated with ER-V (6 μM) for another 30 min; pretreated with Fer-1 (10 μM) for 60 min, following by erastin (10 μM) for 90 min and then incubated with ER-V (6 μM) for another 30 min, respectively. λ_{ex} = 405 nm, λ_{em} = 560 nm ~ 670 nm.

Animal imaging experiment

KM mice (20 g ~ 25 g, 6 weeks old) and female Balb-c nude mice (18 ~ 20 g, 6 weeks old) (provided by the Laboratory Animal Centre of Shanxi Province Cancer Hospital) were used for constructing acute alcoholic gastric injury (AAGI) model and tumor-bearing mice model, respectively. The animal experiments were approved by the Animal Care and Use Committee of Shanxi University and performed in compliance with the Animal Management Rules of the Ministry of Health of the People's Republic of China (document no. 55, 2001). We have taken great efforts to reduce the number of animals used in this work and also taken efforts to reduce animal suffering from pain and discomfort.

For imaging of viscosity in ferroptosis-mediated AAGI model. The experimental KM mice were divided into four groups (Control group, AAGI group, CMTD + AAGI group, and Fer-1 + AAGI group). The AAGI model mice were given 56% alcohol-containing Chinese spirits (3 g/kg) gavage at 9:30 am for 3 consecutive days; while control group mice were given with an equal volume of saline solution. The treatment group mice for AAGI were administered intragastrically with cimetidine (CMTD, 40 mg/mL) or Fer-1 at 2:30 pm for 3 consecutive days. After sacrificing the mice, the stomachs were extracted, and preserved in 10% paraformaldehyde solution and encased in paraffin for HE (Hematoxylin and Eosin) staining. For ER-V staining, the stomachs were sectioned as 5 μm thicknesses,

incubated with ER-V (20 μM) for 30 min, washed with saline three times, and finally subjected to a confocal laser scanning microscope (λ_{ex} = 405 nm, λ_{em} = 560 nm ~ 670 nm).

For imaging of viscosity in tumor model. Tumor-bearingmice were prepared through subcutaneous injection of 4T1 cells into the right axillae of nude mice for about 7 days. After injecting ER-V (200 μM, 100 μL) into the right axillae (tumor site) and left axillae (control site), *in vivo* imaging were performed on the mice using a Bruker small animal *in vivo* Xtreme imaging system with an excitation filter of 440 nm and an emission filter of 570 nm. Then, the normal organs (including heart, liver, spleen, lung, and kidney) and tumor were isolated from the mice for *ex vivo* imaging, using a Bruker small animal *in vivo* Xtreme imaging system with an excitation filter of 440 nm and an emission filter of 570 nm.

For living human tissue slices imaging. The harvested surgical specimens of patients, including the malignant tissues (e.g. papillary thyroid carcinoma and lung adenocarcinoma) and their corresponding normal tissues, as well as H&E staining were kindly provided by Department of Pathology in Shanxi Provincial People's Hospital. Informed consent was obtained for any experimentation with human subjects. All the human tissues slices harvested from surgical specimens of patients and determined by doctors, were cryosectioned as 5 μm thicknesses, respectively. The slices were incubated with ER-V (20 μM) for 30 min, and finally subjected to a Zeiss LSM880+Airyscan laser scanning confocal microscope (λ_{ex} = 405 nm, λ_{em} = 560 nm ~ 670 nm).

^{1}H NMR and ^{13}C NMR spectra of compound 1 and ER-V

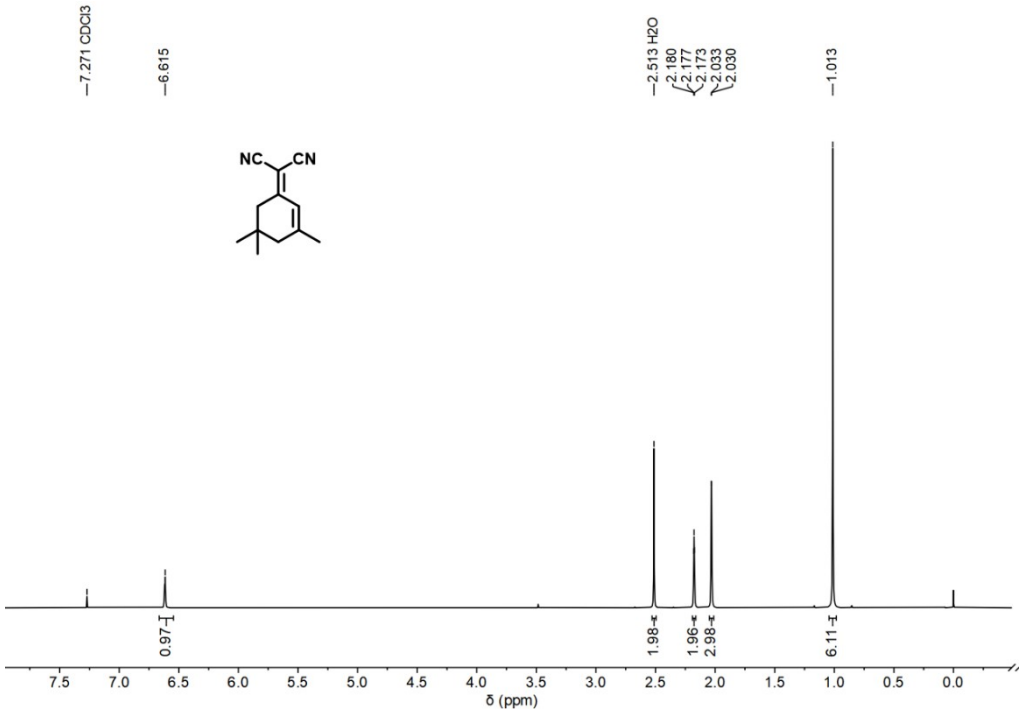


Fig. S1 ^{1}H NMR spectrum of compound 1 in $CDCl_3$.

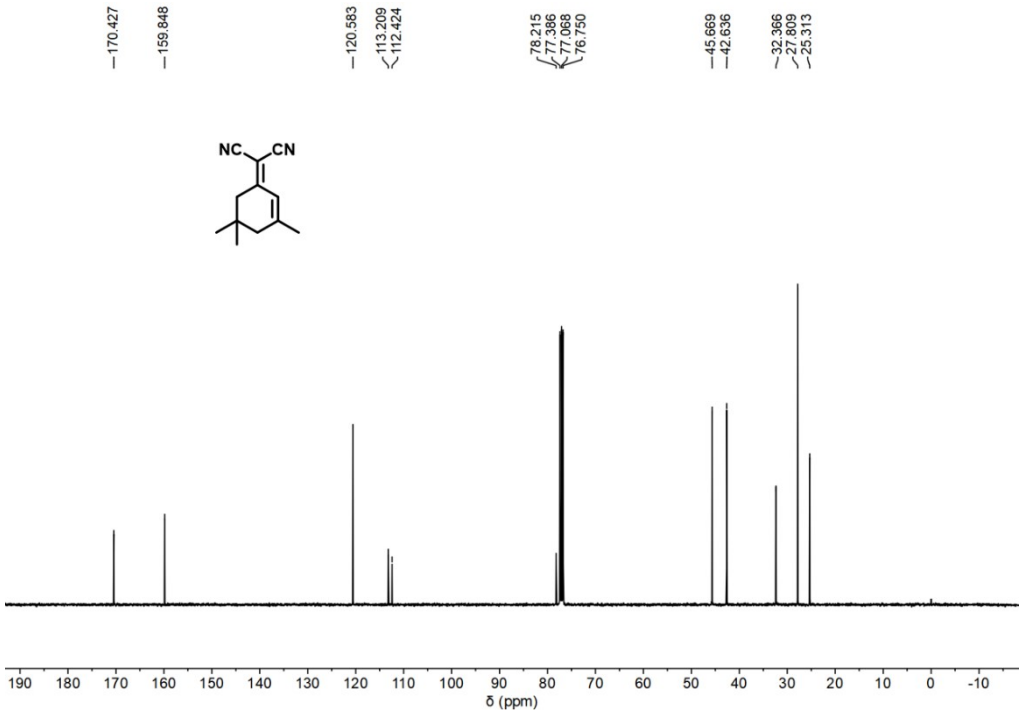


Fig. S2 ^{13}C NMR spectrum of compound 1 in $CDCl_3$.

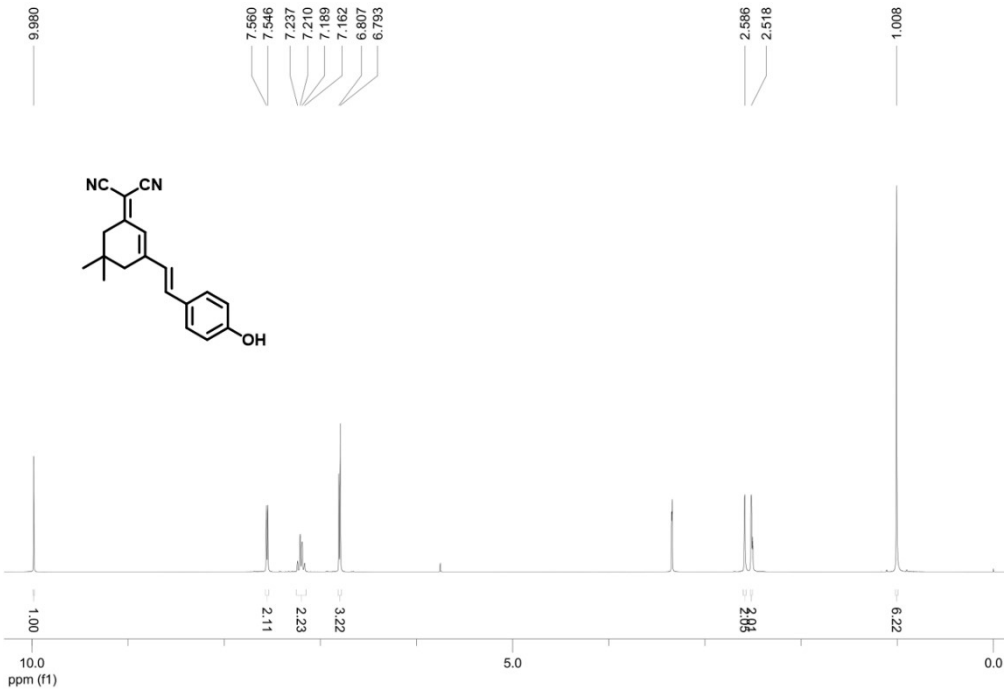


Fig. S3 ^{1}H NMR spectrum of ER-V in DMSO-d_6.

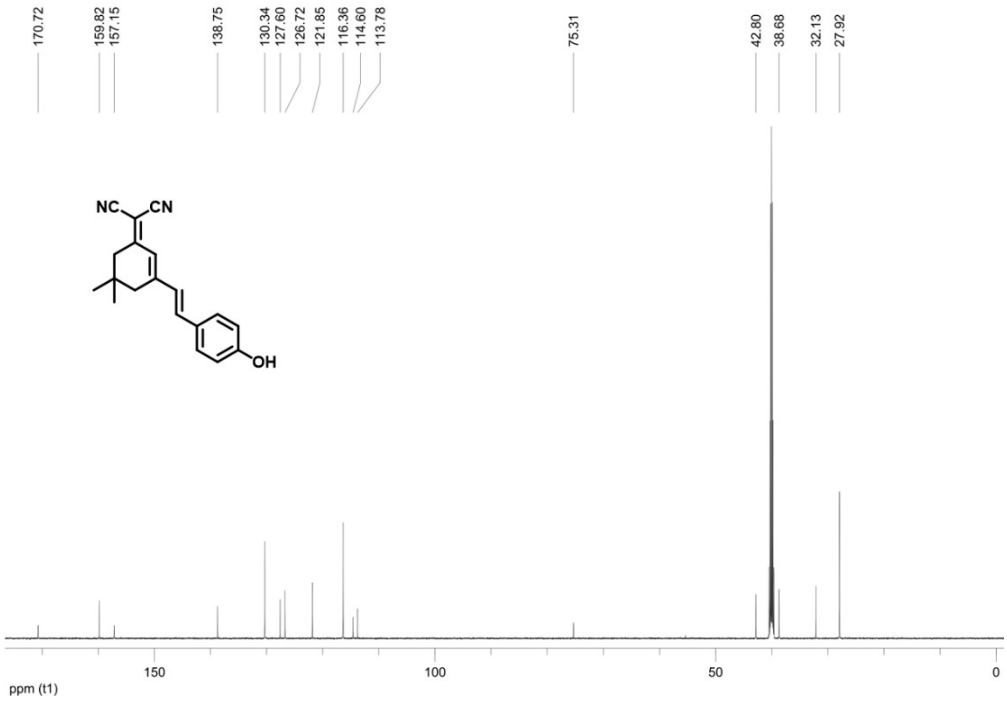
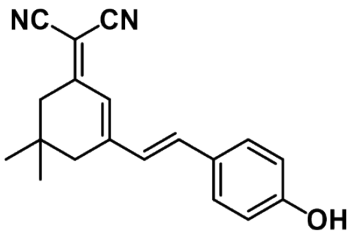


Fig. S4 ^{13}C NMR spectrum of ER-V in DMSO-d_6.



Chemical Formula: $C_{19}H_{18}N_2O$

Exact Mass: 290.1419

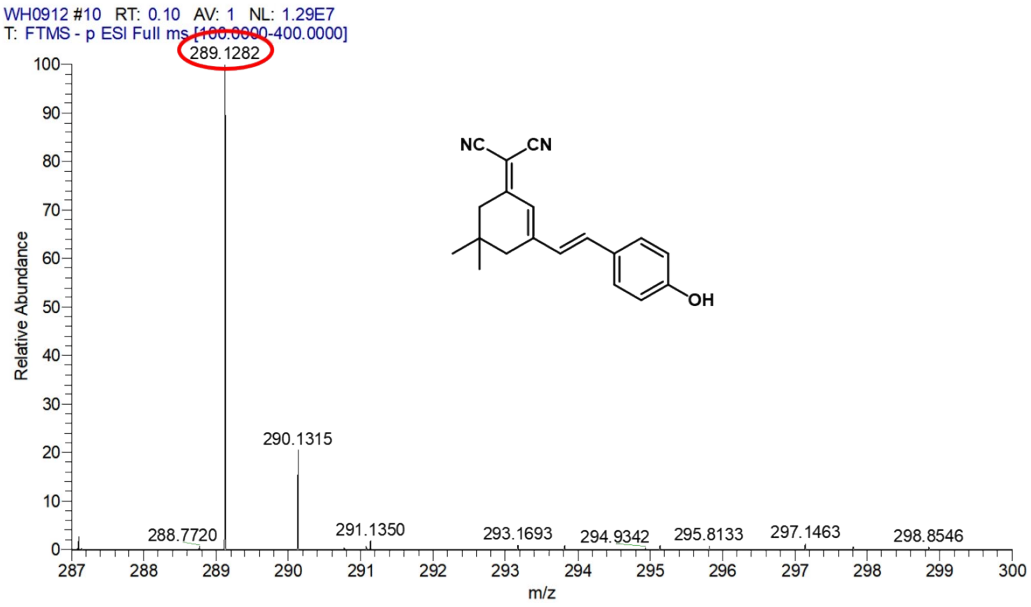


Fig. S5 HR-MS spectrum of ER-V.

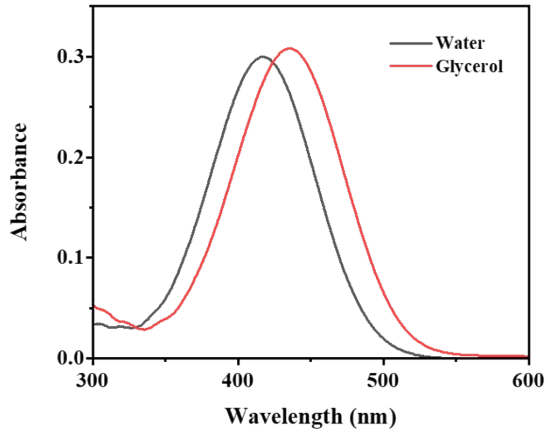


Fig. S6 Absorption spectra of ER-V (10 μM) in water and glycerol.

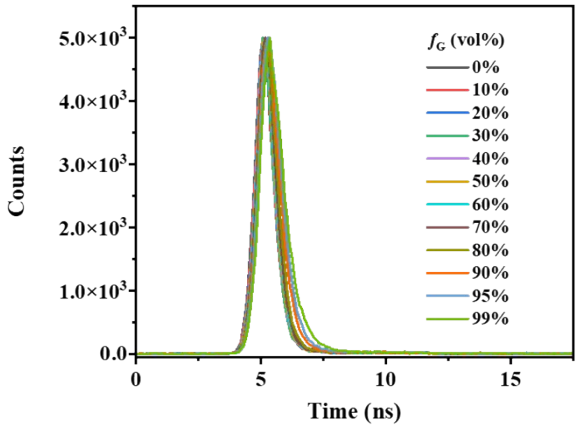


Fig. S7 Fluorescence lifetime spectra of ER-V (10 μM) in water/glycerol mixtures with f_G raising from 0 % to 99 %. The fluorescence lifetime increased from 0.839 ns (0% glycerol), 0.999 ns (20% glycerol), 1.102 ns (40% glycerol), 1.209 ns (60% glycerol), 1.477 ns (80% glycerol) to 1.651 ns (99% glycerol).

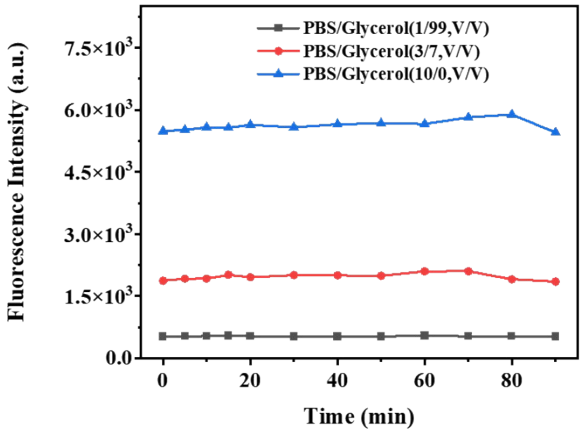


Fig. S8 Photostability of ER-V (10 μM) in water/glycerol mixtures with f_G (0 %, 70% and 90 %), λ_{ex} = 435 nm.

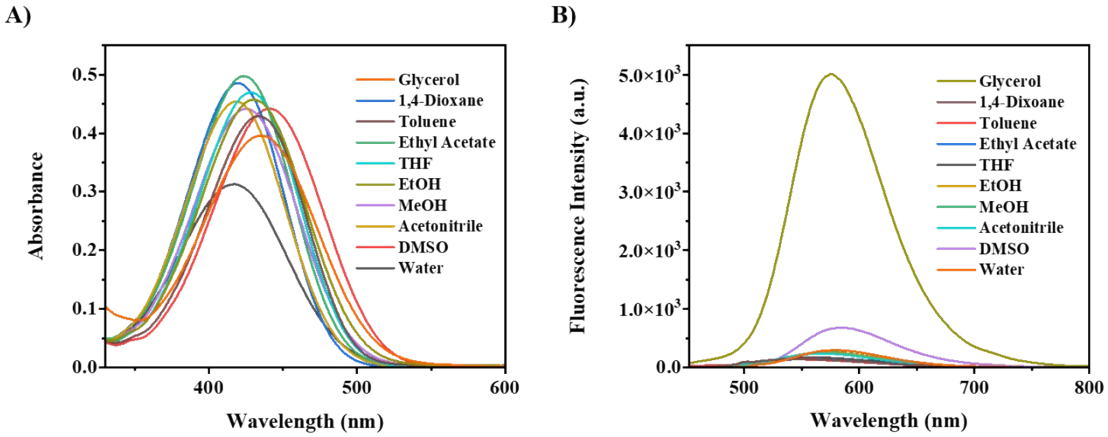


Fig. S9 Absorption spectra (A) and Fluorescence spectra (B) of **ER-V** (10 μM) in various polarity solvents, λ_{ex} = 435 nm.

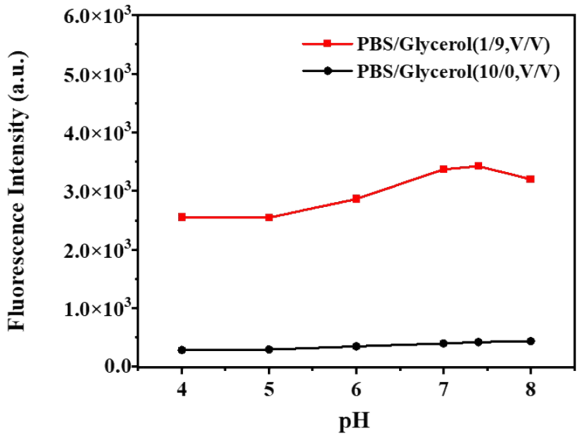


Fig. S10 pH effect on the fluorescent intensity of **ER-V** (10 μM) in PBS/glycerol mixtures with f_G (0 % or 90 %), λ_{ex} = 435 nm.

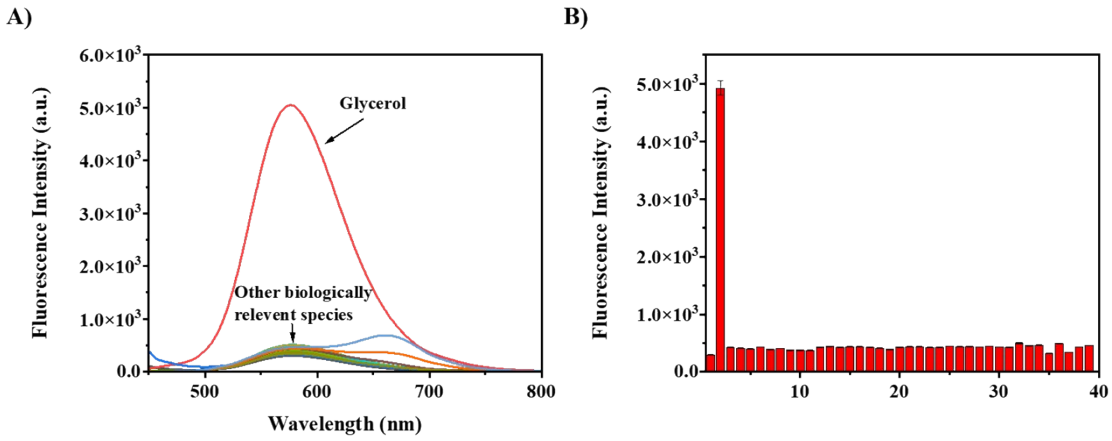


Fig. S11 (A) Fluorescence spectra of ER-V (10 μM) in glycerol and various species in PBS (pH = 7.4). λ_{ex} = 435 nm. (B) Fluorescent intensity at 577 nm of **ER-V** (10 μM) in the presence of various species, 1, Blank; 2, Glycerol; 3, Na^+ (100 μM); 4, K^+ (100 μM); 5, Cu^{2+} (100 μM); 6, Mg^{2+} (100 μM); 7, Ca^{2+} (100 μM); 8, Zn^{2+} (100 μM); 9, Fe^{2+} (100 μM); 10, Fe^{3+} (100 μM); 11, Ag^+ (100 μM); 12, Al^{3+} (100 μM); 13, Ba^{2+} (100 μM); 14, $H_2PO_4^-$ (100 μM); 15, HPO_4^{2-} (100 μM); 16, CO_3^{2-} (100 μM); 17, HCO_3^- (100 μM); 18, Br^- (100 μM); 19, Cl^- (100 μM); 20, I^- (100 μM); 21, $S_2O_3^{2-}$ (100 μM); 22, SO_3^{2-} (100 μM); 23, SO_4^{2-} (100 μM); 24, NO_3^{2-} (100 μM); 25, PO_4^{3-} (100 μM); 26, Hcy (100 μM); 27, Cys (100 μM); 28, GSH (100 μM); 29, Thr (100 μM); 30, Pro (100 μM); 31, $ONOO^-$ (100 μM); 32, H_2O_2(100 μM); 33, ClO^- (100 μM); 34, •OH (100 μM); 35, $^1O_2$ (100 μM); 36, $O_2•^-$ (100 μM); 37, Esterase (1 U/mL); 38, BSA (100 μg/mL); 39, HSA (100 μg/mL).

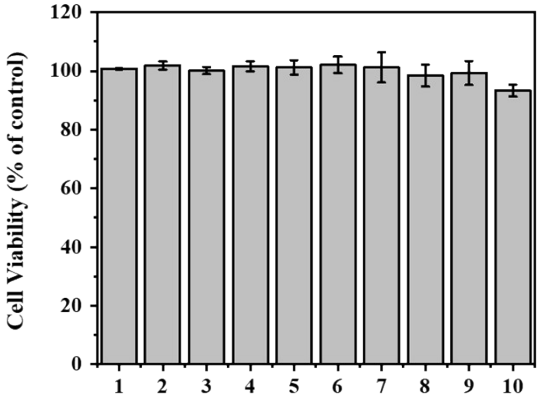


Fig. S12 Cell viability of ER-V on HeLa cells. 1, 0 μM; 2, 0.5 μM; 3, 1μM; 4、2 μM; 5, 5μM; 6, 8 μM, 7, 10 μM; 8, 12μM; 9, 15 μM; 10, 20 μM.

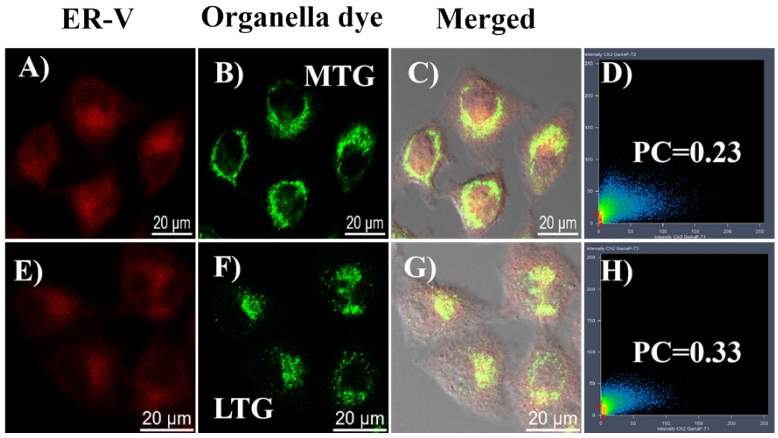


Fig. S13 Co-localization images of cells co-stained with ER-V (6 μM) and MTG or LTG. (A, E) Red channel of ER-V (6 μM, λ_{ex} = 405 nm, λ_{em} = 560 nm - 700 nm); (B, F) Green channel of MTG or LTG (1 μM, λ_{ex} = 488 nm, λ_{em} = 500 nm - 530 nm); (C, G) Merged image. (D, H) Scatter plot. Scale bar: 20 μm.

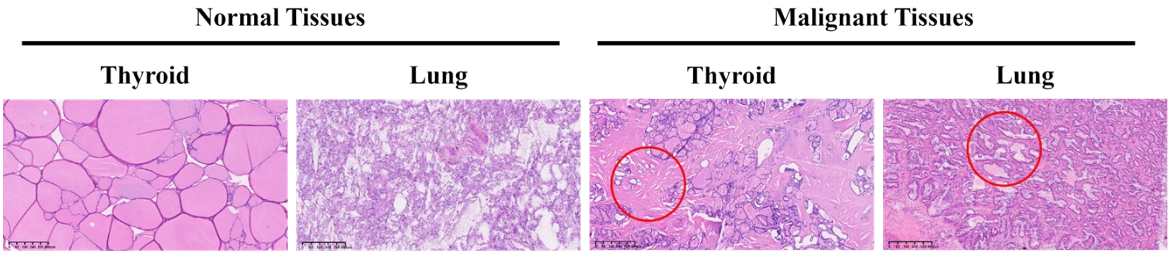


Fig. S14 H&E staining of normal tissues (thyroid and lung) and malignant tissues (papillary thyroid carcinoma and lung adenocarcinoma). Scale bar: 400 μm.