Supporting Information for

HClO activated endoplasmic reticulum fluorescent probe for visualization of ferroptosis-mediated acute alcoholic gastric and liver injury

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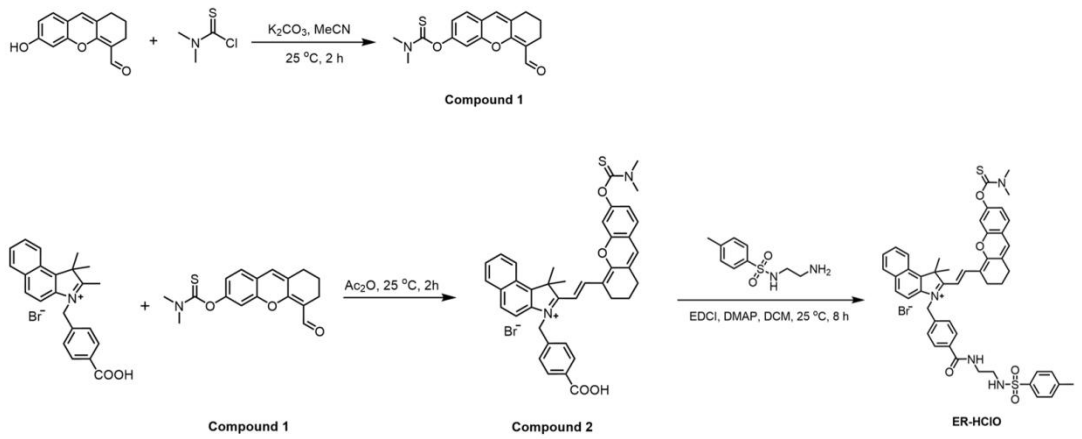
Experimental section

Materials and apparatus

6-Hydroxy-2, 3-dihydro-1H-xanthene-4-carbaldehyde, 3-(4-carboxybenzyl)-1, 1, 2-trimethyl-1H-benzo[e]indol-3-ium bromide were purchased from Jilin Chinese Academy of Sciences - Yanshen Technology Co., Ltd. Dimethylthiocarbamoyl chloride, N-(2-aminoethyl)-4-methylbenzenesulfonamide, 4-dimethylaminopyridine, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide and other chemicals were purchased from Sigma-Aldrich Co., Ltd. ER-Tracker Green was commercially available from Invitrogen. Lipopolysaccharide (LPS), N-acetylcysteine (NAC), tunicamycin (TM), erastin, ferrostatin-1 (Fer-1), cimetidine (CMTD), and silymarin (SM) were purchased from Shanghai Aladdin Biochemical Technology Co., Ltd.

NMR spectra were acquired with a Bruker instrument (BrukerBioSpin AG Ltd., Beijing, China) of 400 MHz for ^{1}H NMR and 101 MHz for ^{13}C NMR, respectively. HR-MS was measured by an Agilent Accurate-Mass-Q-TOF MS 6520 system equipped with an electrospray ionization (ESI) source (Agilent, USA). 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. A Phs-3c pH meter (Germany Sartorius) was used to adjust the pH value. The UV-visible absorption spectra were taken on a TU-1901 double-beam UV-vis spectrophotometer (Beijing Purkinje General Instrument Co., LTD, Beijing, China). Fluorescence spectra 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. Cells fluorescence images were captured on a confocal laser scanning microscope (Zeiss, LSM880, Germany). *In vivo* images were taken by using a PerkinElmer IVIS Lumina LT III *in vivo* imaging system.

Synthesis routine of ER-HClO



Scheme S1. The synthesis routine of **ER-HClO**.

Synthesis of Compound 1. 6-hydroxy-2,3-dihydro-1H-xanthene-4-carbaldehyde (427 mg, 1.87 mmol) and K_2CO_3 (776 mg, 5.621 mmol) were dissolved in MeCN (20 mL) and reacted for 0.5 h in a round-bottomed flask. After that, dimethylthiocarbamoyl chloride (694 mg, 5.612 mmol) was added into the reaction mixture for stirring 2 h at room temperature. After monitoring the reaction process by thin-layer chromatography (TLC), H_2O (20 mL) was added, and the mixture was extracted with CH_2Cl_2 (100 mL × 3). The organic phases were combined, dried over anhydrous sodium sulfate, and concentrated. The crude product was then purified by silica gel column chromatography (PE/CH_2Cl_2 = 1:1, v/v) to obtain compound 1 as a yellow solid (140 mg, 24%). ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 10.23 (d, J = 2.2 Hz, 1H), 7.37 (dq, J = 6.3, 2.7 Hz, 1H), 7.05 – 6.97 (m, 2H), 6.92 – 6.85 (m, 1H), 3.37 – 3.35 (s, 3H), 3.32 (s , 3H), 2.59 (d, J = 6.4 Hz, 2H), 2.29 (d, J = 6.4 Hz, 2H), 1.64 (q, J = 6.2 Hz, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ (ppm): 186.72, 185.76, 159.37, 154.58, 151.44, 128.57, 127.21, 126.34, 118.86, 118.58, 112.50, 110.34, 42.85, 29.06, 21.10, 19.77.

Synthesis of Compound 2. 3-(4-carboxybenzyl)-1,1,2-trimethyl-1H-benzo[e]indol-3-ium bromide (242 mg,

0.571 mmol) and compound 1 (90 mg, 0.286 mmol) were dissolved in acetic anhydride (10 mL). After reaction for 2 h at room temperature, the solvent was evaporated, and the residue was purified by silica gel column chromatography (CH_2Cl_2/MeOH = 25:1, v/v) to obtain compound 2 as a blue solid (174 mg, 84% yield). ^{1}H NMR (400 MHz, CD_3OD) δ (ppm): 8.82 (d, J = 15.0 Hz, 1H), 8.31 (d, J = 8.5 Hz, 1H), 8.15 – 8.01 (m, 4H), 7.76 (t, J = 7.7 Hz, 1H), 7.64 (d, J = 8.2 Hz, 2H), 7.52 (d, J = 8.2 Hz, 1H), 7.41 (d, J = 8.0 Hz, 2H), 7.36 (s, 1H), 7.10 (s, 1H), 6.54 (d, J = 14.9 Hz, 1H), 5.83 (s, 2H), 3.52 (s, 3H), 3.47 (s, 3H), 2.79 (t, J = 6.1 Hz, 2H), 2.62 (t, J = 6.0 Hz, 2H), 2.17 (s, 6H), 1.94 (p, J = 6.1 Hz, 2H). ^{13}C NMR (101 MHz, CD_3OD) δ (ppm): 187.12, 180.74, 161.98, 156.95, 153.65, 146.52, 139.64, 138.13, 136.32, 133.73, 133.00, 132.21, 131.47, 130.86, 130.51, 129.01, 128.72, 128.38, 127.20, 126.82, 122.99, 121.73, 120.48, 115.90, 112.00, 111.53, 104.81, 54.08, 53.42, 43.68, 39.34, 29.69, 28.20, 24.51, 20.71.

Spectral response of ER-HClO to HClO

ER-HClO was dissolved in DMSO to prepare a stock solution (2.0 mM). The spectroscopic responses of **ER-HClO** to HClO (final concentration: 10 μM) were tested in PBS/CH_3CN (v/v = 3/7, pH 7.4, 10 mM) mixtures, in which 10 μL of the **ER-HClO** stock solution was added in 1990 μL of a mixture of PBS/CH_3CN (7/3, v/v, pH 7.4). The spectral data were recorded with $\lambda_{ex}/\lambda_{em}$ = 710/733 nm, and slit widths of 2.5 nm (both excitation and emission).

Preparation of ROS/RNS

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. H_2O_2 was generated by dilution of a 30% H_2O_2 solution in deionized water. For this purpose, hydrogen peroxide was added to a solution of Ferrous sulfate in deionized water. Peroxynitrite solution ($ONOO^-$) was synthesized as the previous report (*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. 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.

Cell culture and cell cytotoxicity assay

Human cervical cancer cells (HeLa) and iving HT22 cells were kindly provided by Modern Research Center for Tradition Chinese (Shanxi University, Taiyuan, China). All the cell lines were cultured in DMEM (Dulbecco's modified eagle medium) supplemented with 10% fetal bovine serum and 1% antibiotics at 37°C in a 5% CO_2 atmosphere.

The cell cytotoxicity of **ER-HClO** to living HT22 cells was performed by a standard MTT assay. About 1×10^4 cells/well in 200 μL cell culture medium was seeded in 96-well microplate and then the medium was replaced with fresh medium that containing **ER-HClO** with various concentrations of 1, control; 2, 0.1 μM; 3, 0.5 μM; 4, 1 μM; 5, 2 μM; 6, 5 μM; 7, 8 μM; 8, 10 μM for 3 h, respectively. Six replicate wells were used for each control and test concentration. After washing the cells with fresh medium three times, 20 μL MTT in 180 μL medium was loaded to each well for another 4 h. The MTT solution was discarded, 200 μL DMSO were added to each well and oscillated at a low speed on a shaker for 10 minutes. Then each well was analyzed with an ELISA microplate reader and the absorbance was detected at 570 nm. The cell viability was expressed as relative to the control cells taken as 100% metabolic activity.

$$\text{Cell viability (\%)} = (A_{\text{with probe}} - A_{\text{blank}}) / (A_{\text{control}} - A_{\text{blank}}) \times 100\%.$$

Cell imaging experiment

For co-localization imaging. HeLa cells were co-incubated with **ER-HClO** (5 μM) and ER-Tracker Green (0.5 μM) for 30 min. The fluorescence images were obtained on a confocal laser scanning microscope with a green channel (λ_{ex} = 488 nm, λ_{em} = 450 nm ~ 550 nm) for ER-Tracker Green; a red channel (λ_{ex} = 561 nm, λ_{em} = 650 nm ~ 750 nm) for **ER-HClO**, respectively.

For imaging of HClO in living cells. HeLa cells were incubated with

ER-HClO (5 μM) for 30 min as control; pretreated with HClO (20 μM) for 60 min and then incubated with **ER-HClO** (5 μM) for another 30 min; pretreated with H_2O_2 (100 μM) for 60 min and then incubated with **ER-HClO** (5 μM) for another 30 min; pretreated with LPS (1mg/mL) for 60 min, then incubated with **ER-HClO** (5 μM) for 30 min; pretreated with NAC (1 mM) for 30 min, LPS (1mg/mL)for 60 min, then incubated with **ER-HClO** (5 μM) for 30 min. HeLa cells were incubated with **ER-HClO** (5 μM) for 30 min as control; pretreated with TM (10 μM) for 30 min and 60 min, respectively, then incubated with **ER-HClO** (5 μM) for 30 min. HeLa cells were incubated with **ER-HClO** (5 μM,) for 30 min as control; pretreated with erastin (10 μM) for 60 min, then incubated with **ER-HClO** (5 μM) for 30 min; pretreated with Fer-1 (10 μM) for 60 min, erastin (10 μM) for 60 min, then incubated with ER-HClO (5 μM) for 30 min. **ER-HClO** channel: λ_{ex} = 561 nm, λ_{em} = 650-750 nm. Laser power: 0.8 W

For imaging of HClO in zebrafish. Zebrafish were incubated with **ER-HClO** (5 μM) as control; pretreated with erastin (10 μM) for 60 min, then incubated with **ER-HClO** (5 μM, 30 min); pretreated with Fer-1 (5 μM) for 60 min, erastin (10 μM) for 60 min, then incubated with **ER-HClO** (5 μM) for 30 min.**ER-HClO** channel: λ_{ex} = 561 nm, λ_{em} = 650 nm ~ 750 nm. Laser power: 0.8 W

Animal and Disease model imaging experiment

Six-week-old female Kunming (KM) mice were supported by the Laboratory Animal Centre of Shanxi Cancer Hospital. All the animal experiments involved in this work were approved by the Committee of Scientific Research in 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 effort to reduce animal suffering from pain and discomfort.

Construction of acute alcoholic gastric injury (AAGI) and liver injury (AALI) mice model and fluorescence imaging. The model mice (e.g., AAGI and AALI)

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. The treatment group mice for AALI were administered intragastrically with silymarin (SM, 100 mg/kg) or Fer-1 (5 mg/kg) at 2:30 pm for 3 consecutive days. After sacrificing the mice, stomachs and livers were extracted, preserved in 10% paraformaldehyde solution, and encased in paraffin, The stomachs and liver slices were examined underan optical microscope after being stained with hematoxylin-eosin (H&E).

For AAGI in vivo imaging. The control group, AAGI group, CMTD group and Fer-1 group were injected with **ER-HClO** (200 μM, 200 μL) via tail vein, and then subjected to *in vivo* imaging after 10 min. In addition, the control mice were euthanized the abdomen was dissected for fluorescence imaging, so as to observe the differences in fluorescence intensity among tissues and organs. **ER-HClO** channel: λ_{ex} = 690 nm, λ_{em} = 720 nm. Exposure time: Auto.

For AALI ex vivo imaging. The control group, AAlI group, SM group and Fer-1 group were injected with **ER-HClO** (200 μM, 200 μL) via tail vein. After for 10 min, the mice were euthanized and the hearts, livers, spleens, lungs and kidneys were isolated for *ex vivo* imaging. **ER-HClO** channel: λ_{ex} = 690 nm, λ_{em} = 720 nm. Exposure time: Auto.

^{1}H NMR and ^{13}C NMR spectra of compound 1, compound 2, ER-HClO.

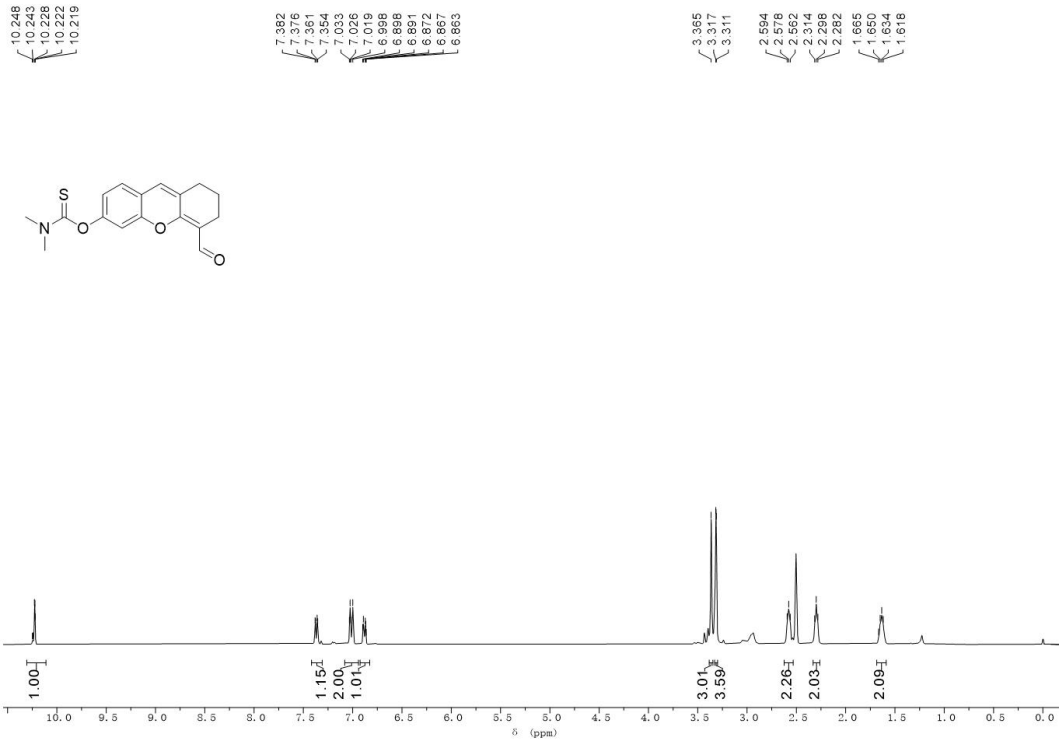


Fig. S1 ^{1}H NMR spectrum of compound 1 in DMSO-d_6.

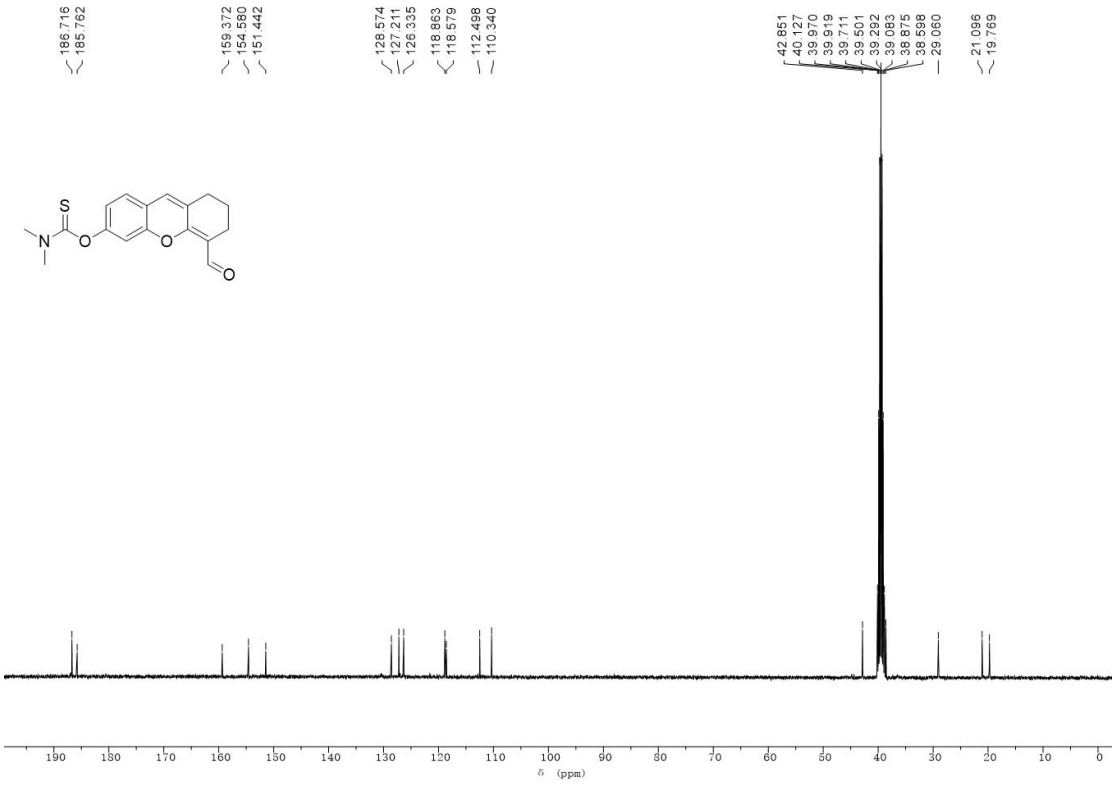


Fig. S2 ^{13}C NMR spectrum of compound **1** in DMSO-d_6.

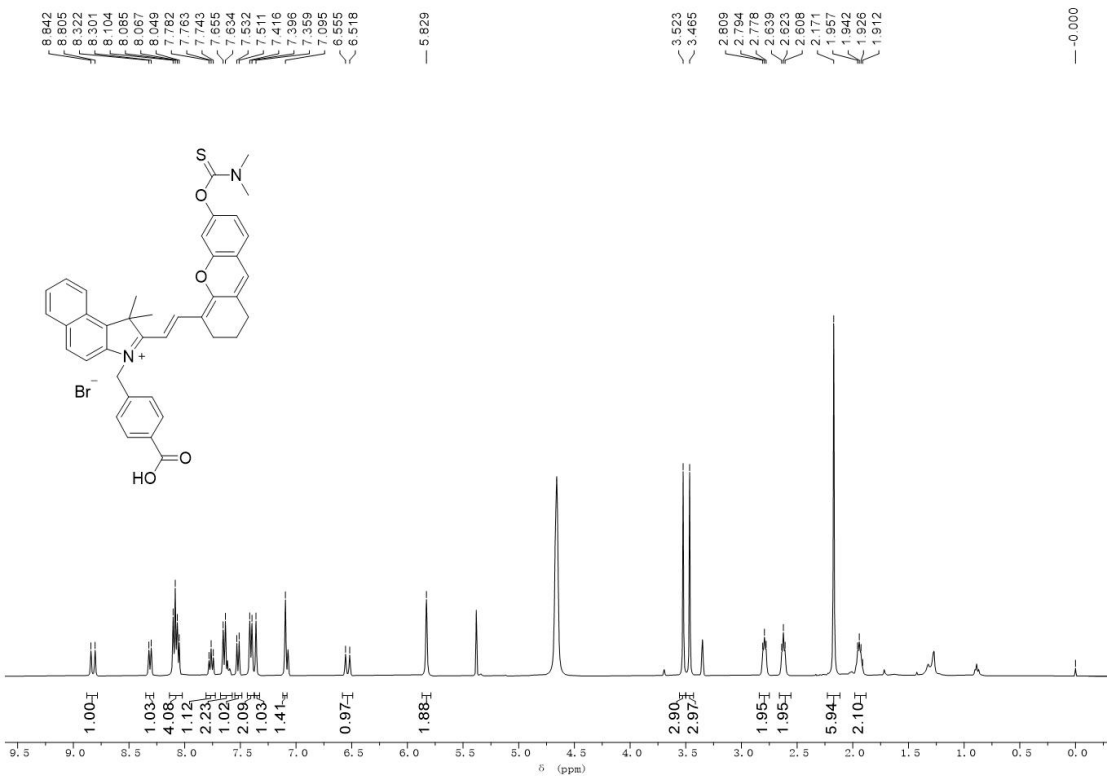


Fig. S3 ^{1}H NMR spectrum of compound 2 in CD_3OD.

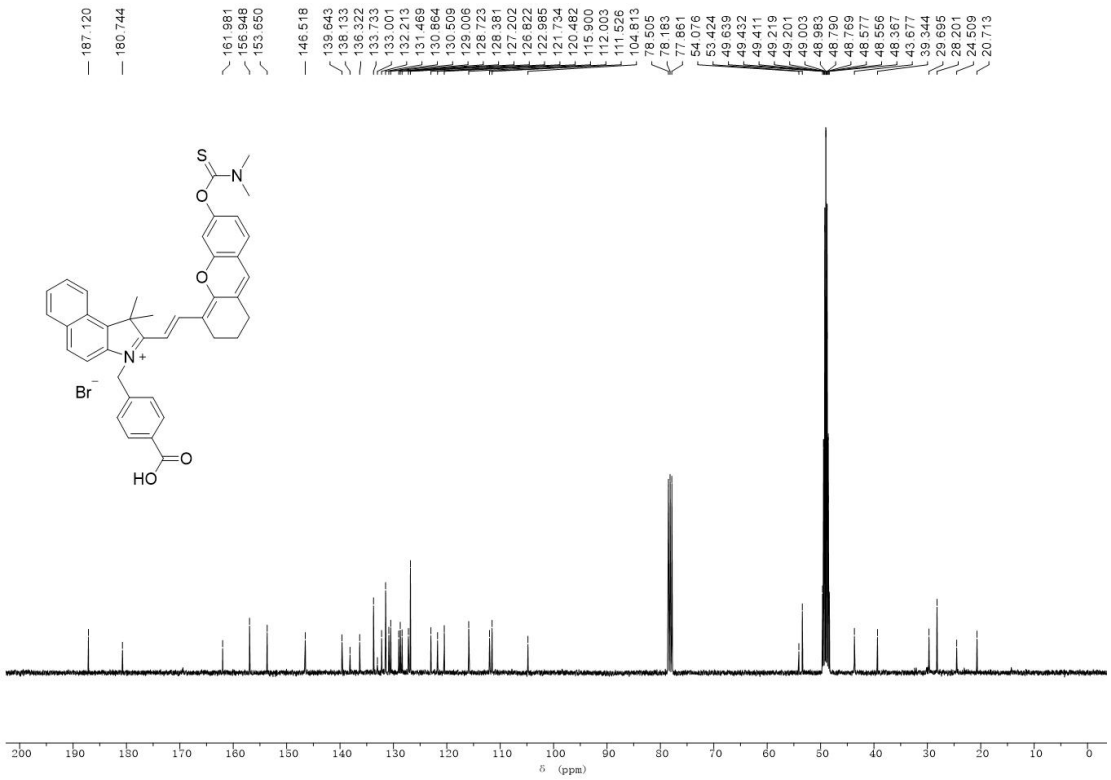


Fig. S4 ^{13}C NMR spectrum of compound 2 in CD_3OD.

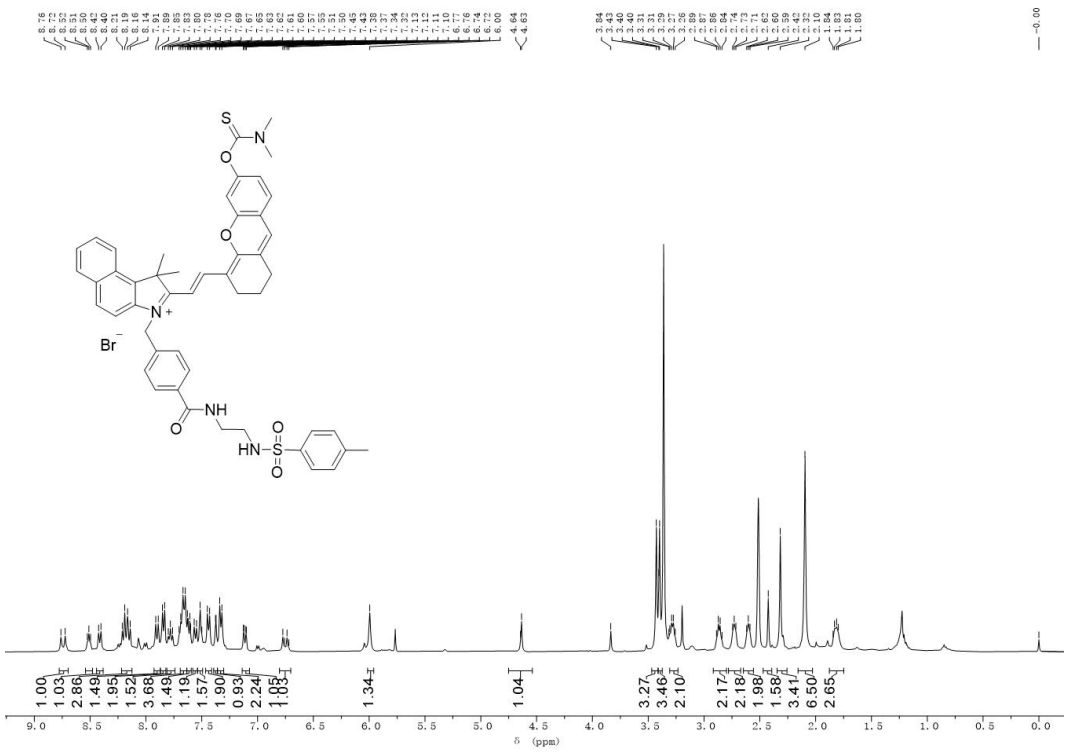


Fig. S5 ^{1}H NMR spectrum of ER-HClO in DMSO-*d*$_6$.

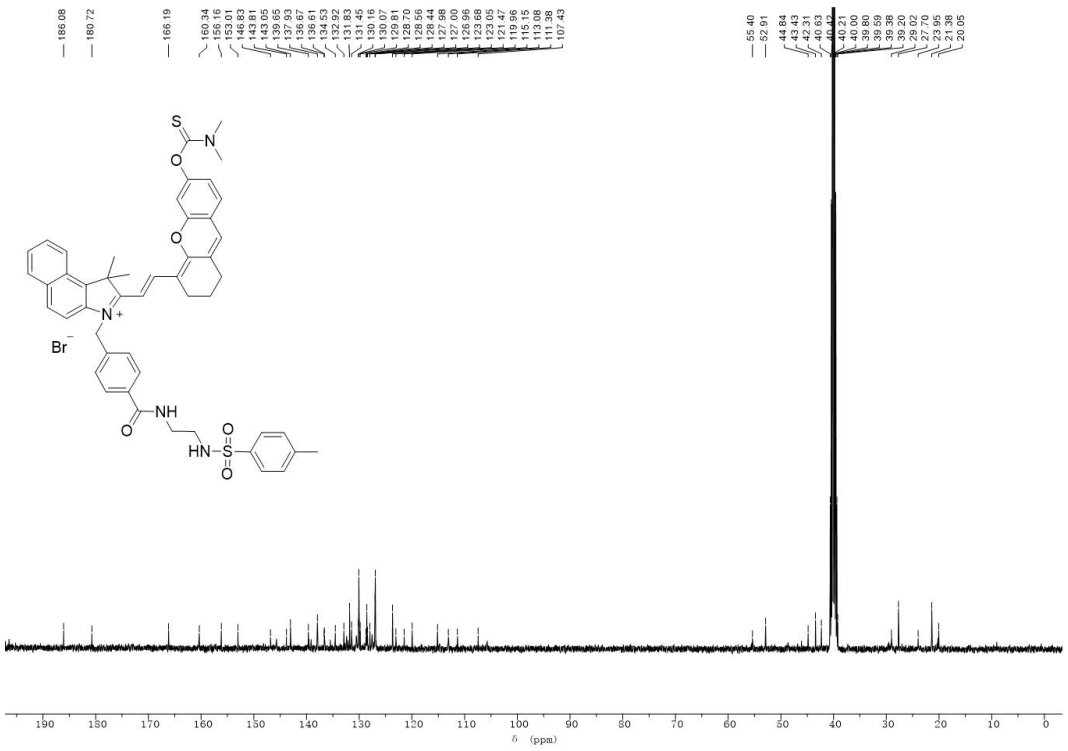


Fig. S6 ^{13}C NMR spectrum of ER-HClO in DMSO-*d*$_6$.

HR-MS spectra of ER-HClO in the presence of HClO

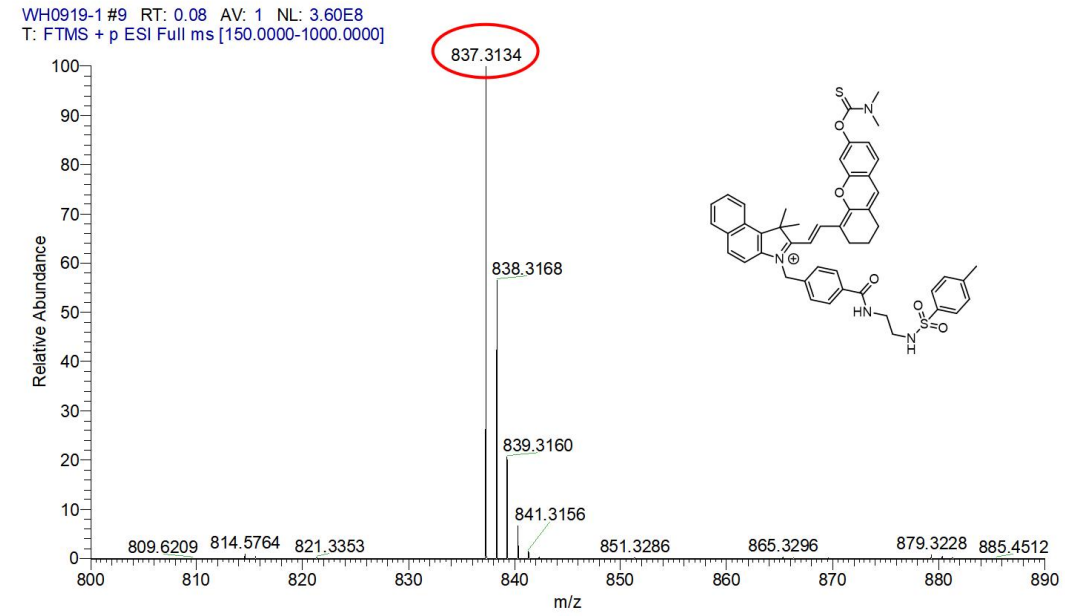


Fig. S7 HR-MS spectrum of ER-HClO.

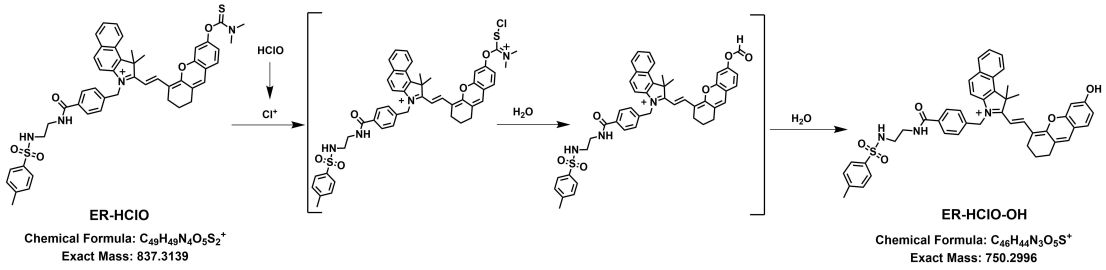


Fig. S8 Schematic illustration of ER-HClO in response to HClO.

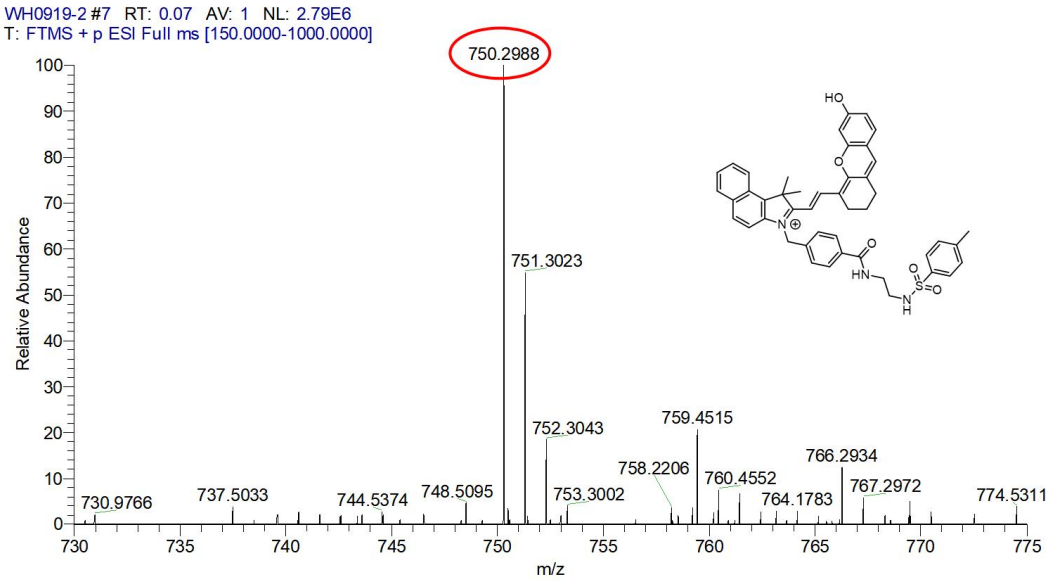


Fig. S9 HR-MS spectrum of ER-HClO + HClO.

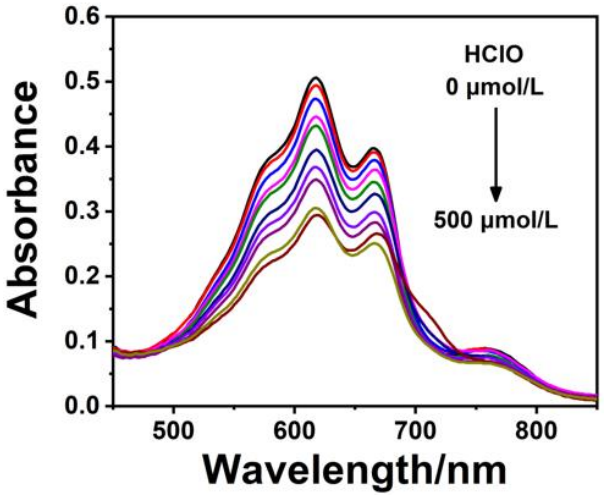


Fig. S10 Absorption spectra of ER-HClO (10 μM) in response to HClO (0 ~ 500 μM) in CH_3CN/PBS (v/v = 3/7, pH 7.4, 10 mM). λ_{ex} = 710 nm.

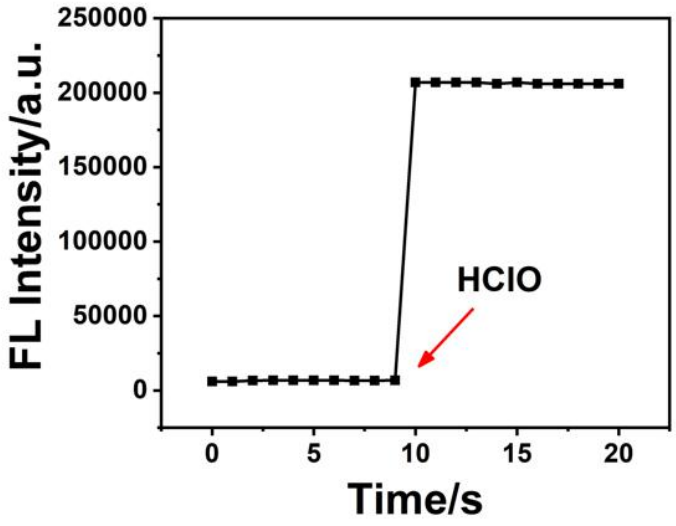


Fig. S11 Response time of **ER-HClO** (10 μM) in the presence of HClO (500 μM). λ_{ex} = 710 nm; λ_{em} = 734 nm.

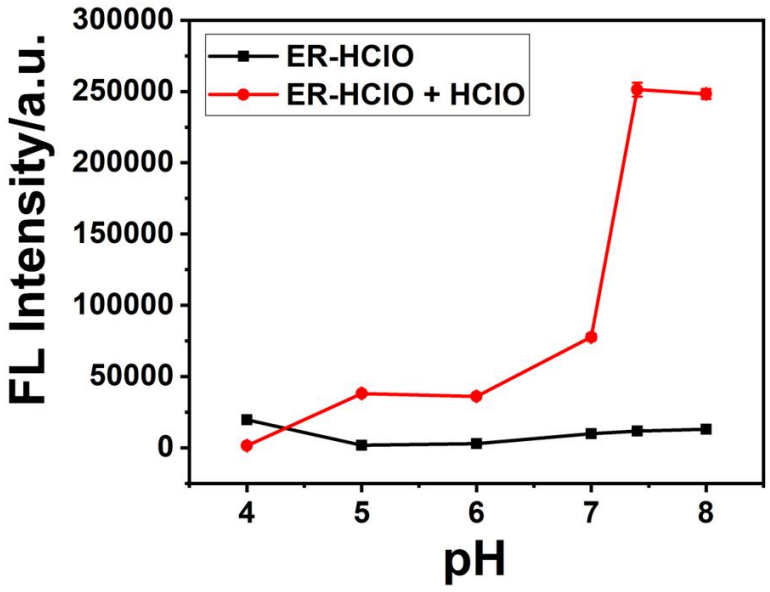


Fig. S12 Fluorescence intensity of **ER-HClO** (10 μM) with or without HClO (500 μM) in a range of pH 4.0 ~ 8.0. λ_{ex} = 710 nm; λ_{em} = 734 nm.

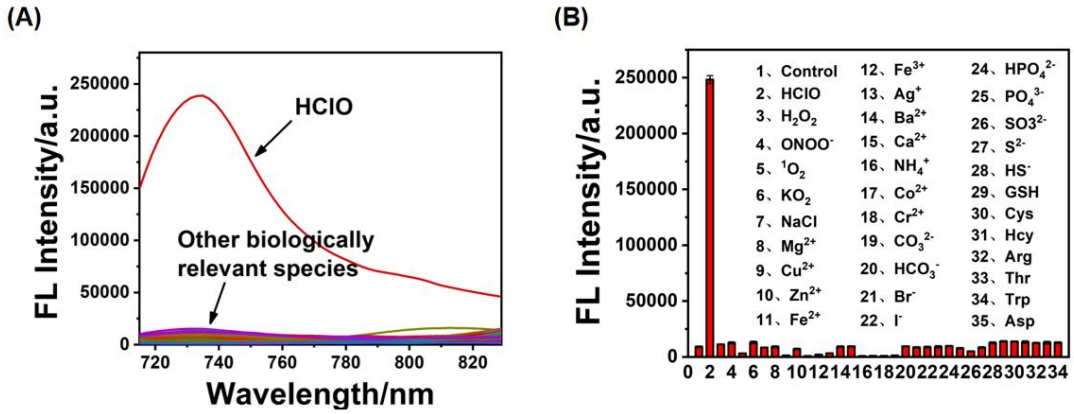


Fig. S13 (A) Fluorescence spectra and (B) fluorescence intensity (I_{734} nm) of ER-HClO (10 μM) in the presence of various biologically relevant specie in CH_3CN/PBS (3/7, *v*/*v*, pH 7.4). λ_{ex} = 710 nm; λ_{em} = 734 nm.

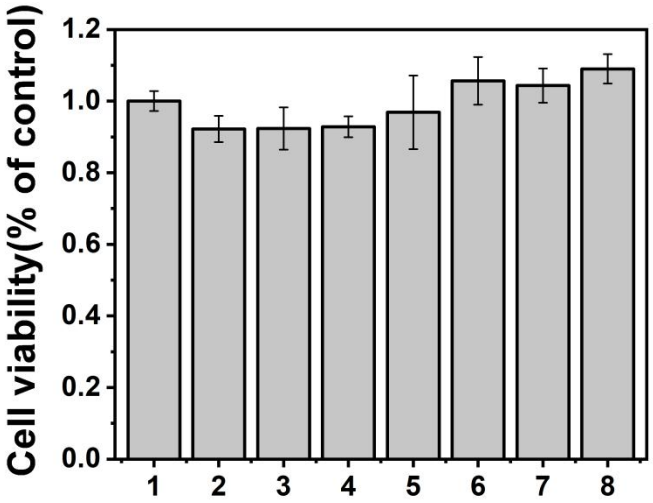


Fig. S14 Cell viability of ER-HClO on HeLa cells by a standard MTT assay. 1, control; 2, 0.1 μM ; 3, 0.5 μM; 4, 1 μM; 5, 2 μM; 6, 5 μM; 7, 8 μM; 8, 10 μM. Data are expressed as mean values ± standard error of the mean of three independent experiments, each performed in three triplicates.

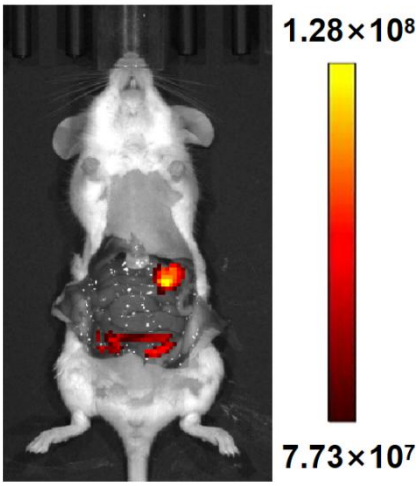


Fig. S15 Fluorescent images of the abdominal cavity of mice exposed by laparotomy at 10 min post-injection of ER-HClO (200 μM, 200 μL). λ_{ex} = 660 nm, λ_{em} = 710 nm.