Supporting Information

A combined viscosity-restricted intramolecular motion and mitochondrial targeting leads to selective tumor visualization.

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Materials and Methods

All reagents were purchased from commercial sources and were used without further purification. Flash chromatography was carried out on silica gel (300-400 mesh). ^{1}H NMR spectra were recorded using a BRUKER DRX 400 spectrometer; ^{13}C NMR spectra were recorded using a BRUKER DRX 400 spectrometer; mass spectrometry was recorded with an Agilent 1100 LC-MSD/TOF mass spectrometer. Monensin and NaN_3 was purchased from Shanghai Titan Scientific Co. Ltd. The fluorescence spectra were performed on a F97XP FL spectrophotometer with a 1 cm standard quartz cell. Excitation and emission slit widths were both set at 10 nm. The UV-Vis spectra were obtained using a UV-240IPC spectrophotometer. cell images were observed with a fluorescence confocal microscope (Olympus FV10i; Olympus, Tokyo, Japan) which is equipped with fluorescence mirror unit (U-MWU2). The mounted nematodes were imaged using an Olympus BX51 inverted fluorescence microscope. The ex vivo biodistribution were imaged using Caliper IVIS Lumina II (Ivis Lumina Xr).

All animal care and experimental protocols involved in this article comply with the animal management regulations of the Ministry of Health of the People's Republic of China and have been approved by the Chinese Academy of Science, Kunming Institute of Zoology, with the approval number of *SMKX-20180523-144*. In addition, there is no any experimentation with human subjects.

General Procedure for the Synthesis of V-M1

Compound 4 [S1]**:** Acenaphthene (1 g, 6.5 mmol) and $AlCl_3$ (1.2 g) were dissolved in 5 mL 1,2-dichloroethane. Under a constant temperature of 0 to 5 ℃, acetic anhydride (614 μL) was added. After being cooled in an ice-bath for 4 h, concentrated hydrochloric acid (1.7 mL) was added followed by ice (8.34 g) and the mixture was stirred until the ice was completely melted. The resulting mixture was extracted using chloroform (5 mL) twice, and the pooled organic phase was washed by water (5mL) twice. Using activated carbon to remove colored impurities, the crude product was purified by chromatography on a silica gel, using CH_2Cl_2: petroleum ether (1:3) as an eluent, resulting in a yellow oily liquid. The oil liquid was recrystallized with methanol and compound 5 (35 mg) was obtained. ^{1}HNMR (400 MHz, $CDCl_3$) δ (ppm) 8.73 (d, J = 8.5 Hz, 1H), 8.08 (d, J = 7.3 Hz, 1H), 7.61 (dd, J = 7.0, 8.5 Hz, 1H), 7.38 (d, J = 6.9 Hz, 1H), 7.31 (d, J = 7.3 Hz, 1H), 3.43 (s, 2H), 3.42 (s, 2H), 2.74 (s, 3H).

Compound 3 [S1]**:** Compound 4 (0.5 g, 2.56 mmol) and acetic acid (9.375 mL) were added to a flask and stirred until fully dissolved. Then sodium chromate (3.75 g, 22.5 mmol) was added to the solution. The resulting suspension was heated for 2 h with the oil bath maintained at 90 ℃ to 100 ℃, after which the reaction mixture was stirred for 30 min at room temperature then mixed with ice water (12.5 mL), resulting in the precipitation of a green solid that was filtered off. The

green solid was washed by a large amount of water, resulting in a white solid, compound 3. ^{1}HNMR (400 MHz, $CDCl_3$) δ (ppm) 8.74 (d, *J* = 8.0 Hz, 1H), 8.09 (d, *J* = 8.0 Hz, 1H), 7.63 (t, *J* = 8.0 Hz, 1H), 7.38 (d, *J* = 8.0 Hz, 1H), 7.32 (d, *J* = 8.0 Hz, 1H).

Compound 2 [S2]**:** Compound 3 (200 mg, 1.25 mmol) was dissolved in ethanol (12.5 mL) and *n*-butylamine (1.45 mL, 1.4 mmol) was added, then refluxed and stirred for 6 h. The crude product was purified by chromatography on silica gel, using ethyl acetate: petroleum ether (1:2) as the eluent, resulting in a white solid, compound 2 (50 mg). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 8.98 (d, *J* = 8.6 Hz, 1H), 8.79 (m, 2H), 8.15 (d, *J* = 7.6 Hz, 1H), 7.93 (m, 1H), 4.26 (m, 2H), 2.84 (s, 3H), 1.75 (tt, *J* = 7.7, 6.6 Hz, 2H), 1.48 (dd, *J* = 15.2, 7.5 Hz, 2H), 1.01 (t, *J* = 7.4 Hz, 3H).

V-M1: 4-(Diethylamino)Salicylaldehyde (58 mg, 0.3mmol) and compound 2 (85.2 mg, 0.3 mol) were dissolved in 2 ml concentrated sulfuric acid. Then the mixture was heated and stirred at 90°C for 6 h. After the reaction solution was cooled to room temperature, ice water (20 g) and 70% $HClO_4$ (0.5 mL) were added respectively, then the mixture was filtered, washed with water and the organic layer was dried over Na_2SO_4. The crude product was purified by chromatography on a silica gel, using $CHCl_2$: methanol (10:1) as an eluent, resulting in a purple solid, V-M1 (30 mg, yield:13.4%). ^{1}H NMR (400 MHz, 298K, $CDCl_3$): δ (ppm) 8.708 (d, 1H, *J* = 8.0 Hz), 8.623 (m, 3H), 8.257 (d, 1H, *J* = 7.6 Hz), 7.999 (d, 1H, *J* = 9.6 Hz), 7.953 (m, 1H, *J* = 7.6 Hz, 8.8 Hz), 7.689 (d, 1H, *J* = 4.0 Hz), 7.440 (m, 1H, *J* = 2.4 Hz, 9.6 Hz), 7.052 (d, 4H, *J* = 1.6 Hz), 4.133 (t, 2H, 7.6 Hz), 3.757 (t, 4H, *J* = 7.6 Hz), 1.725 (m, 2H), 1.455 (t, 2H, *J* = 7.6 Hz), 1.398 (t, 6H, *J* = 7.6 Hz), 0.962 (t, 3H, *J* = 7.2 Hz). ^{13}C NMR (100 MHz, 298 K, $CDCl_3$): δ (ppm) 164.49, 163.39, 163.00, 160.61, 157.73, 148.29, 133.71, 133.29, 131.96, 130.78, 130.47, 130.02, 129.48, 128.46, 128.38, 125.76, 123.05, 121.24, 120.06, 114.79, 96.34, 47.14, 46.88, 40.51, 30.11, 20.36, 13.83, 13.25, 12.15. TOF MS: 453.2176.

General Procedure for the Solvent Tests and Biological Tests

Viscosity analysis

Viscous solutions were prepared by mixing methanol or ethanol and glycerol in different volume proportions. The viscosity of each sample was measured with an NDJ-5S rotational viscosimeter. 50 μL of a **V-M1** stock solution (1.0 mM in methanol/ethanol) was added to the methanol/ethanol-glycerol mixture (5 mL) to give a final concentration of 10 μM. The resulting solutions were shaken for 30 min and then kept still for 30 min at 26 °C, after which the absorbance and fluorescence was recorded. The quantitative relationship between fluorescence intensity and the viscosity of solution was fitted by the Förster-Hoffmann equation 1 as follows: [S3].

$$\log (I_f) = C + x \log \eta$$

Where I_f is the fluorescence intensity; η stands the viscosity of solution; C is a concentration- and temperature-dependent constant and x is a sensor- and temperature-dependent constant.

In addition, the fluorescence at 650 nm upon excitation at 590 nm of a solution of **V-M1** (10 μM) in 70% methanol/ethanol-glycerol was recorded between -5 °C and 40 °C at 5 °C increments.

Fluorescence lifetime detection

Solvents of variable viscosity were prepared as mentioned above. A fluorescence lifetime measuring apparatus (Shimadzu) was used to acquire the fluorescence lifetimes of **V-M1**, with the excitation wavelength at 580 nm and emission at 650 nm. An excellent straight fitting was obtained and the quantitative relationship between the fluorescence lifetime of **V-M1** and the viscosity of the solution is described by Förster–Hoffmann equation 2: [S3]

$$\text{Log } \tau = C + x \log \eta$$

Where τ is the fluorescence lifetime; η stands the viscosity of solution; C is a concentration- and temperature-dependent constant and x is a sensor- and temperature-dependent constant.

Effect of different pH and polarity

The effects of pH and polarity on the fluorescence response of **V-M1** (10 μM) were investigated. The experiment of pH stability was carried out at the pH range 3-8.5 in water/glycerol system (4:6, v/v, 0.01 M PBS buffer). The range of pH from 3-8.5 (0.5 interval) was determined by an accurate range pH test paper. The experiment of polarity stability was conducted by using 1,4-dioxane/ water in different volume proportions.

The experiment of selectivity

Considering the complexity of an intracellular environment, interferences of various bio-analytes towards **V-M1** was also monitored, including glutathione (GSH), cysteine (Cys), Homocysteine (Hcy), nitrite (NO_2^-), hydrogen peroxide (H_2O_2), peroxynitrite anion ($ONOO^-$), hypochlorous acid (HClO), hydrogen sulfifide (H_2S), dipotassium pentasulfifide (K_2S_5), leucine aminopeptidase (LAP), nitroreductase (NTR), azo reductase (AZOR), diaphorase (DIA), cations (Na^+, K^+, Fe^{3+}, Cu^{2+}, Ca^{2+}, Mg^{2+}) and serum albumin. The Stock solutions of the biologically relevant analytes (1.0 mM each, except serum albumin) were prepared in trice distilled water. Stock solutions of V-M1 (1.0 mM) were prepared in methanol. For the measurements of fluorescence spectra, the excitation wavelength was set at 505nm with excitation slit widths at 5 nm, and emission at slit widths 5 nm. The selective experiments were performed using 10μM of **V-M1** in PBS solutions (pH 7.4, 0.01 M, 1% methanol, 27 °C) with 50 μM of each analyte (2 mg/mL of serum albumin).

Fluorescence quantum yields measurements.

The relative fluorescence quantum yields were identified with Rhodamine B (Φ_s = 0.97) in pure ethanol as a reference and calculated utilizing the following equation [3]:

$$\Phi_x = \Phi s(F_x/F_s)(A_s/A_x)(\lambda_{exs}/\lambda_{exx})(n_x/n_s)^2$$

where Φ represents quantum yield; F is the integrated area under the corrected emission spectrum; A stands for absorbance at the excitation wavelength; n is the refractive index of the solvent (because of the low concentrations of the solution (10^{-6}-10^{-7} mol/L), The change of refraction coefficient in solution can be ignored); λ_{ex} is the excitation wavelength; and the subscripts x and s represent for the unknown and the reference, respectively.

Cell-culture and confocal microscopy experiments

HeLa cells were all cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10 % FBS (fetal bovine serum) in an atmosphere of 5% CO_2 and 95% air at 37 °C. The cells were seeded onto 24-well flat-bottomed plates (1.0×10^4 per well). When the density of the cell reached to 70-80% of confluence, the sub-culturing was conducted. The culture medium was replaced every 2-3 days after PBS washing.

For the confocal imaging, HeLa cells were seeded onto 35mm-confocal dish (1.5×10^6) (glass bottom dish) and then incubated for 24 h at 37 °C under 5% CO_2 and 95% air. Then the cells were incubation with 0 μM, 5 μM, 10 μM and 15 μM of monensin for 2 h at 37 °C, respectively. The cells were washed twice with 1 mL PBS at room temperature. The cells were incubation with final concentration of 10.0 μM of **V-M1** (PBS/Methanol, 100/1, v/v) for 1 h at 37 °C. Then HeLa cells were stained with DAPI (1.0 μM) and Mitotracker Green dye (MTG) (1.0 μM) for 20 min. The cells were washed once with 1 mL PBS at room temperature before fluorescence imaging. Fluorescence cell imaging was performed with an (OLYMPUS FV10i) confocal microscopy equipped with a 40 × objective lens. Fluorescence images of HeLa cells were monitored at 405-440 nm, 460-550nm and 585-610 nm for blue, green and red channels, respectively.

For FLIM experiments, HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% FBS (fetal bovine serum) in an atmosphere of 5% CO_2 and 95% air at 37 °C. The cells were seeded onto 35mm-confocal dish (1.5×10^6) and then incubated for 24 h at 37 °C under 5% CO_2 and 95% air. Then the cells were incubation with 0 μM, and 10 μM monensin for 2 h at 37 °C, respectively. The cells were washed twice with 1 mL PBS (0.01 M) at room temperature. The cells were incubated with final concentration of 10.0 μM of **V-M1** (PBS/Methanol, 100/1, v/v) 1 h at 37 °C. The cells were washed once with 1 mL PBS at room temperature before fluorescence imaging. Fluorescence cell imaging was performed with a

confocal-lifetime microscope equipped with a 100× objective lens. Fluorescence life-time images of cells were monitored in the 585-610 nm spectral window.

Cytotoxicity test

To investigate the cytotoxicity of **V-M1**, five cell lines: leukemic cells (HL-60), lung carcinoma cells (A549), liver hepatocarcinoma cells (SMMC-7721), breast adenocarcinoma cells (MCF-7), colonic cancer cells (SW480) and normal cell lines (BEAS-2B) were used. IC_{50} was evaluated by utilizing the commercial MTS assay (a new derivative of MTT), 3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethoxyphenyl)-2-(4-sulfopheny)-2H-tetrazolium. Firstly, cell suspensions were made by using medium containing 10% fetal bovine serum (DMEM or RMPI1640) and cells were seeded into 96-well plates (100 μL of medium and 3×10^3 to 1.5×10^4 each well,). All cells were seeded and cultured 24 hours in advance. Stock solutions of **V-M1** (10.0 mM) were prepared in DMSO. **V-M1** was added at a final concentration of 40 μM, 8 μM, 1.6 μM, 0.32 μM, 0.064 μM, respectively and the final volume of the medium in each well was 200 μL. Three duplicate wells were set for each treatment. After 48 h incubation (at 37 ℃), 20 μL of MTS solution (50 mM) was added to each well. As positive controls, 20 μL of the MTS stock solution was added per well in the presence of DPP and Taxol (40 μM, 8 μM, 1.6 μM, 0.32 μM, 0.064 μM). After 4 h incubation with MTS (at 37 ℃), the absorbance of each well was monitored by using a microplate spectrophotometer (MULTISKAN FC) at a wavelength of 492 nm. Finally, the IC_{50} of each cell line was calculated by using the method of Reed and Muench.

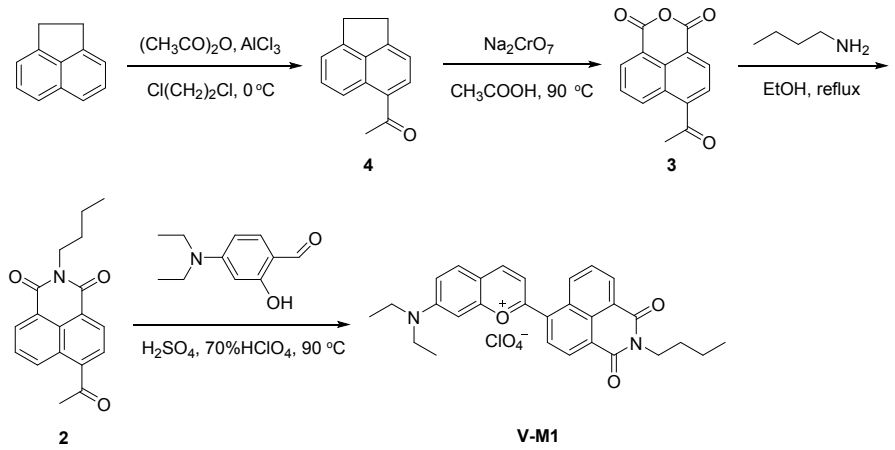
The fluorescence imaging of the C. elegans

The *C. elegans* wild type strain N_2 was acquired from the Kunming Institute of Zoology. The larval stage 4 (L4) of *C. elegans* was used. Each petri dish was first filled with 1 mL of M9 buffer supplemented with 0 M, 0.4 M, 0.8 M, 1.2 M NaN_3 respectively at 20 ℃ for 30 min. Then monensin was added (1×10^{-5} M) to every petri dish for 2 h. After the incubation, the exposed nematodes were washed three times with M9 buffer and collected by centrifugation at 3000 r/min for 2 min. For imaging of accumulations of viscosity in the nematode, the previously exposed worms were incubated in Petri dishes, containing final concentration of 10.0 μM of **V-M1** (M9/Methanol, 100/1, v/v), at 20 ℃ for 30 min. For imaging of accumulations of viscosity in the nematode, the previously used fluorescence microscope (Olympus IX71) equipped with a 20× objective lens was used to observe the fluorescence.

4T1 murine mammary tumor-bearing BALb/c mice model

The cultivation of the nude mouse: all the animal studies were carried out in the Kunming

Institute of Zoology, with the approval number of SMKX-20180523-144. 4-6-week-old female BALb/c nude mice were purchased from the Kunming Institute of Zoology and maintained under standard conditions (temperature 25 °C and humidity is 50%). Each nude mouse was injected with 0.2 mL 4T1 cell sap (1×10^7 cells/mL) into the selected positions to establish the 4T1 tumor model of BALb/c mice. Observing the living situation of nude mice and the tumor size. Volume of tumor was calculated according to the following formula: V (mm3) =1/2 × a × b^2, (a: long diameter of the tumor, b: Short diameter of tumor).



Scheme S1. Synthesis of **V-M1**

^{1}H, ^{13}C and ESI-TOF Mass spectra

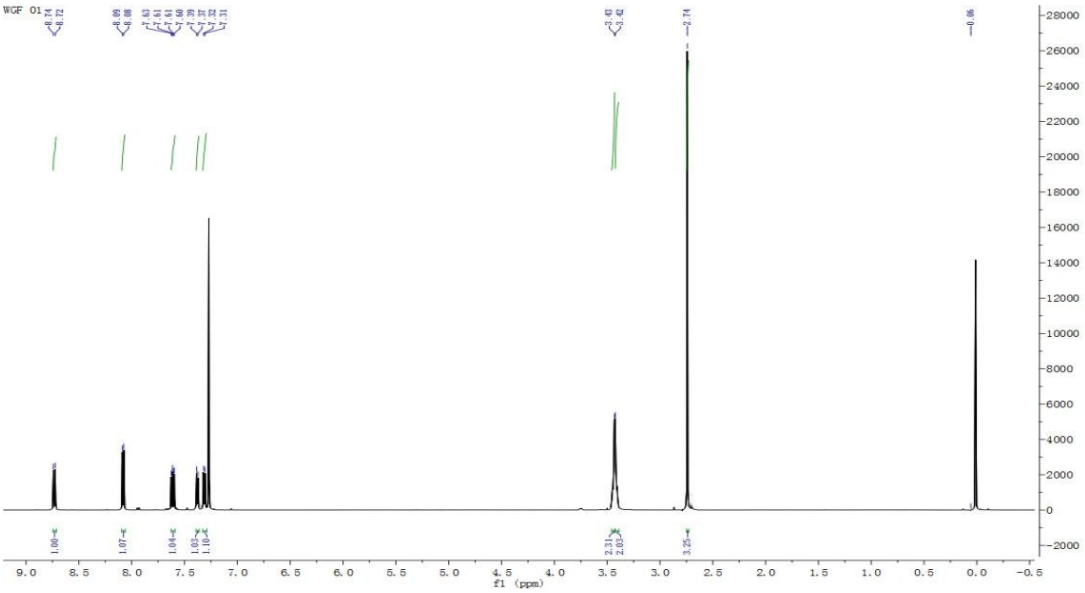


Fig. S1. ^{1}H-NMR (d_6-$CDCl_3$, 400 MHz) spectrum of compound 4**.**

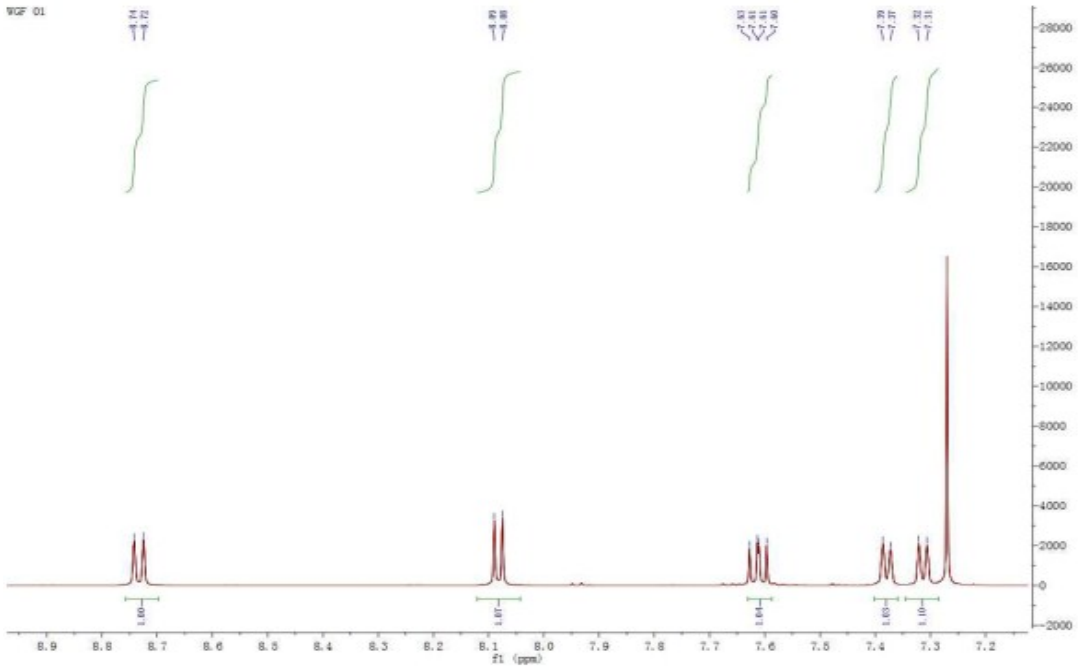


Fig. S2. ^{1}H-NMR (d_6-$CDCl_3$, 400 MHz) spectrum of compound 3.

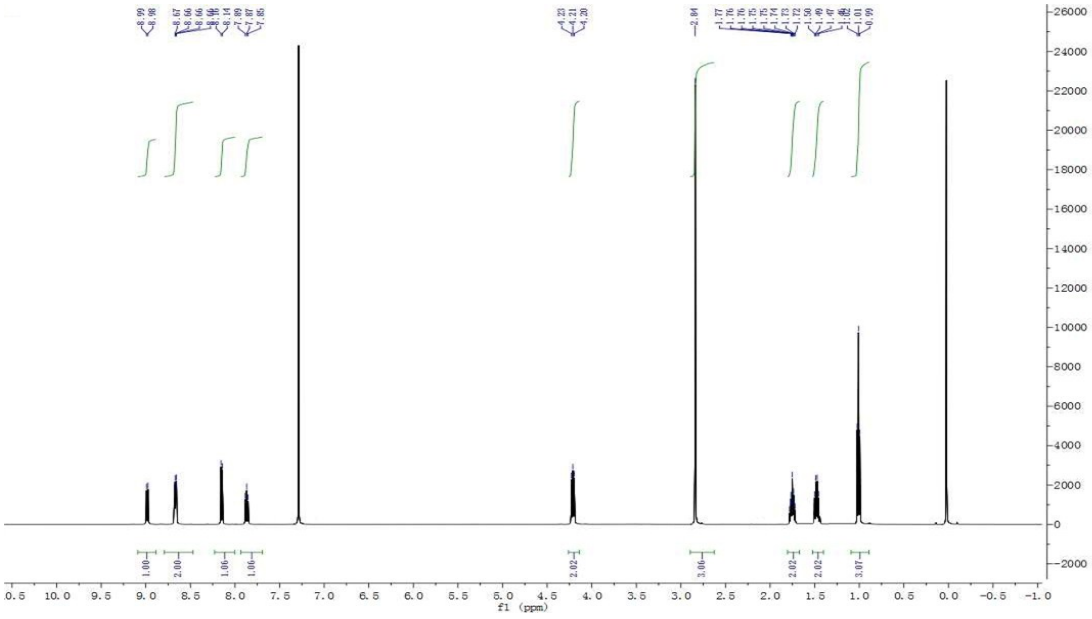


Fig. S3. ^{1}H-NMR (d_6-$CDCl_3$, 400 MHz) spectrum of compound 2**.**

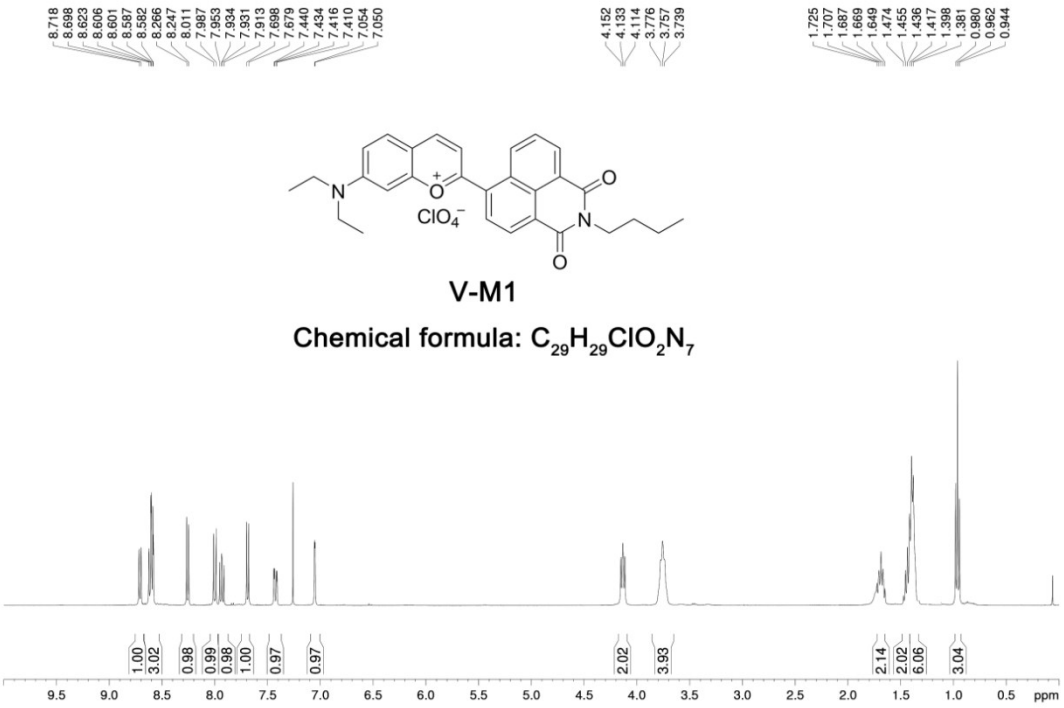


Fig. S4. ^{1}H-NMR (d_6-$CDCl_3$, 400 MHz) spectrum of **V-M1.**

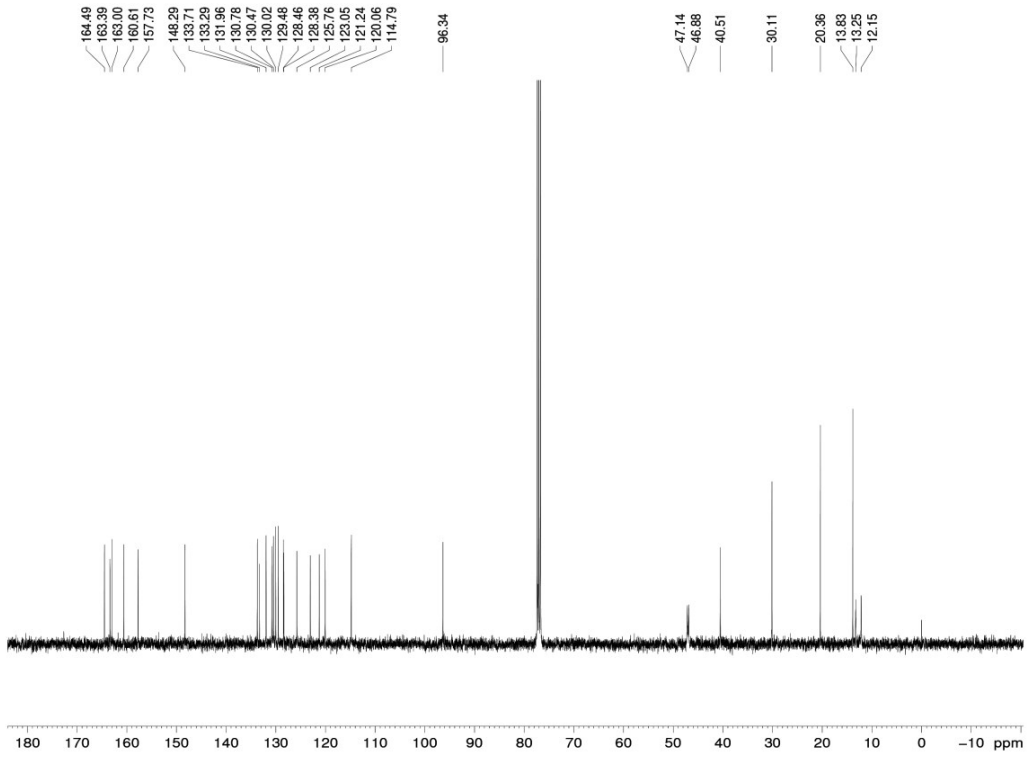


Fig. S5. ^{13}C-NMR (d_6-$CDCl_3$, 100 MHz) spectrum of **V-M1.**

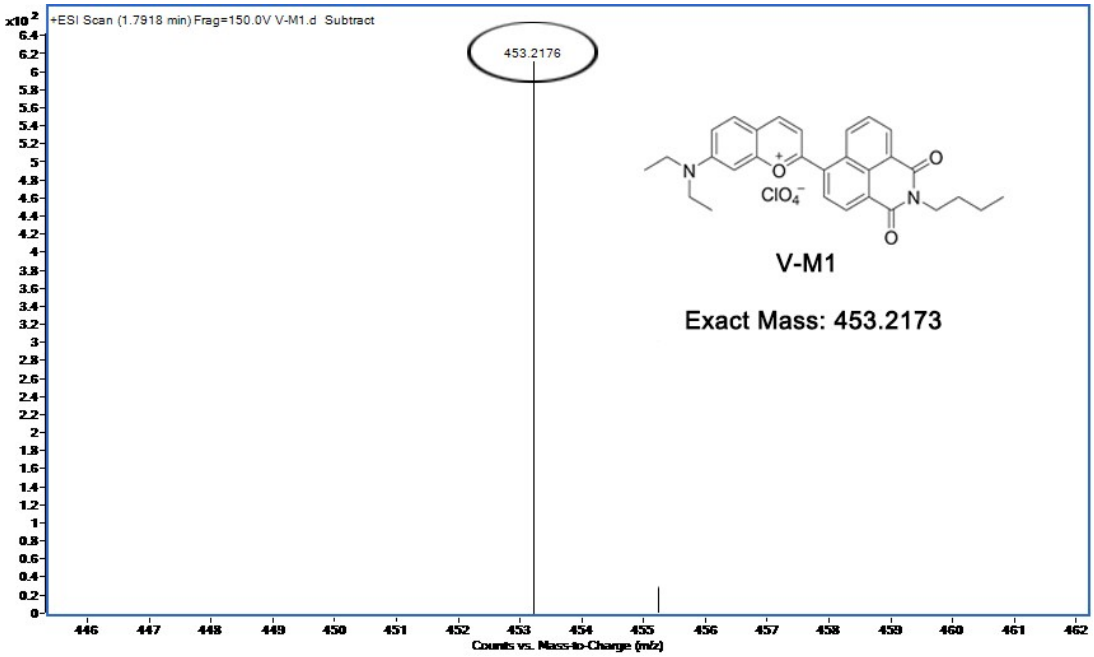


Fig. S6. ESI-TOF spectrum of **V-M1**.

The viscosity of the solvents:

Table S1. The viscosity of glycerol/ethanol mixtures in centipoise (cP) at 25 ℃

V%		Viscosity / cP
glycerol	ethanol	
90%	10%	310.0
80%	20%	186.0
70%	30%	117.0
60%	40%	51.9
50%	50%	19.7
40%	60%	9.7
30%	70%	4.5
20%	80%	2.6
10%	90%	1.5
0%	100%	0.8

Table S2. The viscosity of glycerol/methanol mixtures in centipoise (cP) at 25 ℃

V%		Viscosity / cP
glycerol	methanol	
90%	10%	430.0
80%	20%	316.0
70%	30%	198.0
60%	40%	119.0
50%	50%	46.0
40%	60%	25.2
30%	70%	12.0
20%	80%	7.0
10%	90%	4.4
0%	100%	1.2

Table S3. The viscosity of 1,4-dioxane/water mixtures in centipoise (cP) at 25 ℃

V%		Viscosity / cP
water	1,4-dioxane	
90%	10%	6.2
80%	20%	5.7
70%	30%	5.2
60%	40%	4.8
50%	50%	4.4
40%	60%	3.7
30%	70%	2.9
20%	80%	2.2
10%	90%	1.4
0%	100%	1.1

Spectra of Solvent Tests:

Table S4. Spectral data of **V-M1** in methanol/ethanol

Solvents	dielectric constant	λ_{abs} (nm)	λ_{em} (nm)	ε (L×mol^{-1}×cm^{-1})	Φ_f
Methanol	32.61	512	650	4.09 ×10^4	0.051
Ethanol	24.85	515	649	4.17 ×10^4	0.041

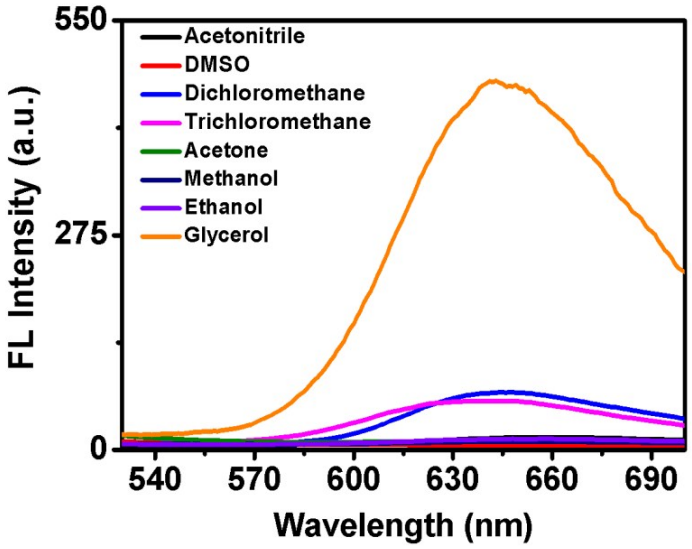


Fig. S7 Fluorescence emission spectra of probe **V-M1** (10 μM) in different solvents at 25 ℃, excited at 505 nm.

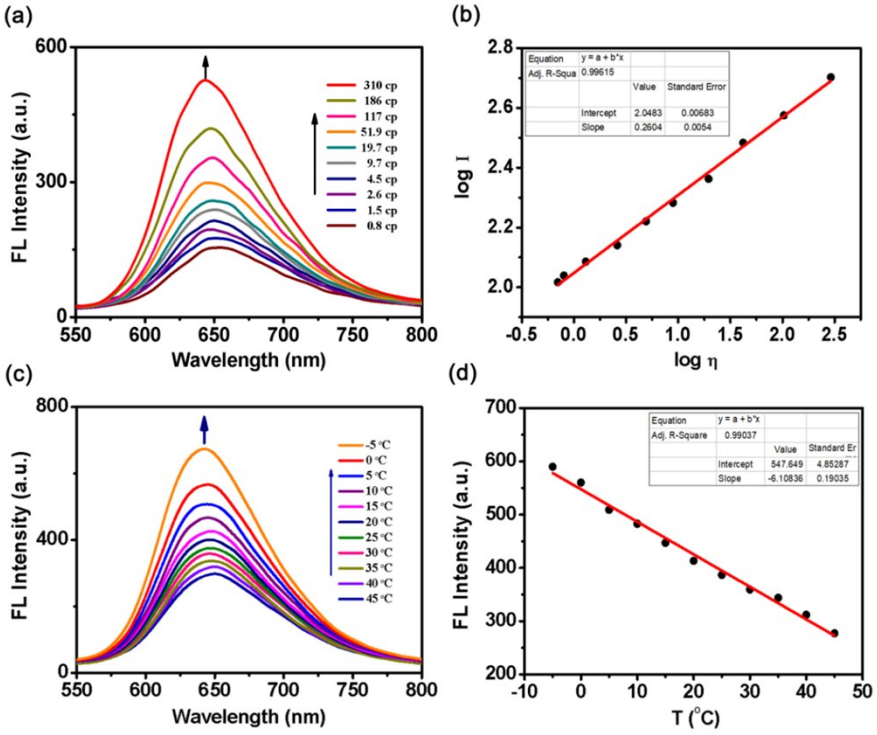


Fig. S8 (a) Fluorescence spectra of **V-M1** (10 μM, excited at 505 nm) in the mixed solvents with different proportions of ethanol-glycerol at 25 ℃. (b) The linearity of relationship between the logarithms of the fluorescence intensity and the viscosity for solvents with different proportions of ethanol-glycerol, R^2 = 0.9961. (c) Fluorescence spectra and ratio of **V-M1** (10 μM) at different temperatures in ethanol-glycerol (3/7, *v/v*) at 25 ℃, excited at 505 nm. (d) The linearity of the plot of the fluorescence intensity versus the temperature in ethanol-glycerol (3/7, *v/v*), R^2=0.9903.

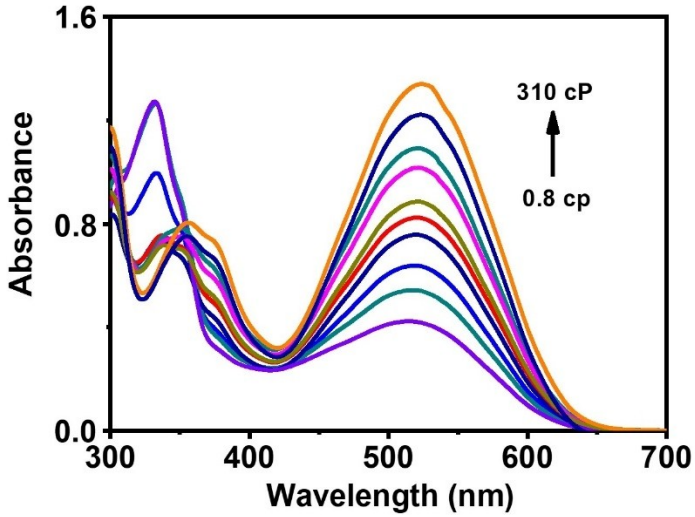


Fig. S9 Absorption spectra of **V-M1** (10 μM) in the mixed solvents with different proportions of ethanol-glycerol at 25 ℃.

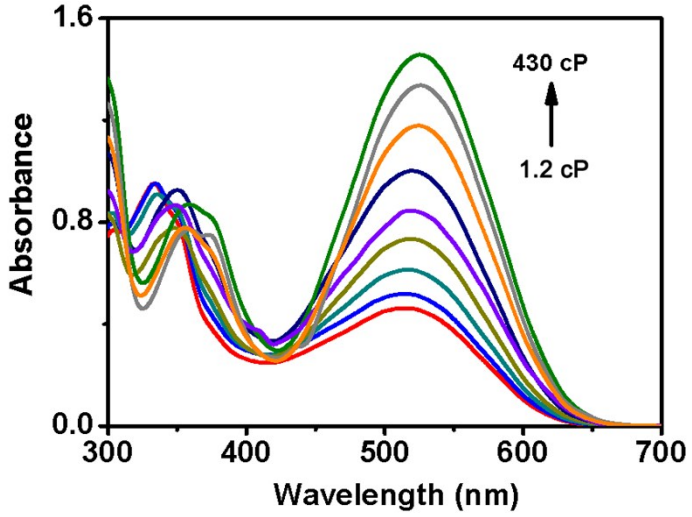


Fig. S10 Absorption spectra of **V-M1** (10 μM) in the mixed solvents with different proportions of methanol-glycerol at 25 ℃.

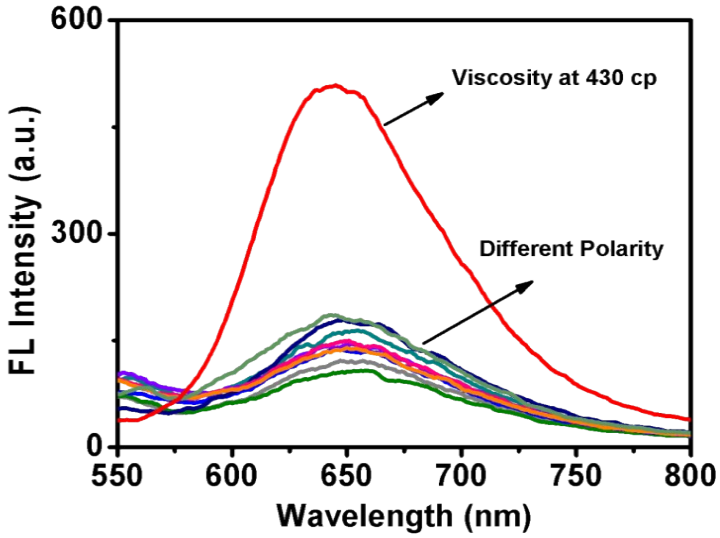


Fig. S11 Fluorescent spectra of **V-M1** (10 μM) under variable solvent polarity in the 1,4-dioxane/water system and the emission at a viscosity at 430 cp as a comparison (at 25 ℃).

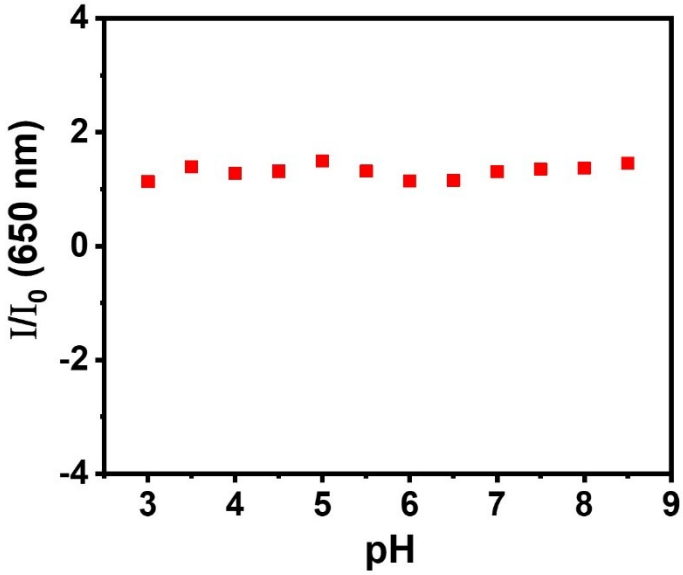


Fig. S12 Relative fluorescence emission intensity (I/I_0, λ_{em} = 650 nm) of **V-M1** (10 μM) at different pH values in water/glycerol system (4:6, *v/v*, 0.01 M PBS buffer, λ_{ex} = 505 nm) at 25 ℃.

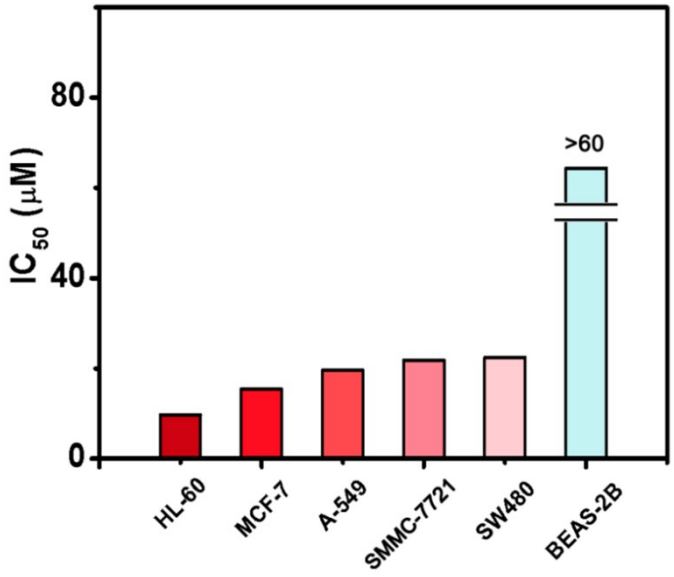


Fig. S13 Cytotoxicity of compounds **V-M1** by using MTS. IC_{50} of leukemic cells (HL-60), lung carcinoma cells (A549), liver hepatocarcinoma cells (SMMC-7721), breast adenocarcinoma cells (MCF-7), and colonic cancer cells (SW480) and a normal cell line (BEAS-2B) upon treatment with **V-M1**, as determined by an MTS assay.

Biological Imaging:

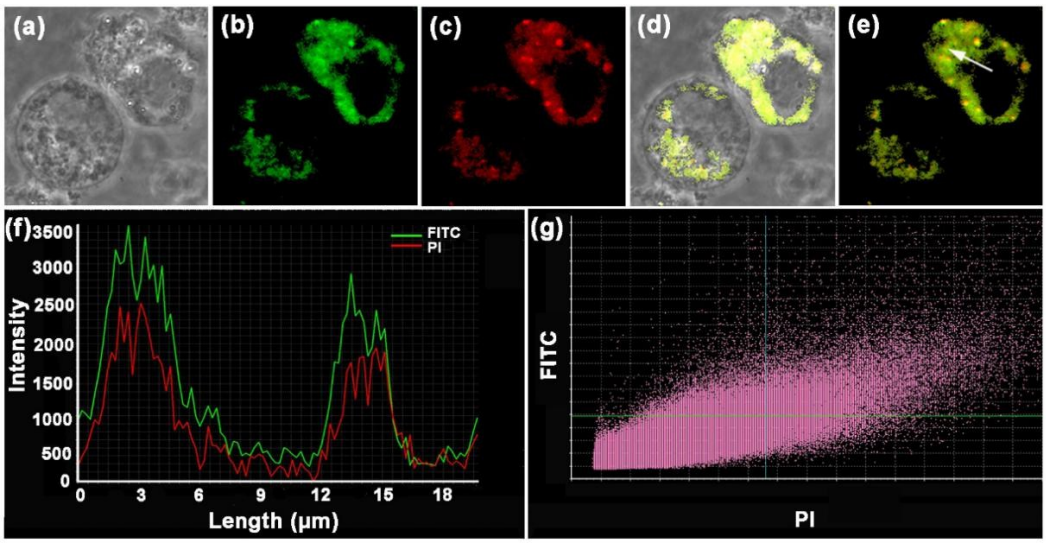


Fig. S14 Bright field and fluorescence images of HeLa cells stained with **V-M1** (10 μM) and a mitochondrial dye. (a) bright field image; (b) from the FITC channel (fluorescence image Mito Tracker Green); (c) from the PI channel (**V-M1** staining); (d) overlay of FITC and PI channels; (e) overlay of bright field FITC and PI channels; (f) Intensity profile of linear region of interest across the HeLa cell co-stained with the FICT channel of MTG and PI channel of **V-M1**; (g) Intensity scatter plot of FICT and PI channels.

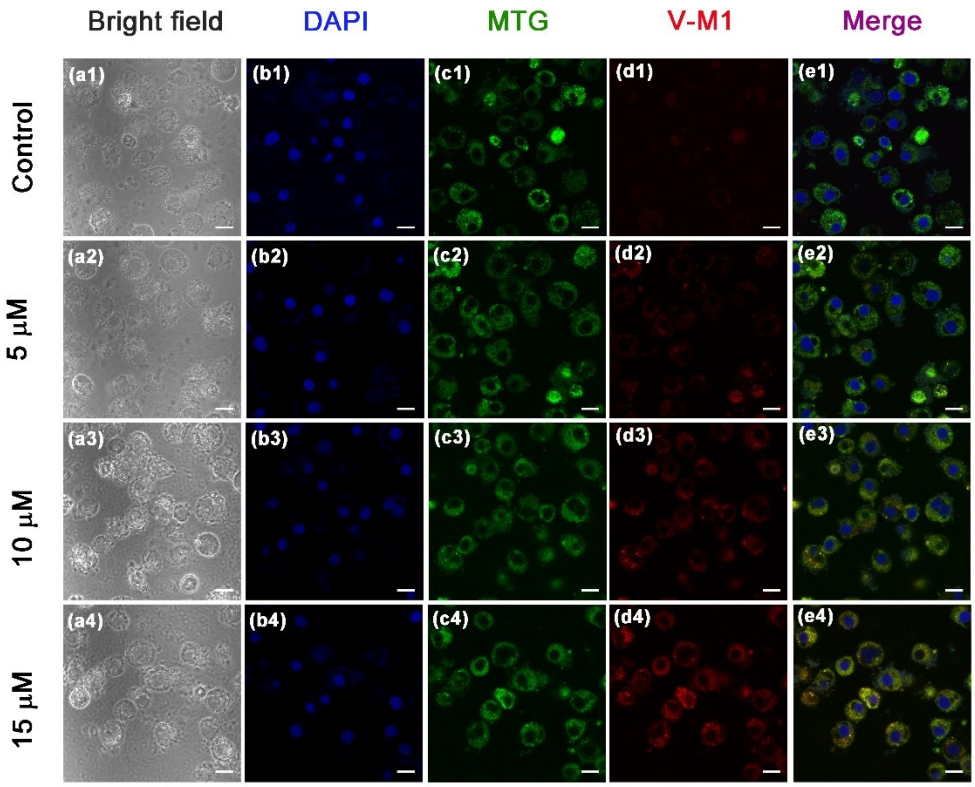


Fig. S15 Confocal laser fluorescence imaging of HeLa cells stained with **V-M1** (10 μM), DAPI (DAPI Staining Solution), and MTG (Mito Tracker Green). Cells were treated with different concentration (0 μM, 5 μM, 10 μM, 15 μM) of monensin.

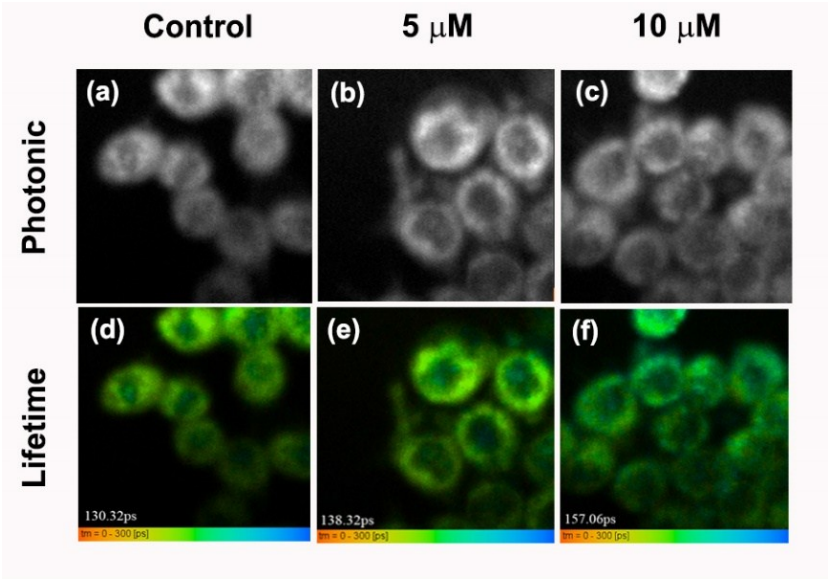


Fig. S16 Imaging studies of **V-M1** stains in HeLa cells. (a-c) Photonic imaging of V-M1 in cells upon treatment with different volume of Monensin (10 μM) for 2 h. (d-f) Fluorescence lifetime imaging of **V-M1** in cells upon treatment with different volume of Monensin (10 μM) for 2 h.

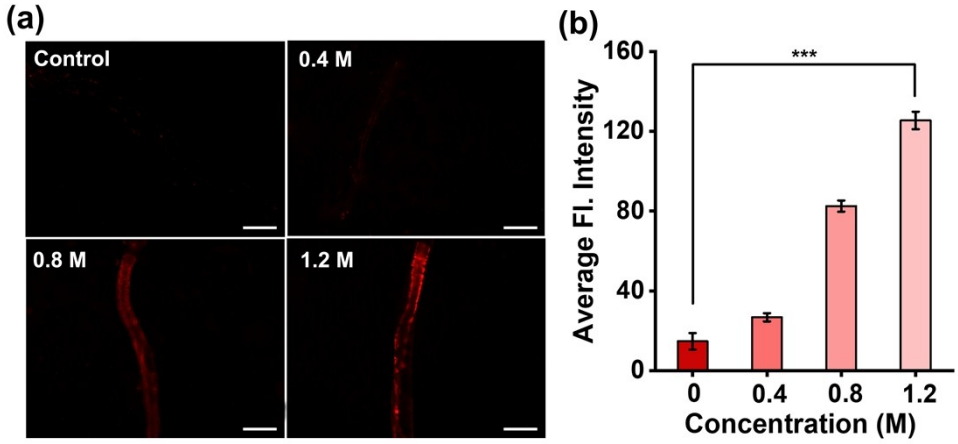


Fig. S17 (a) Fluorescence imaging of C. elegans stained with V-M1, in nematodes subjected to increasing concentrations of NaN3. Images were obtained using an excitation wavelength of 505 nm, with the emission being monitored in a spectral window encompassing 600-650 nm. Scale bar: 20 μm; (b) Average fluorescence intensity of each group from panel (a). Bars denote the average of n = 3 measurements of similar regions of interest from the different part of the same nematode, while error bars denote the standard deviation. ***$p < 0.001$.

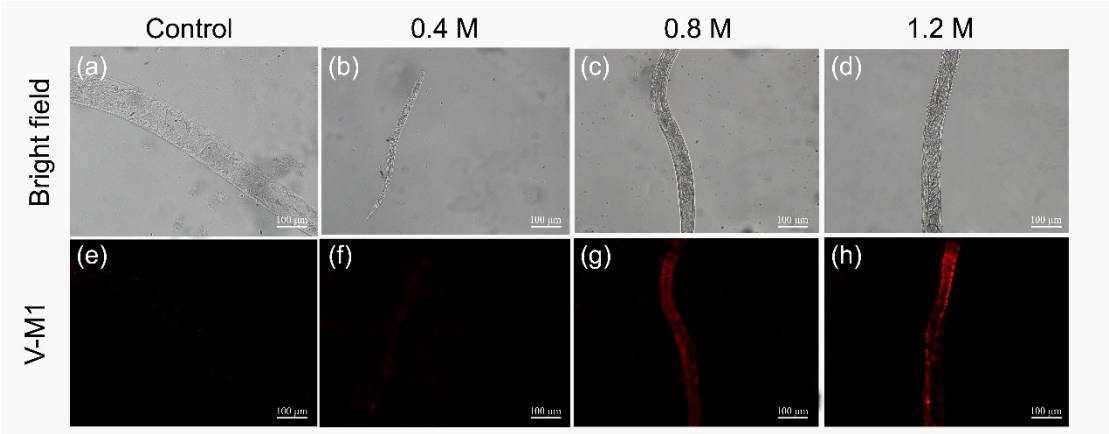


Fig. S18 Brightfield image of and phase contrast of C. elegans with different concentrations of NaN_3 in vitro. (Scale bar: 100 μm.)

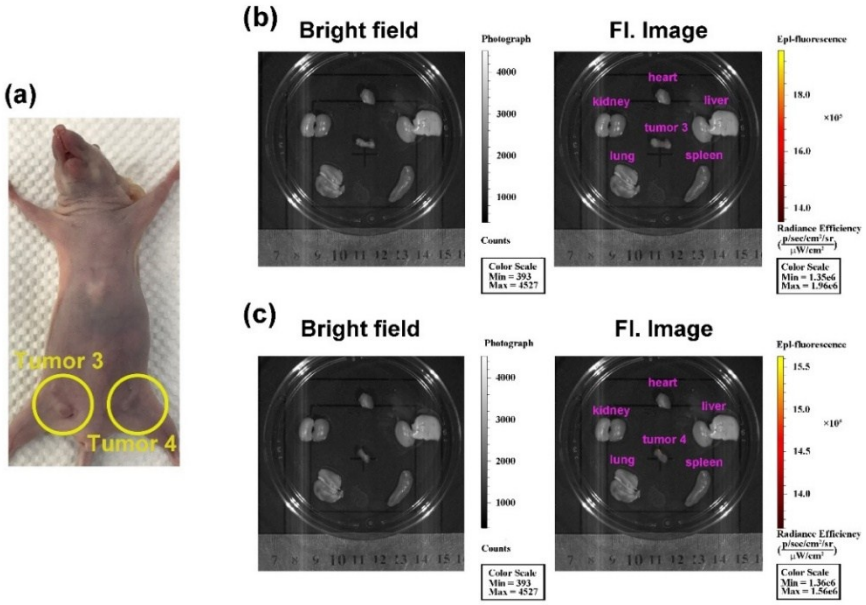


Fig. S19 (a) Photo of the mouse used in the experiment. (b) Bright field and fluorescence signal imaging of 4T1 xenographt tumor 3 (the left one). (c) Bright field and fluorescence signal imaging of the 4T1 xenographt tumor 4 (the right one). All organs and tumors were incubated in an ethanol-PBS system (1:10, v/v) for 4 h at 37 ℃. Images were obtained using excitation wavelength 530 nm, with the emission being monitored in the 600-650 nm spectral window.

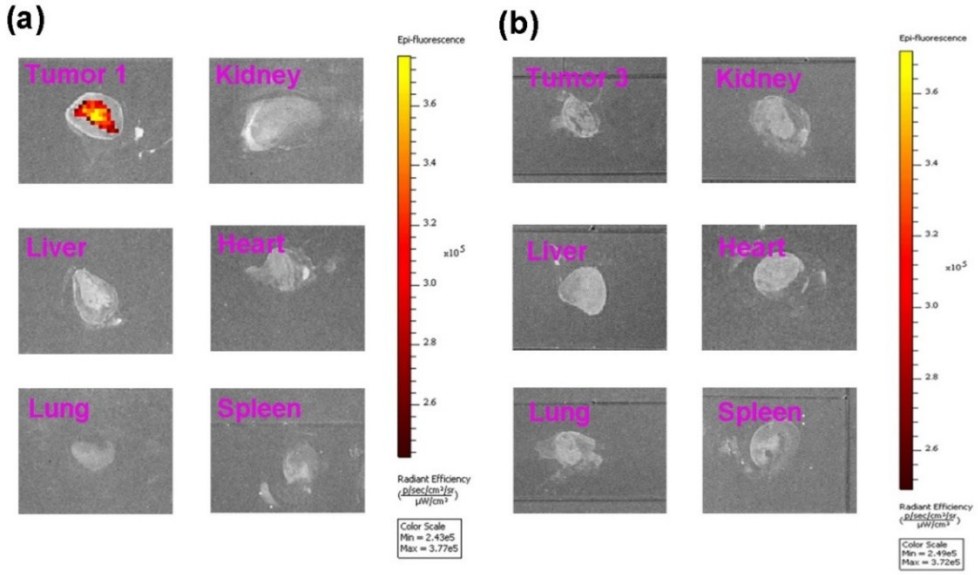


Fig. S20 (a) Fluorescence signal imaging of a slice of tumor 1 as well as slices from vital organs. (b) Fluorescence signal imaging of a slice of tumor 3 as well as slices from vital organs. Images were obtained using excitation wavelength 530 nm, with the emission being monitored over 600-650 nm regions.

Reference

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