Supporting Information

Assessment of cancer cell migration using a viscosity-sensitive fluorescent probe

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1. Experimental section

Materials and methods. 3-Hydroxy-4-methoxybenzaldehyde, isophoron, malononitrile and thiazolyl blue were purchased from Energy Chemical (Shanghai, China). Piperidine, tetrahydrofuran, anhydrous ethanol, dimethyl sulfoxide and acetonitrile were from Sinopharm Chemical Reagent (Shanghai, China). Resveratrol was from Dalin Meilun Biotecnology (Dalian, China). Rotenone and nystatin were from MedChemExpress. 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphnyl-2H-tetrazolium bromide (MTT) was from Sigma-Aldrich. ^{1}H NMR and ^{13}C NMR spectrum were taken on a nuclear magnetic resonance spectrometer (Advance 400 MHz, Bruker, Switzerland). Fluorescence spectra were carried out on a fluorescence spectrometer (Edinburgh Instruments, U.K.). High-resolution mass spectral analyses (HRMS) were recorded on a High-Resolution Quadrupole-time of flight mass spectrometer (Bruker, Germany). The fluorescence imaging was obtained with a confocal (LSM 880, Zeiss, Germany).

In vitro detection of viscosity. The stock solution of NV1 (2 mM) was prepared in DMSO. Stock solutions of various interferential reagents (K^+, Na^+, Ca^{2+}, Mg^{2+}, Fe^{3+}, Fe^{2+}, F^-, Br^-, I^-, DTT, GSH, Cys, Hcy, H_2O_2, ClO^-, HSO_3^-, S^{2-}) were prepared in double-distilled water. For spectral measurements, the stock solution of the NV1 probe was diluted with PBS buffer or glycerol to give a final concentration of 10 μM. Various analytes were added to PBS-glycerol mixed solutions, and then, the NV1 (10 μM) was added. The fluorescence emission spectra were recorded in a range from 450-750 nm (λex = 440 nm, λem = 590 nm).

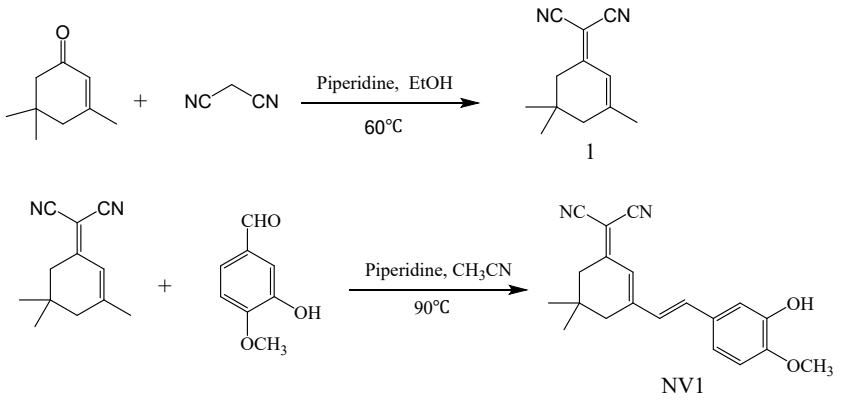
Cell culture. HepG2 and MDA-MB-231 cells were cultured in DMEM supplemented with 10% FBS and 1% antibiotics (100 U/mL penicillinand 100 mg/mL streptomycin) at 37°C in a humidified atmosphere of 95% air (20% O_2) and 5% CO_2. To simulate the hypoxic tumor microenvironment, cells were cultured in a mixture containing 94% N_2, 1% O_2 and 5% CO_2 using a low oxygen incubator (Bugbox M, Ruskinn, England).

Cytotoxicity assay of NV1. HepG2 cells (1×10^4 cells/well) were added to 96-well plates with 200 μL DMEM culture medium and incubated for 24 h at 37°C. Then the cells supernatant was removed and incubated with different concentrations of NV1 for 12 h. 20 μL MTT solution (5 mg/mL) was added into each well. After 4 h, the supernatant was discarded, and 100 μL dimethyl sulphoxide (DMSO) was added to each well to dissolve the formazan crystals. Absorbance was recorded at a wavelength of 490 nm with a microtiter plate reader.

Fluorescence imaging in living cells. The fluorescence of NV1 was detected by confocal microscopy. Briefly, HepG2 and MDA-MB-231 cells were seeded on glass-bottom culture dishes (15 mm). After treatment, the cells were incubated with 20 μM NV1 probe for 30 min in FBS-free DMEM medium at 37°C, and then, cells were washed three times with PBS buffer and imaged immediately using a confocal microscope. NV1 probe was excited at 405 nm and the fluorescence emission range was set from 450-750 nm.

Cell migration detection. HepG2 and MDA-MB-231 cells were diluted with serum-free DMEM medium to 10×10^4/mL. Added 600 μL serum-containing DMEM medium to 24-well plate, and gently placed the Transwell chamber into it. 200 μL cell suspension was added into the upper chamber and cultured for 24 h. Then, the upper chamber medium was abandoned, and new serum-free medium or different concentrations of resveratrol were added, respectively, and cultured under hypoxia or normoxia for 24 h. After the incubation time was over, taken out the Transwell chamber and added 1 mL of methanol to the lower chamber to fix for 15-20 min. Then, washed with PBS for 3 times, and added 0.1% crystal violet to stain for 15-20 min. After soaking the cells in PBS for 3-5 times, the membrane penetration of the cells was observed.

2. Synthesis of probe NV1



Scheme S1. Synthesis of NV1

Synthesis of compound 1. Malononitrile (0.33 g, 5 mmol) and isophoron (0.75 mL, 5 mmol) were added into 10 mL anhydrous ethanol respectively to form a solution, then, the catalytic amount of piperidine was dropped, and the solution reacted at 60°C for 8 h. After cooling to room temperature, the precipitate was filtered to give compound 1 (0.67 g, yield: 72%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 6.55 (d, J = 1.3 Hz, 1H), 2.53 (s, 2H), 2.23 (s, 2H), 2.04 (d, J = 0.9 Hz, 3H), 0.95 (s, 6H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 170.88, 161.96, 118.80, 112.88, 112.08, 75.39, 44.20, 41.36, 31.37, 26.68, 24.37; HRMS: Calculated for [$C_{12}H_{14}N_2$], [M–H]$^-$: 185.1073, found 185.1058.

Synthesis of NV1. Compound 1 (200 mg, 1.1 mmol) and 3-Hydroxy-4-methoxybenzaldehyde (170 mg, 1.1 mmol) were dissolved in 5 mL anhydrous ethanol, catalytic amount of piperidine was added and the solution was refluxed for 5 h at 90°C. After cooling to room temperature, the solvent was removed by rotary evaporation, and the crude product was separated and purified by silica gel column (dichloromethane: methanol = 200:1) and vacuum dried for 12 h. Finally, the purified product NV1 was obtained (172 mg, yield: 48%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.11 (s, 1H), 7.15 (d, J = 14.3 Hz, 4H), 6.97 (s, 1H), 6.82 (s, 1H), 3.81 (s, 3H), 2.58 (s,

2H), 1.01 (s, 6H).; ^{13}C NMR (101 MHz, DMSO-d_6) δ 170.75, 156.92, 150.03, 147.14, 138.65, 129.49, 127.61, 122.20, 121.18, 114.63, 114.56, 113.71，112.51, 75.60, 56.11, 42.80, 38.67, 32.12, 27.92. HRMS: Calculated for [$C_{20}H_{20}N_2O_2$], [M–H]$^-$: 319.1441, found 319.1378.

3. Supplemental figures

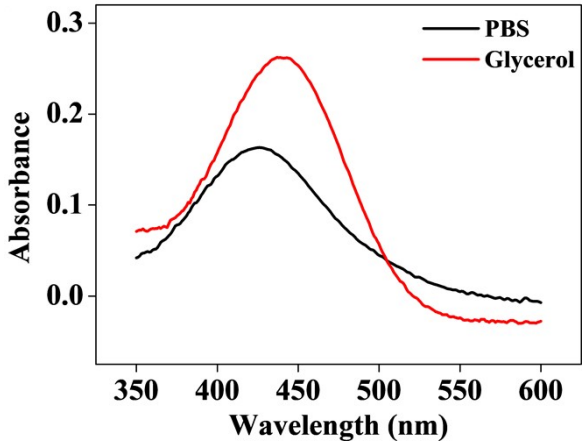


Figure S1. Uv-vis absorption spectra of NV1 (10 μM) in PBS and glycerol.

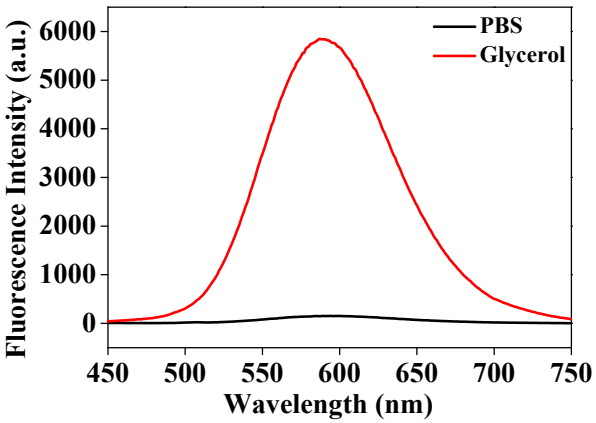


Figure S2. Fluorescence emission spectra of NV1 (10 μM) in PBS and glycerol.

Table S1 Dielectric constant and viscosity of different polar solvents

Solvents	EA	THF	DCM	EtOH	MeOH	ACN	Gly	H_2O
Dielectric constant	6.02	7.58	9.1	25.7	31.2	37.5	42.5	80.4
Viscosity (cP)	0.45	0.55	0.44	1.07	0.6	0.37	600	1

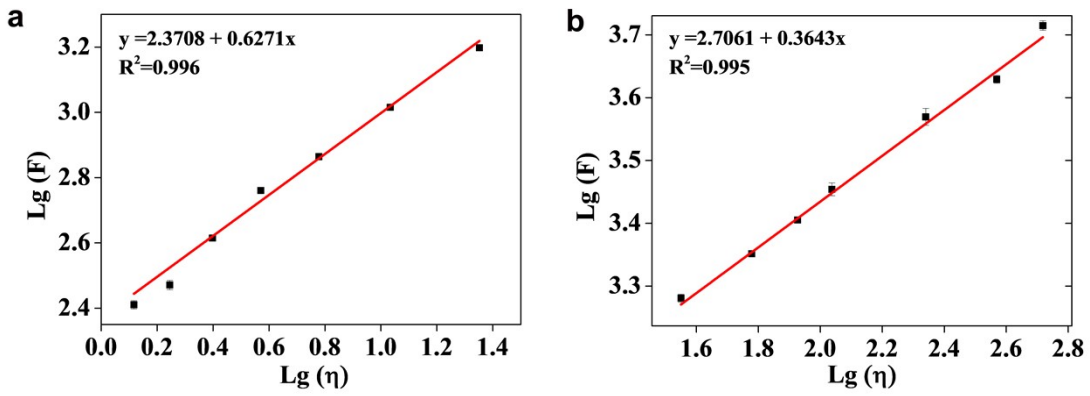


Figure S3. (a) The linear relationship between fluorescence intensity Lg (F) and viscosity Lg (η) of NV1 at glycerin ratio of 0%-70%. (b) The linear relationship between fluorescence intensity Lg (F) and viscosity Lg (η) of NV1 at glycerin ratio of 70%-99%. λ_{ex} = 440 nm, λ_{em} = 590 nm.

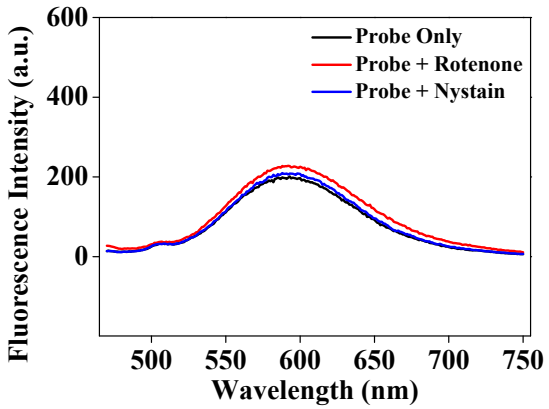


Figure S4. Fluorescence emission spectra of NV1 (10 μM) coexisting with rotenone (10 μM) or nystatin (10 μM). λ_{ex} = 440 nm, λ_{em} = 590 nm.

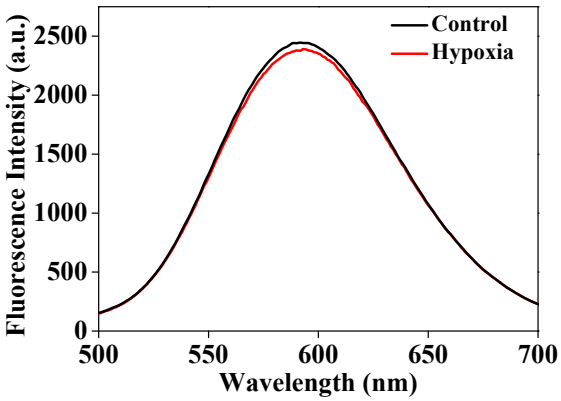


Figure S5. Fluorescence emission spectra of the NV1 (10 μM) under normoxic or hypoxic conditions in 80% glycerol system. λ_{ex} = 440 nm, λ_{em} = 590 nm.

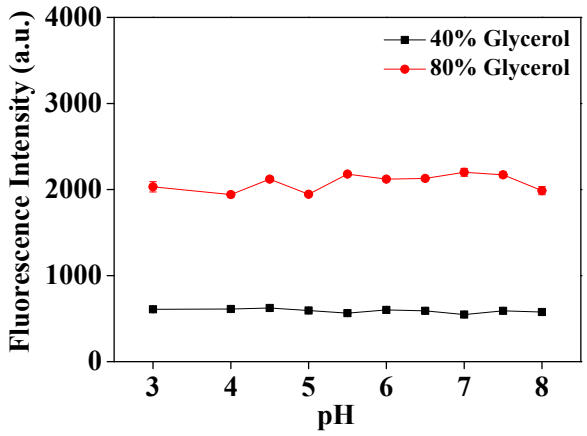


Figure S6. Fluorescence intensity changes of NV1 (10 μM) at different pH values in the low viscosity solution (40% glycerol, black) or high viscosity solution (80% glycerol, red). λ_{ex} = 440 nm, λ_{em} = 590 nm.

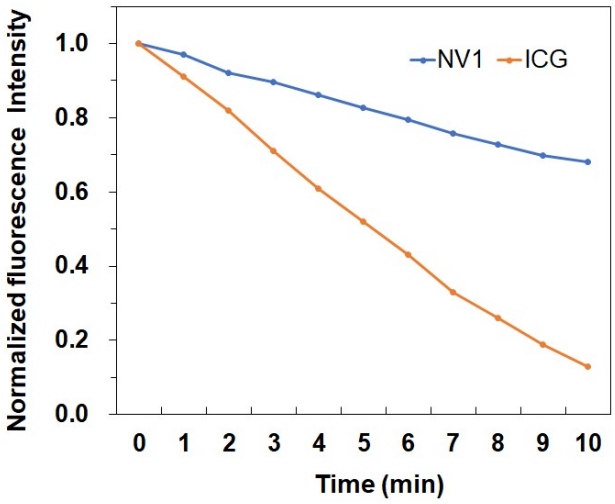


Figure S7. Time-dependent fluorescence intensity of NV1 (10 μM, λ_{ex} = 440 nm, λ_{em} = 590 nm) and ICG (10 μM, λ_{ex} = 780 nm, λ_{em} = 800 nm) under continuous laser irradiation for 10 min.

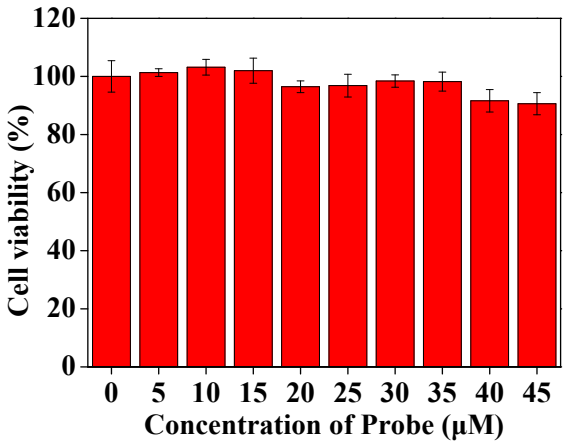


Figure S8. Cell viability estimated by MTT assay. HepG2 cells were incubated with 0-45 μM NV1 for 12 h, then the cell viabilities were determined by an MTT assay.

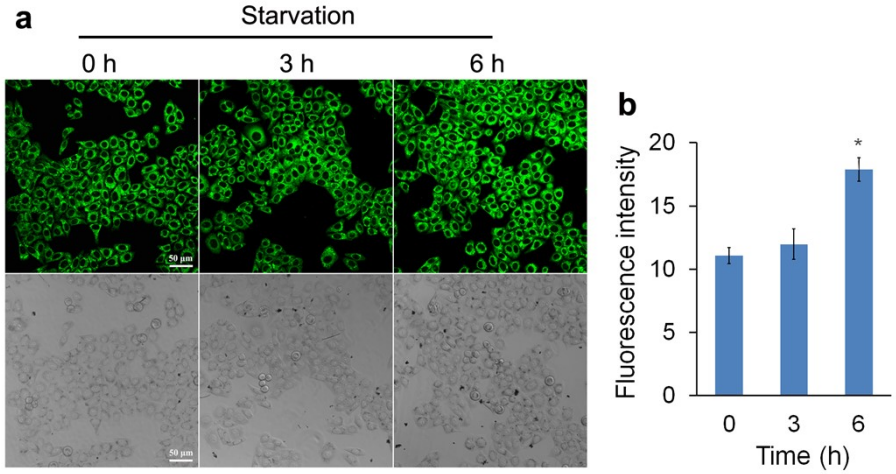


Figure S9. Fluorescence imaging of viscosity in living cells after starvation. HepG2 cells were cultured in a serum-deficient cell culture medium for 3-6 h, and then incubated with 20 μM of the NV1 probe for 30 min. (a) The fluorescence images were obtained using a confocal microscopy. (b) The fluorescence intensity was quantified based on the results of the relative fluorescence intensity of per cell in the scanned area. The scale bar in all fluorescence images of cells is 50 μm. (*P<0.05)

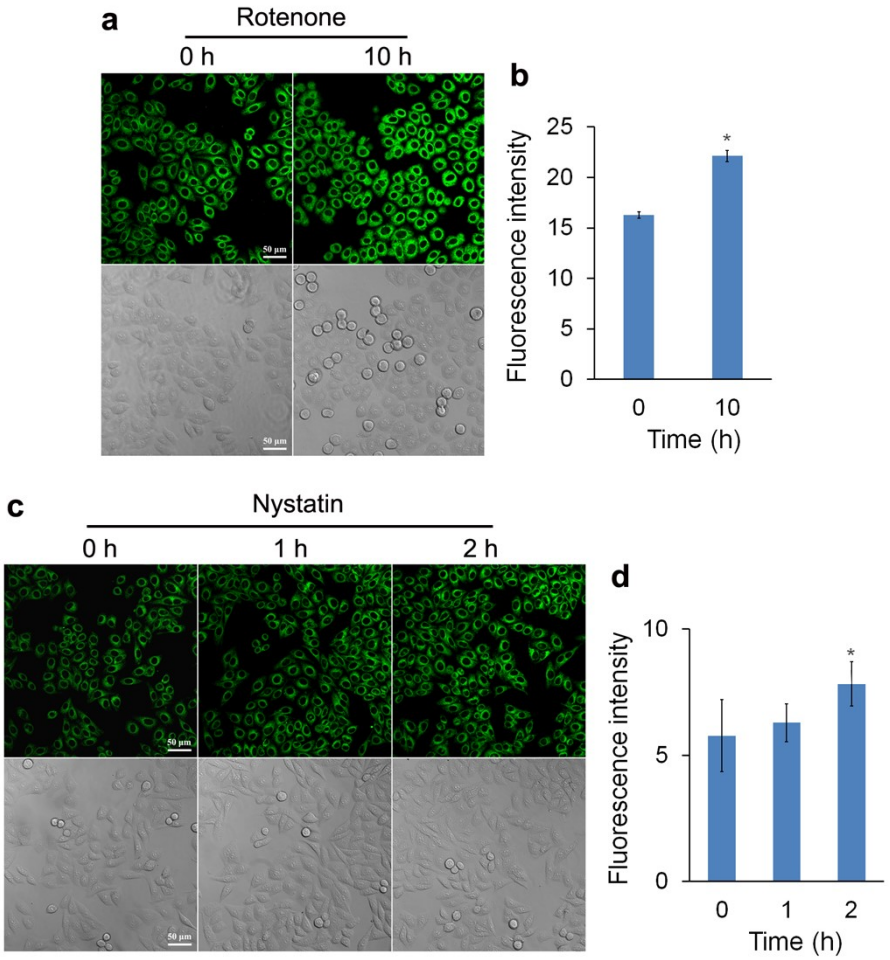


Figure S10. Fluorescence imaging of intracellular viscosity in living cells after treatment with rotenone and nystatin. (a) and (b) HepG2 cells were treated with rotenone (10 μM) for 10 h, and then incubated with 20 μM of the NV1 probe for 30 min. (c) and (d) HepG2 cells were treated with nysta-tin (10 μM) for 1-2 h, then 20 μM of the NV1 was added for 30 min. The fluorescence images were obtained using confocal microscopy (a) and (c). The fluorescence intensity was quantified (b) and (d). The scale bar in all fluorescence images of cells is 50 μm. (*P<0.05)

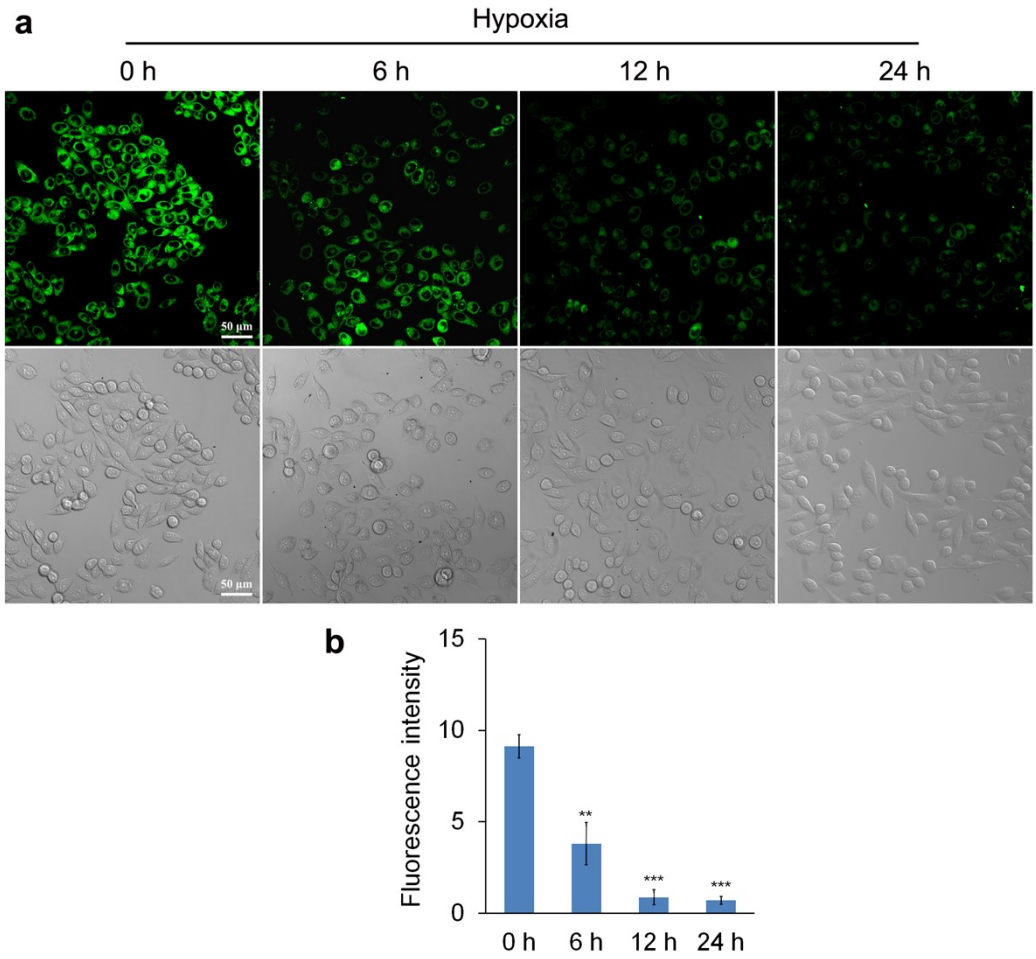


Figure S11. MDA-MB-231 cells were cultured under hypoxia (1% O_2) for 0-24 h, and then incubated with 20 μM of the NV1 probe for 30 min. (a) The fluorescence images were obtained using confocal microscopy. (b) The fluorescence intensity was quantified based on the results of the relative fluorescence intensity of per cell in the scanned area. The scale bar in all fluorescence images of cells is 50 μm. (**P<0.01, ***P<0.001)

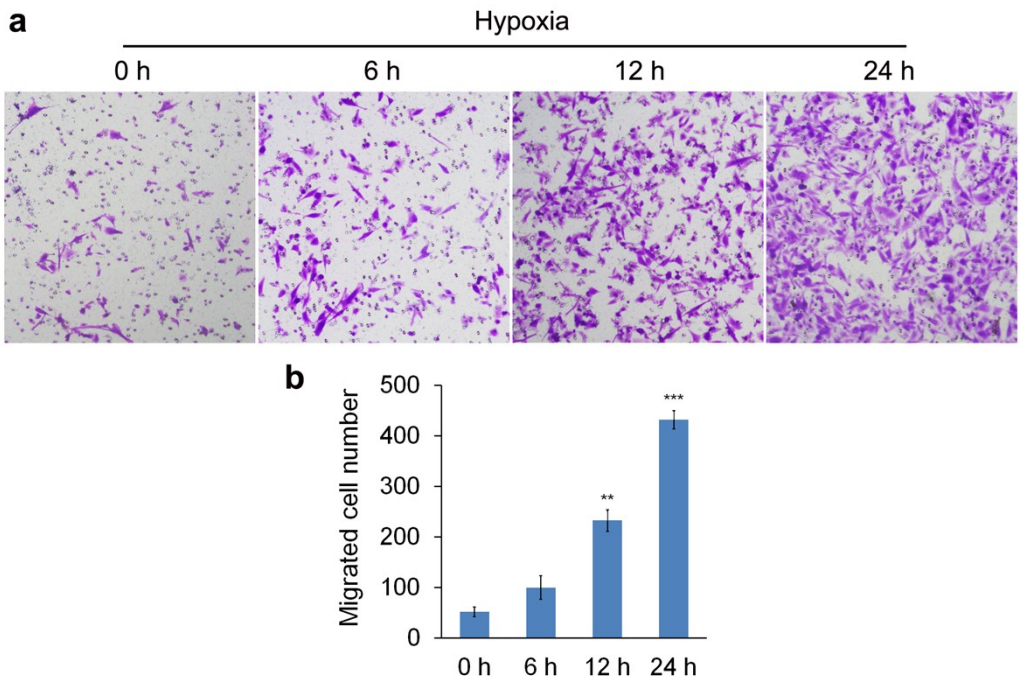


Figure S12. MDA-MB-231 cells were seeded in the Transwell plate and cultured under hypoxia (1% O_2) for 0-24 h, then, the migrated cells were observed under a microscope (a). The migrated cell number was quantified based on the average number of cells in six randomly selected regions (b). All images magnified 200×. (**P<0.01, ***P<0.001)

4. Spectra of compound 1 and NV1

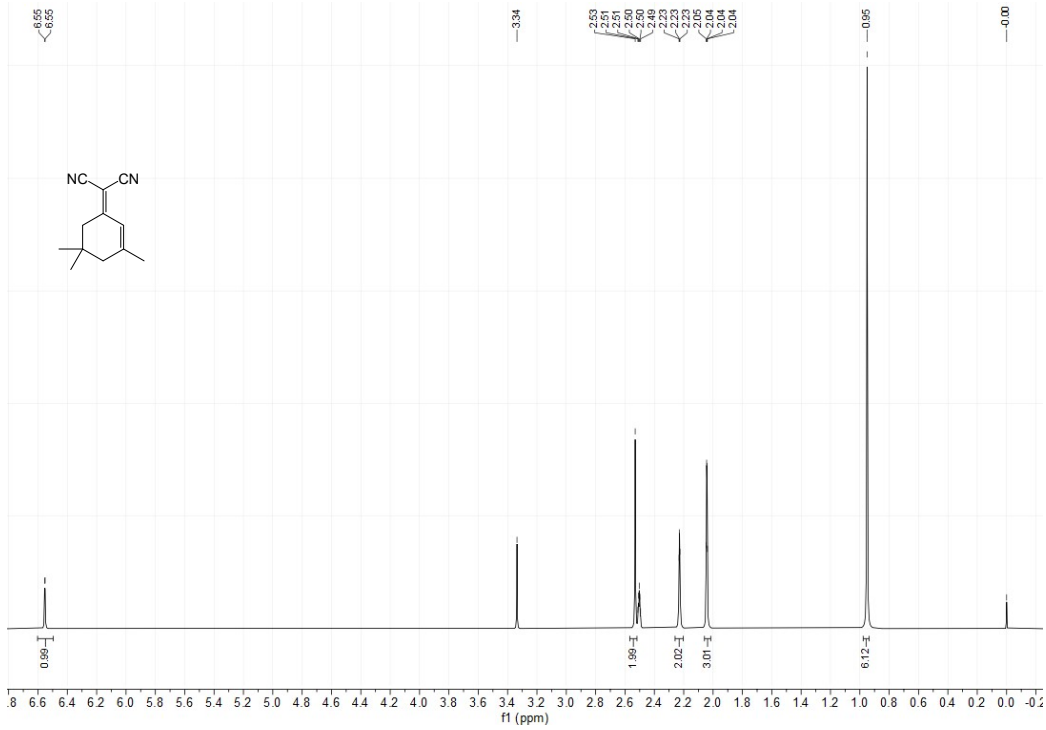


Figure S13. ^{1}H NMR spectra of compound **1**.

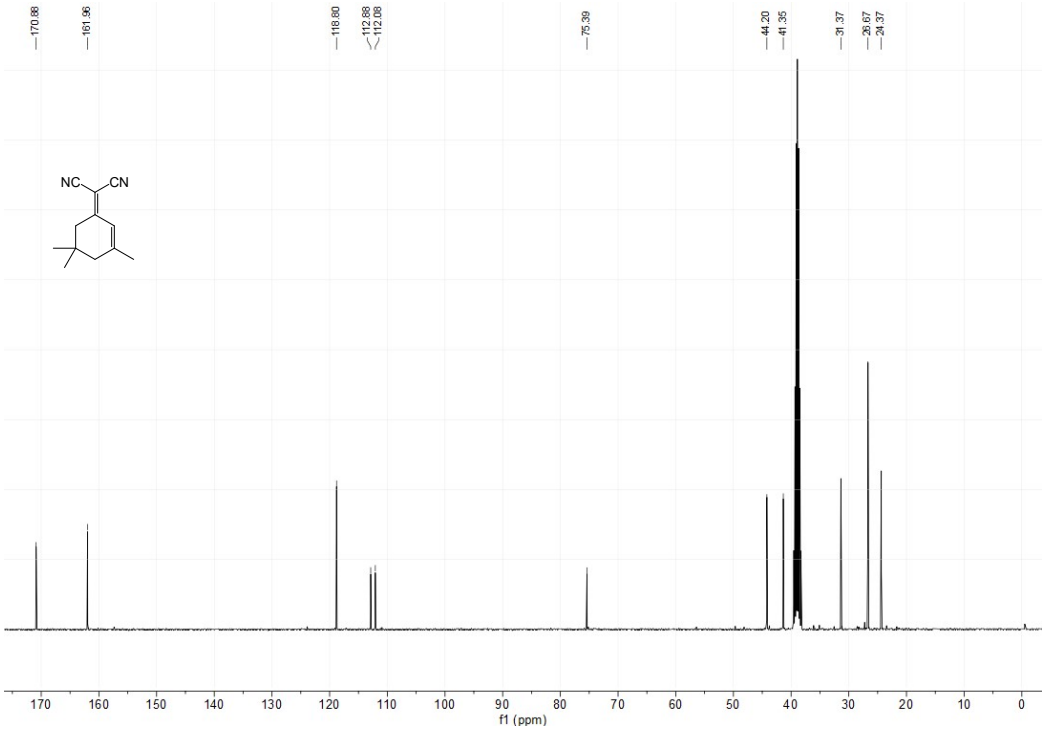


Figure S14. ^{13}C NMR spectra of compound **1**.

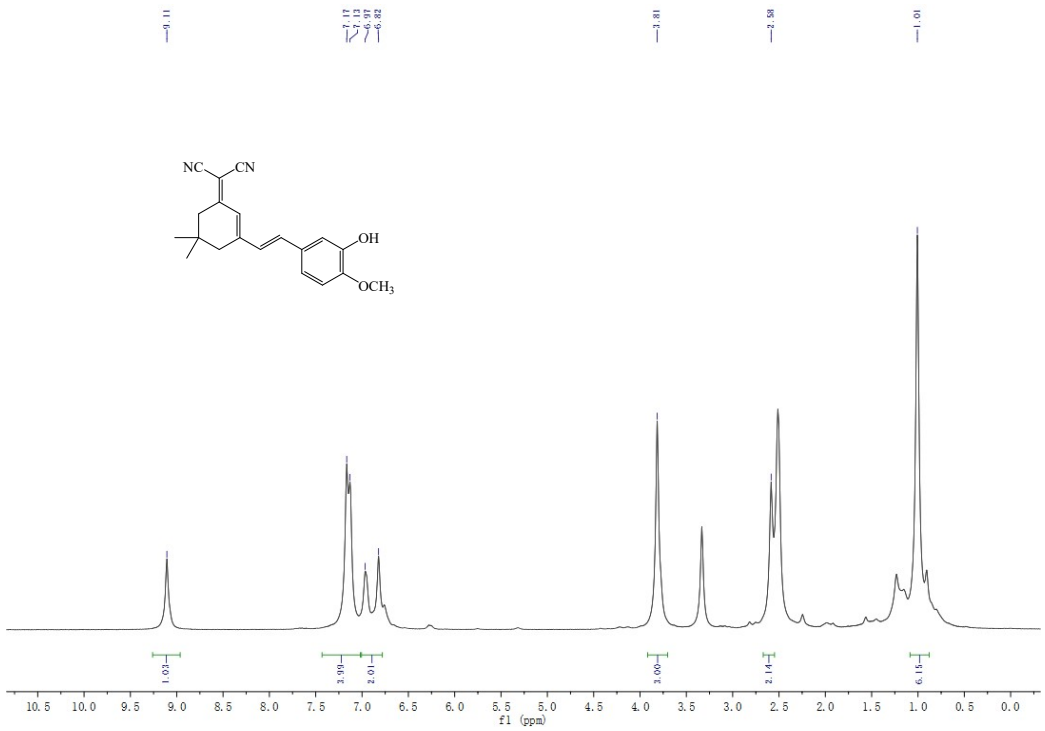


Figure S15. ^{1}H NMR spectra of NV1.

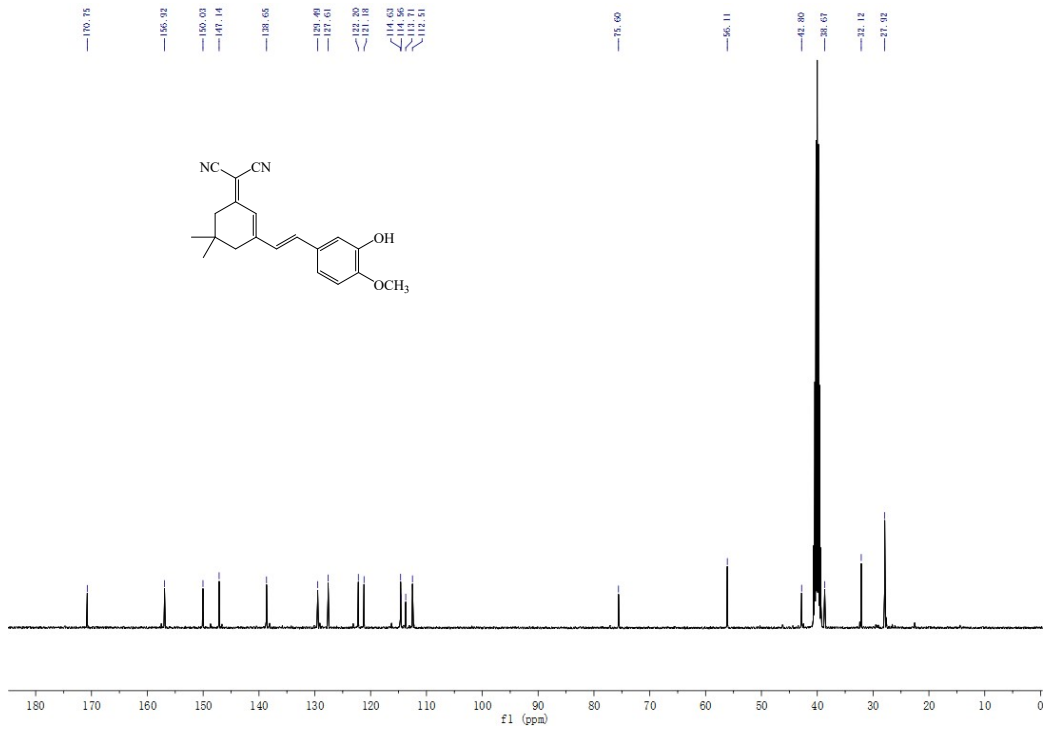


Figure S16. ^{13}C NMR spectra of NV1.

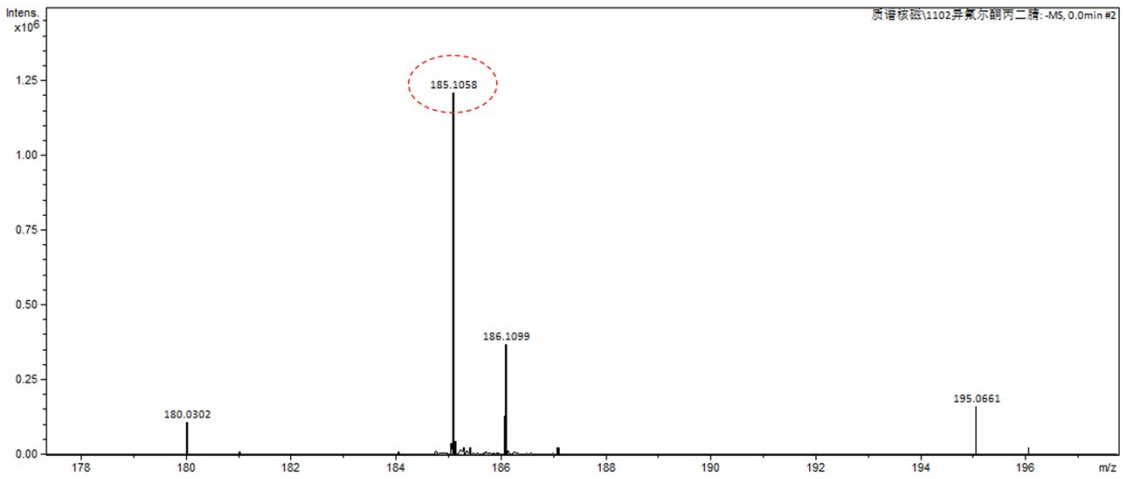


Figure S17. High resolution mass spectrometry of compound **1**.

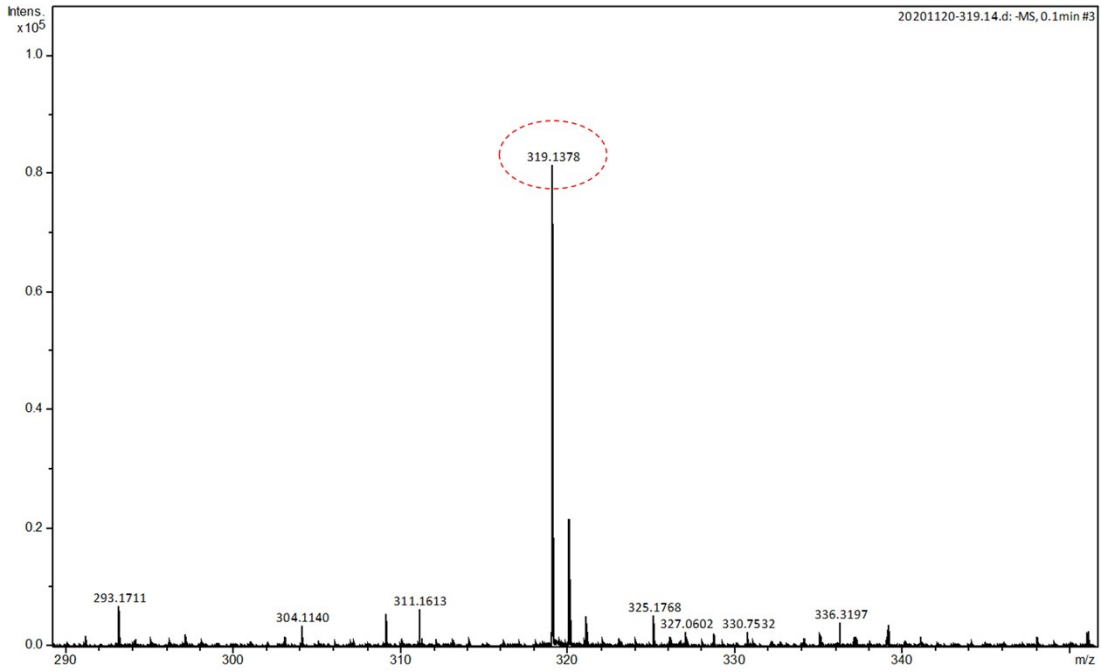


Figure S18. High resolution mass spectrometry of NV1.