Supporting Information

Cys-lighted near-infrared fluorescence for guiding orthotopic liver tumor resection

Miaomiao Zhang,[a,d] Miao Feng,[a] Ying Yang,[a] Sheng Chen,[a] Shusheng Zhang,[a] Yanhao Zhang*[c], and Bin Zhao[b*]

[a]College of Chemistry, Zhengzhou University, Zhengzhou, 450001, China

[b]School of Chemical Engineering, Zhengzhou University, Zhengzhou 450001, China

[c]College of Ecology and Environment, Zhengzhou University, Zhengzhou 450001, China

[d]Center for Advanced Analysis & Gene Sequencing, Zhengzhou University, Zhengzhou 450001, China

Correspondence and requests for materials should be addressed to email:

yhzhang_chem@outlook.com; zb2016@mail.ustc.edu.cn.

1. General methods

Experimental materials and instruments

All the starting materials were obtained from Adamas or Sangon Biotech. Commercially available reagents were used without further purification, unless noted otherwise. All other chemicals were reagent grade or better. All aqueous solutions were prepared using ultrapure water obtained from a Milli-Q water purification system. ^{1}H NMR and ^{13}C NMR spectra were obtained on a 400 MHz Bruker AV 400. Electrospray ionization mass spectrum (ESI-MS) was performed by SCIEX Triple TOF 6600 instrument. The fluorescence spectra were obtained on a Shimadzu RF-6000 spectrometer. The fluorescence imaging of HepG2 cells were obtained using a Leica confocal laser scanning microscope. Fluorescence imaging of mice was performed on an IVIS Lumina XRMS Series III from PerkinELmer Co. Ltd.

Selectivity study in vitro

To study the selectivity of two Cys-activated probes toward biological interfering substances, **MOR-A** or **MOR-F** was incubated with 100 μM biothiols (Cys, Hcy, GSH) or other 19 natural amino acids (Val, Tyr, Trp, Ser, Pro, Phe, Met, Lys, Leu, Arg, Ile, His, Gly, Gln, Glu, Asp, Asn, Ala, Thr) respectively.

Cell incubation

The HepG2 cells were cultured in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% FBS (fetal bovine serum) at 37 °C in a 5% CO_2 atmosphere.

Cell viability assay

The cytotoxicity was measured using 3-(4, 5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) assay with HepG2 cells. Cells growing in log phase were

seeded into 96-well cell culture plate at 5 × 10^3 /well. The cells were incubated overnight at 37 °C under 5% CO_2. The solutions of **MOR-A** or **MOR-F** at concentrations of 5, 10 or 20 μM in 100 μL of medium were added to the wells, respectively. The cells were incubated for 1, 2, or 4 h at 37 °C under 5% CO_2. Ten microliter solution of 5 mg/mL MTT dissolved in PBS buffer (pH 7.4) was added to each well of the 96-well plate. After 4 h incubation, 100 μL DMSO was added to each well to dissolve the formazan. The data were obtained using an enzyme-linked immunosorbent assay (ELISA) reader (VARIOSKAN FLASH) to detect its absorption at 490 nm. The following formula was used to calculate the viability of cell growth: viability (%) = (mean of absorbance value of treatment group/mean of absorbance value of control) × 100.

Fluorescence imaging of mice

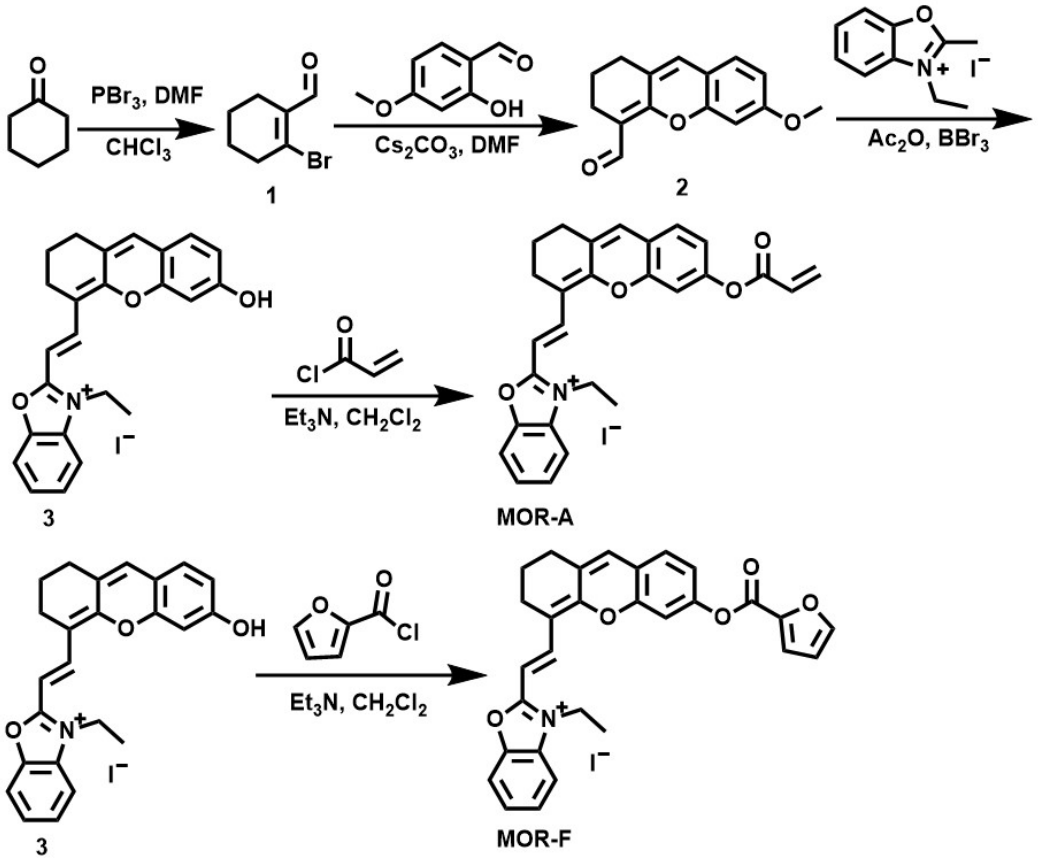
BALB/c nude mice (5-week-old, female) for tumor model building were purchased from SPF Beijing Biotechnology (Beijing, China). All animal experiments adhered to the approved guidelines of Ethical Committee of Zhengzhou University.

To build the HepG2 xenograft models, the mice was randomly divided into three groups, HepG2 cells (1 × 10^7 cells per mouse) were subcutaneously implanted in the thigh of BALB/c nude mice (20 g). When the size of tumor reach 100 mm^3 (3 weeks), **MOR-A** (500 μM, 100 μL) was intratumorally injected. Fluorescence images of the mice were obtained on the multi-mode small animal *in vivo* imaging system (λ_{ex} = 540 nm). To construct the orthotropic hepatocellular carcinoma model, a midline incision of the anterior abdominal wall was made, and 5×10^6 HepG2 cells in 50 μL PBS were directly injected into the left liver lobe of the mouse under anesthesia of tribromoethanol. Three weeks later, the orthotropic liver tumors

were successfully established. After being sacrificed, the tumor of the mice was sprayed with **MOR-A** and imaged with the imaging systems.

2. Syntheses and characterizations of MOR-A and MOR-F.

Scheme S1. The synthetic route for **MOR-A** and **MOR-F**.



***Synthesis of 1*:**

PBr_3 (0.9 mL, 9.5 mmol) was added dropwise to anhydrous DMF (1.2 mL, 15.5 mmol) and chloroform (10 mL) at 0°C under constant stirring for 1 h. Then cyclohexanone (0.4 mL, 3.9 mmol), dissolved in chloroform (10 mL), was introduced. The reaction mixture was then stirred for 18 hours at 25°C. After that, the mixture was neutralized by adding it to a saturated sodium bicarbonate solution, adjusting the pH to 7. The solvent was washed with dichloromethane and then concentrated to afford a yellow oil. Yield: 650 mg (89%). It was observed that compound 1 could be only stored for extended periods under inert gas at -20°C.

***Synthesis of 2*:**

A solution of freshly synthesized compound **1** (2.4 mmol) in 10 mL anhydrous DMF was prepared, to which 2-hydroxy-4-methoxybenzaldehyde (2 mmol) and Cs_2CO_3 (6 mmol) were added sequentially. The reaction mixture was stirred at 25°C for 12 hours. After that, it was extracted with CH_2Cl_2 and concentrated. By column chromatography purification, compound **2** was obtained as a bright yellow crystalline solid. Yield: 288 mg (60%).

***Synthesis of 3*:**

The mixture of compound 2 (96 mg, 0.4 mmol) and 3-ethyl-2-methylbenzoxazolium iodide (144.5 mg, 0.5 mmol) in acetic anhydride (10 mL) was heated and refluxed for 12 h. After that, the solvent was removed, and the residue was dissolved in 20 mL CH_2Cl_2. The solvent was washed with H_2O, then concentrated to yield the intermediate. The crude product was subsequently dissolved in anhydrous dichloromethane (10 mL) and cooled to 0°C. BBr_3 (0.24 mL, 2.5 mmol) was added dropwise, then the reaction mixture was then stirred for 12 hours at 25°C. Then, the mixture was pouring into ice water, followed by neutralization with $NaHCO_3$ solution. After that, the residue was extracted with CH_2Cl_2 and concentrated. After column chromatography purification, using CH_2Cl_2:CH_3OH (20:1, v/v) mixture as the eluent, compound 2 was obtained as a pure purple solid. Yield: 100 mg (50%).

***Synthesis of MOR-A*:**

Compound 3 (25 mg, 0.05 mmol) was dissolved in anhydrous dichloromethane (2 mL). With the solution stirred, triethylamine (Et_3N, 14 μL, 0.1 mmol) and acryloyl chloride (8 μL, 0.1 mmol) were added dropwise. After stirring for 20 min, the reaction mixture was then concentrated under reduced pressure using a rotary evaporator to obtain a crude solid residue.

Purification was achieved through column chromatography, employing a gradient eluent system of dichloromethane/methanol (20:1, v/v). Yield: 15 mg (55%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.64 (d, J = 13.2 Hz, 1H), 7.76 (dd, J = 20.2, 6.9 Hz, 2H), 7.56 (t, J = 5.1 Hz, 2H), 7.29 (d, J = 8.3 Hz, 1H), 7.25 – 7.19 (m, 1H), 7.03 – 6.87 (m, 3H), 6.64 (d, J = 17.2 Hz, 1H), 6.32 (dd, J = 17.2, 10.5 Hz, 1H), 6.08 (d, J = 10.4 Hz, 1H), 5.12 – 4.85 (m, 2H), 2.92 – 2.75 (m, 2H), 2.65 (q, J = 5.7 Hz, 2H), 1.97 – 1.79 (m, 2H), 1.60 (t, J = 5.6 Hz, 3H) (Fig. S1). ^{13}C NMR (101 MHz, $CDCl_3$) δ 163.82, 162.34, 153.38, 151.81, 147.96, 136.89, 136.20, 133.44, 132.74, 130.05, 129.73, 129.71, 128.67, 127.54, 127.25, 124.22, 119.65, 117.84, 113.82, 109.28, 108.61, 55.81, 29.44, 29.14, 27.04, 13.94 (Fig. S2). MS: calc. $[M]^+$ 426.1700, obsvd. ESI-MS: m/z 426.1736 (Fig. S3).

***Synthesis of MOR-F*:**

Above attained **3** (30 mg, 0.06 mmol) was dissolved in 2 mL anhydrous dichloromethane. To this vigorously stirred solution, triethylamine (Et_3N, 17 µL, 0.12 mmol) and furoyl chloride (12 µL, 0.12 mmol) were dropwise added to above solution. After 30 minutes of stirring, the reaction mixture was then concentrated, yielding a crude residue. Purification was performed via column chromatography, with a gradient eluent of dichloromethane/methanol (15:1, v/v). Yield: 20 mg (56%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.64 (d, J = 14.5 Hz, 1H), 7.77 (d, J = 7.4 Hz, 1H), 7.70 (d, J = 6.5 Hz, 2H), 7.59 – 7.50 (m, 2H), 7.40 (d, J = 3.6 Hz, 1H), 7.33 – 7.27 (m, 2H), 7.02 (dd, J = 8.5, 2.2 Hz, 1H), 6.92 (s, 1H), 6.86 (d, J = 14.3 Hz, 1H), 6.66 – 6.55 (m, 1H), 5.05 – 4.79 (m, 2H), 2.76 (d, J = 5.9 Hz, 2H), 2.65 (t, J = 6.1 Hz, 2H), 1.87 (t, J = 6.3 Hz, 2H), 1.64 – 1.48 (m, 3H) (Fig. S4). 13C NMR (101 MHz, $CDCl_3$) δ 179.15, 162.53, 152.97, 151.60, 147.74, 147.38, 145.15, 143.35, 136.38, 130.38, 129.88, 128.85, 127.66,

127.60, 120.27, 118.05, 113.13, 112.47, 111.92, 111.62, 109.50, 108.79, 59.13, 29.68, 29.30, 27.20, 14.17 (Fig. S5). MS: calc. $[M]^+$ 466.1649, obsvd. ESI-MS: m/z 466.1639 (Fig. S6).

3. Supporting figures and tables.

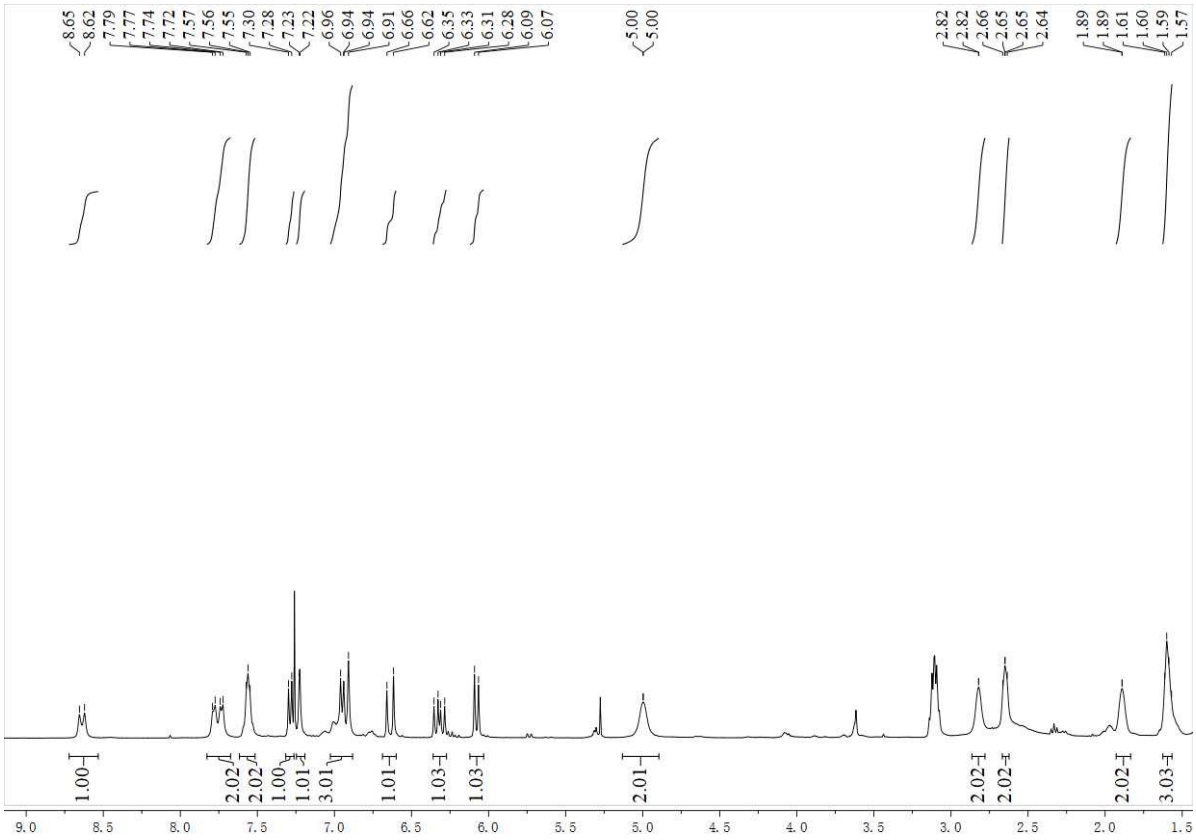


Fig. S1. ^{1}H NMR of **MOR-A** in $CDCl_3$**.**

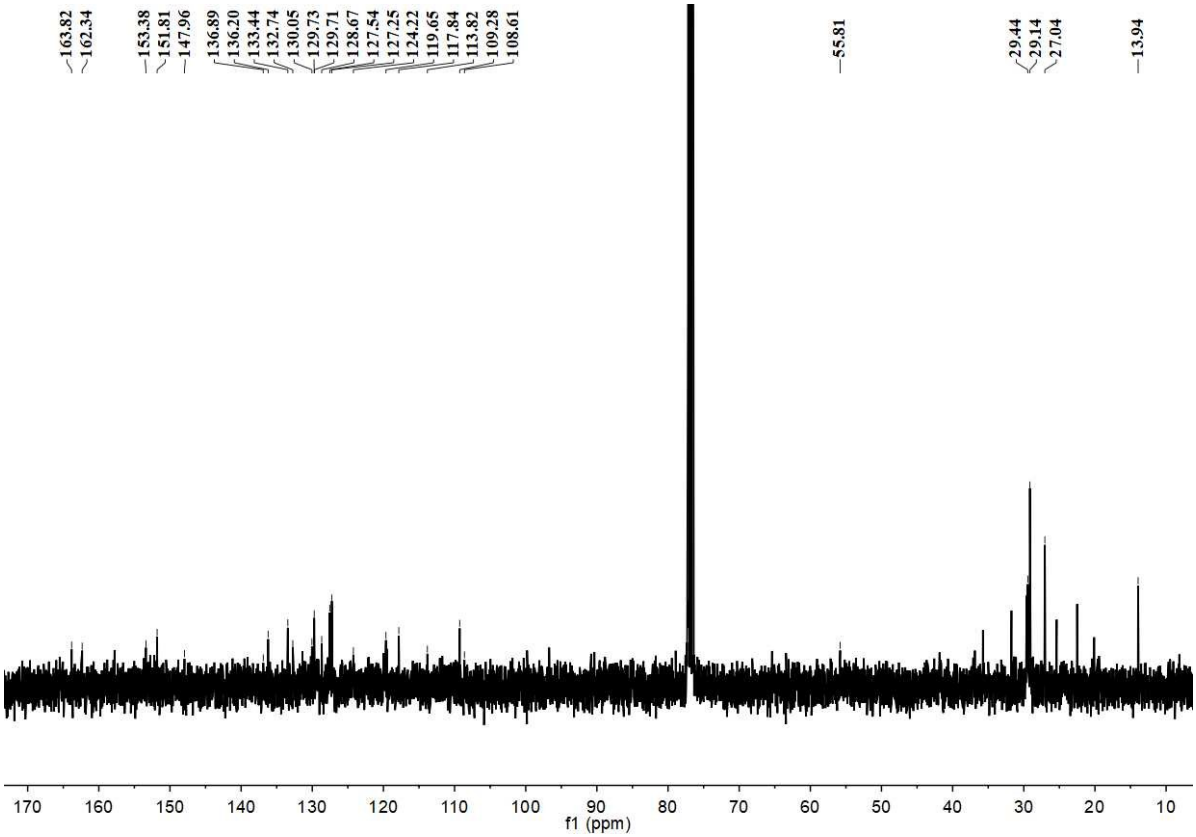


Fig. S2. ^{13}C NMR of **MOR-A** in $CDCl_3$.

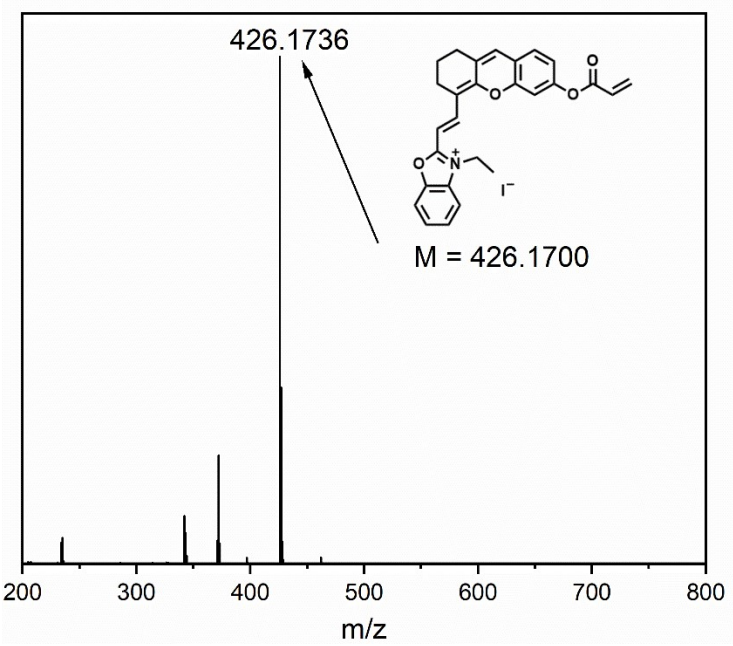


Fig. S3. ESI-MS spectrum of **MOR-A**.

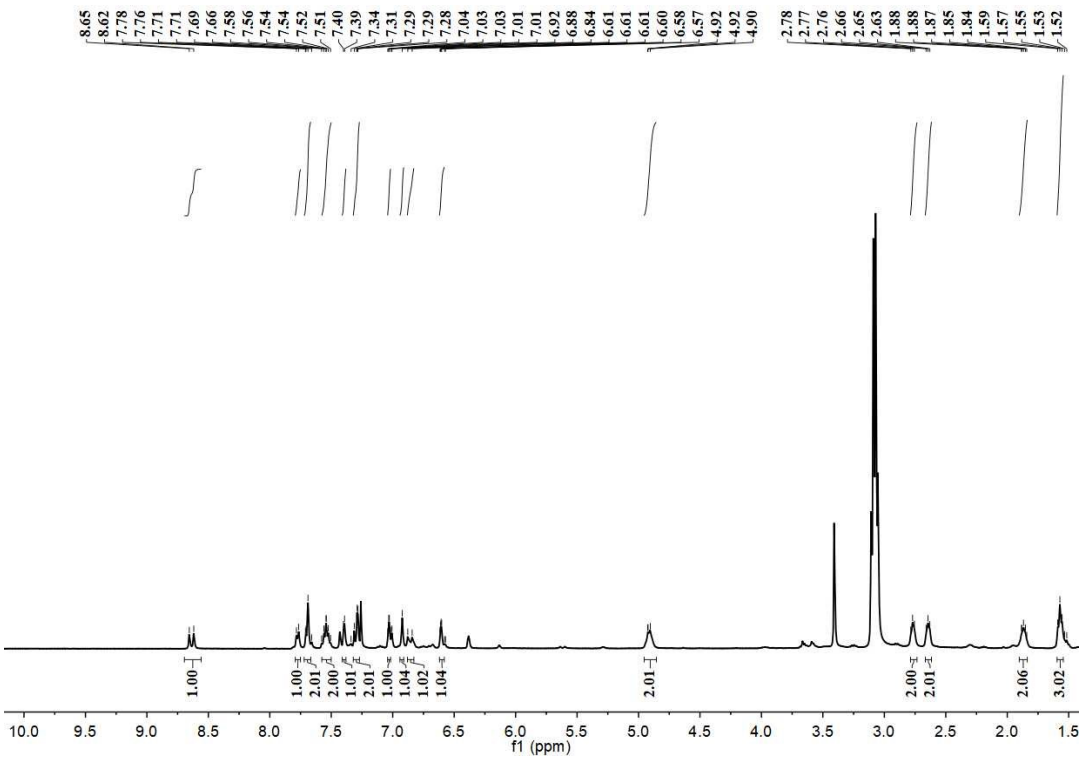


Fig. S4. ^{1}H NMR of **MOR-F** in $CDCl_3$.

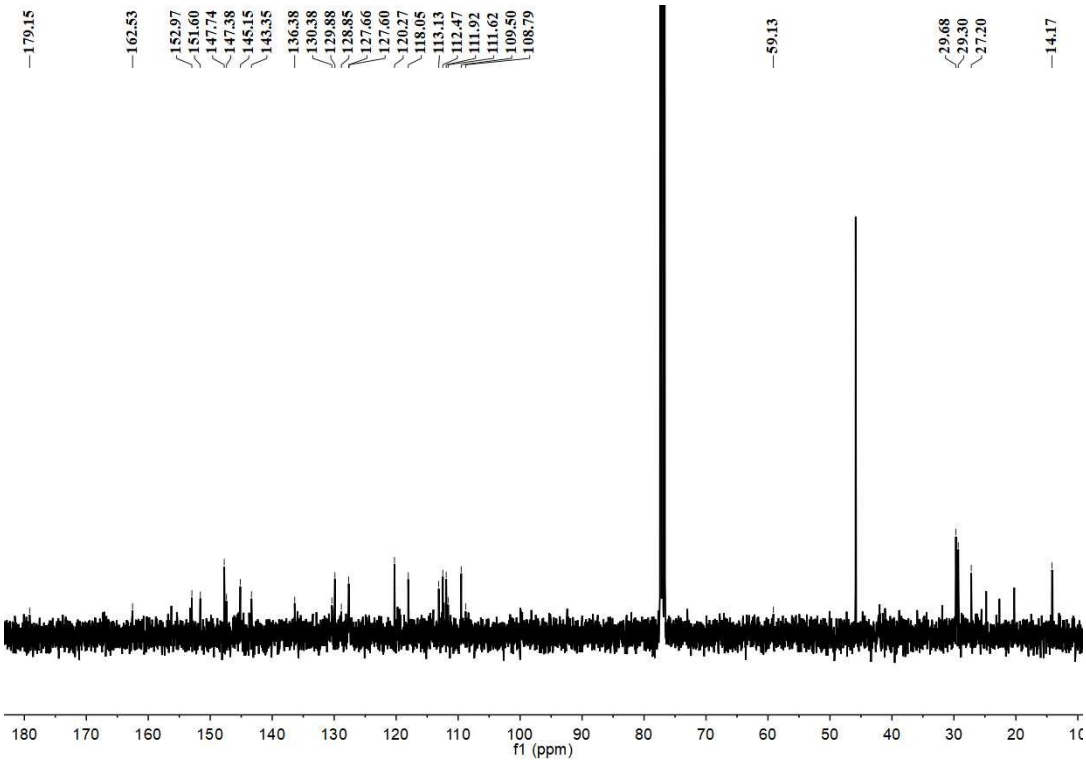


Fig. S5. ^{13}C NMR of **MOR-F** in $CDCl_3$.

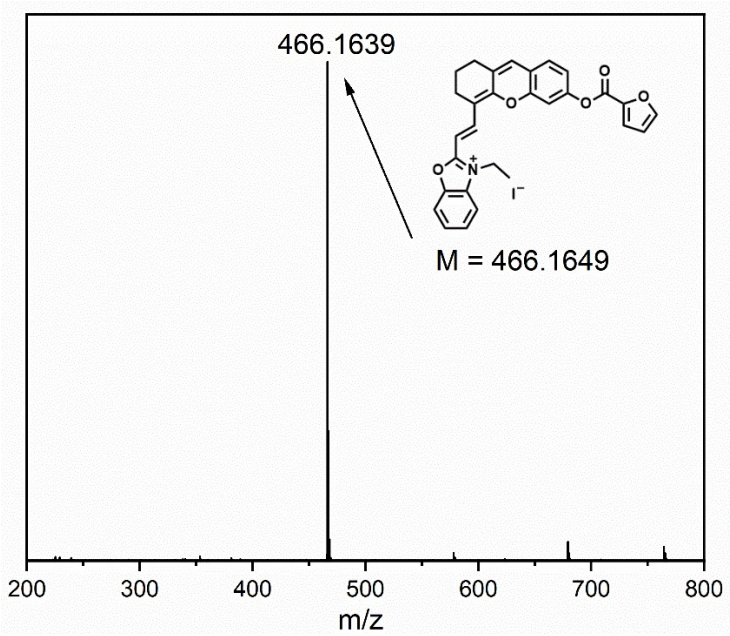


Fig. S6. ESI-MS spectrum of **MOR-F**.

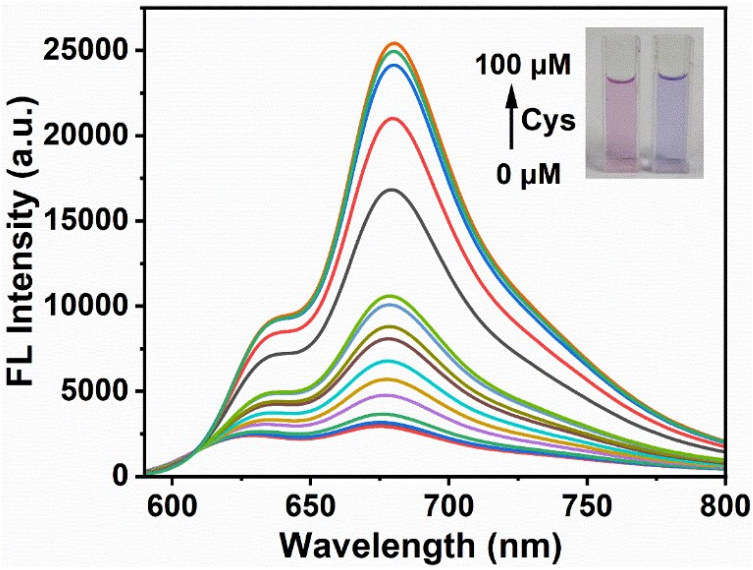


Fig. S7. The fluorescence spectra for **MOR-A** (10 μM) in the presence of different concentrations of Cys (0 - 100 μM).

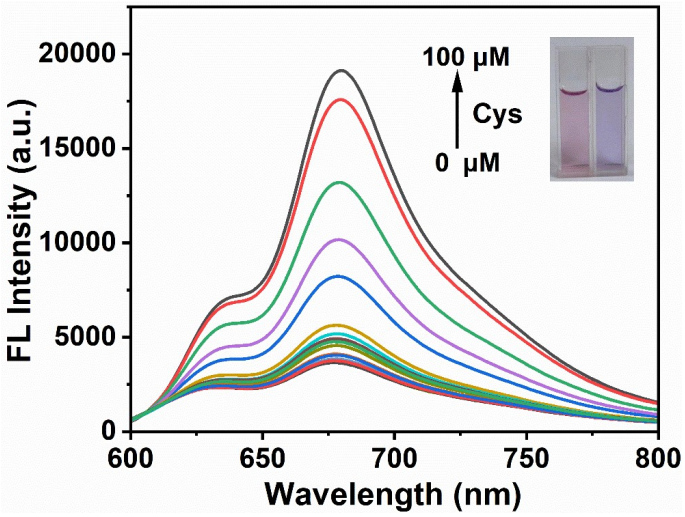


Fig. S8. The fluorescence spectra for **MOR-F** (10 μM) in the presence of different concentrations of Cys (0 -100 μM).

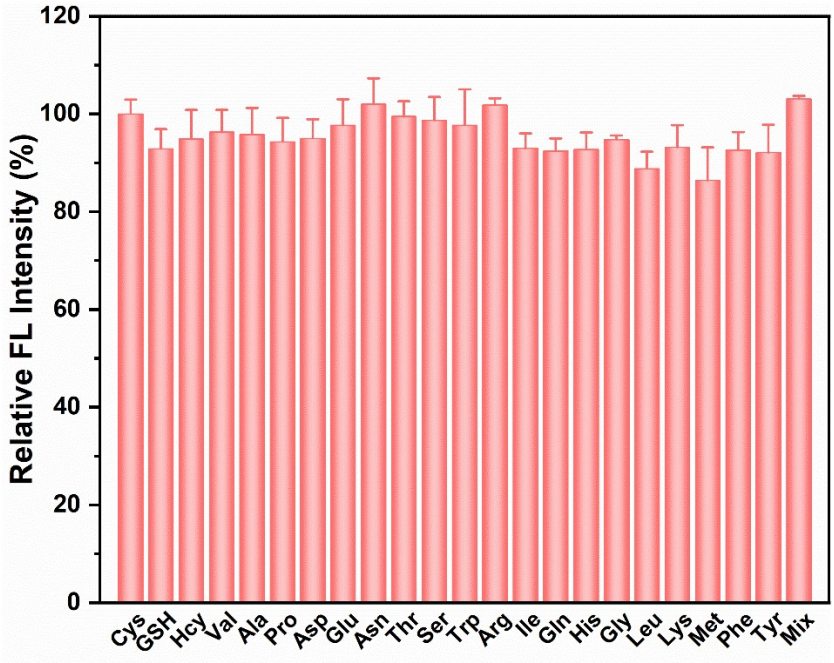


Fig. S9. The relative fluorescence intensity of 10 μM **MOR-A** with 100 μM Cys in the presence of other analytes. The experiments were performed in triplicate.

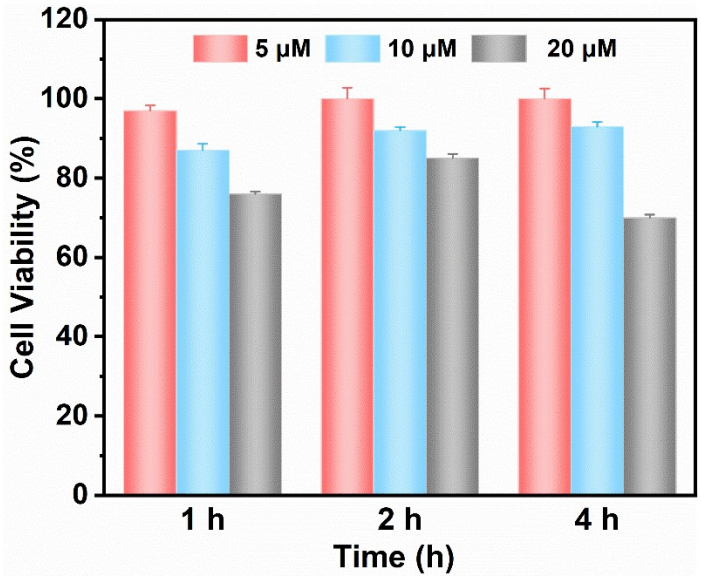


Fig. S10. MTT assay of **MOR-A** on HepG2 cells. The experiments were performed in triplicate. Results are representative of three independent experiments. Error bars represent standard deviations.

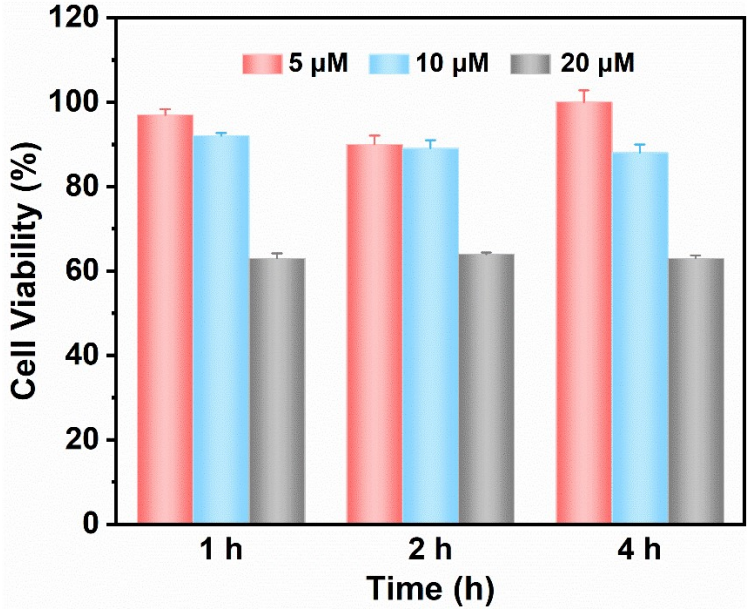


Fig. S11. MTT assay of **MOR-F** on HepG2 cells. The experiments were performed in triplicate. Results are representative of three independent experiments. Error bars represent standard deviations.

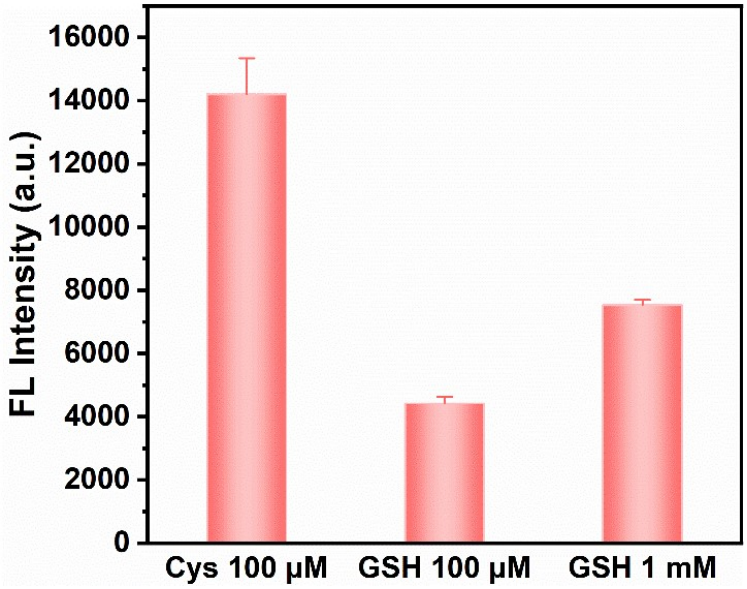


Fig. S12. The fluorescence spectra for **MOR-A** (10 μM) in the presence of different concentrations of Cys and GSH.

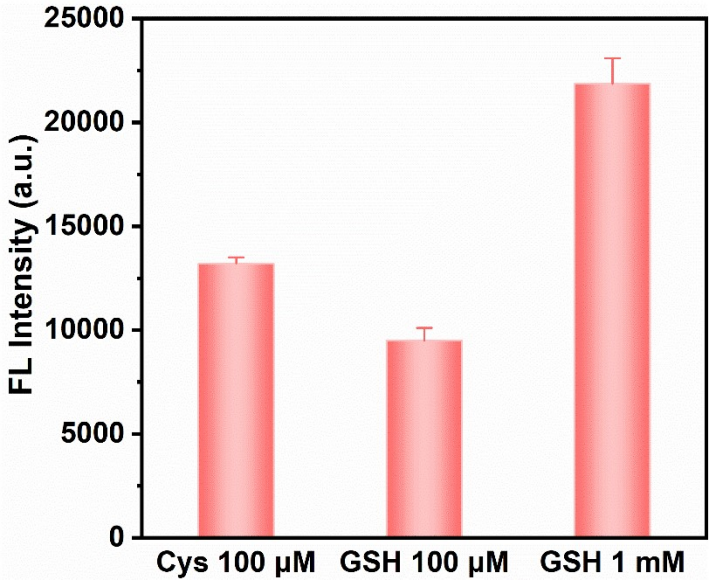


Fig. S13. The fluorescence spectra for **MOR-F** (10 μM) in the presence of different concentrations of Cys and GSH.

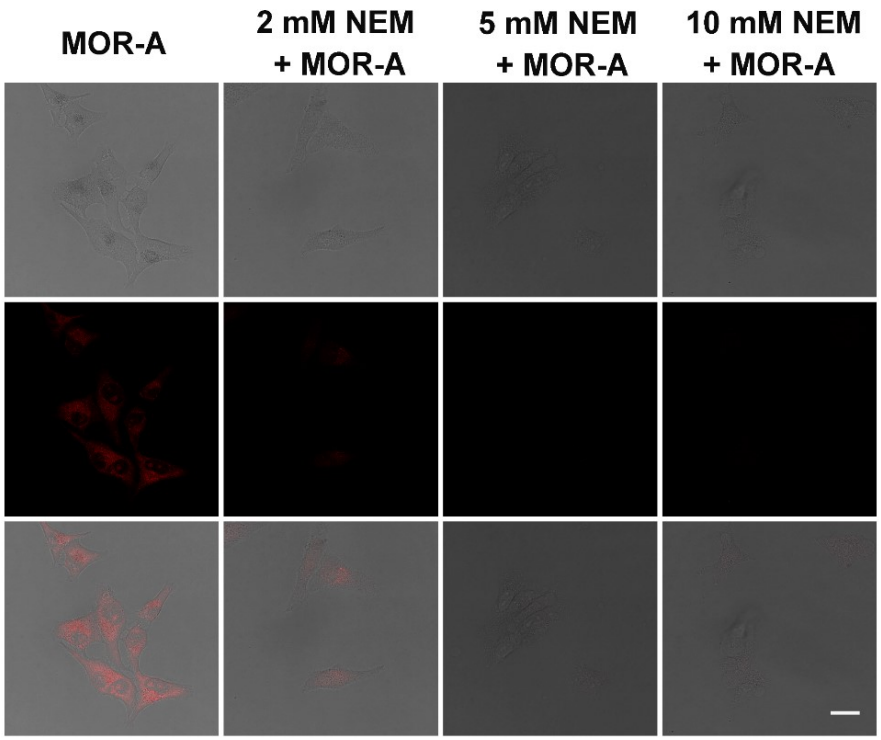


Fig. S14. Fluorescence images of HepG2 cells pre-incubated with different concentration NEM (2, 5, 10 mM) and then incubated with **MOR-A** for 10 min. Scale bar = 25 μM.

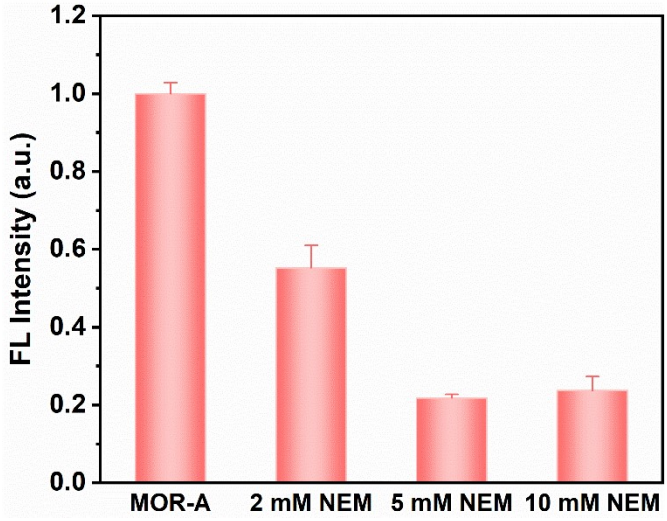


Fig. S15. Quantification of fluorescence intensities of Fig.S14 (each point represents Mean ± SD, n = 3).

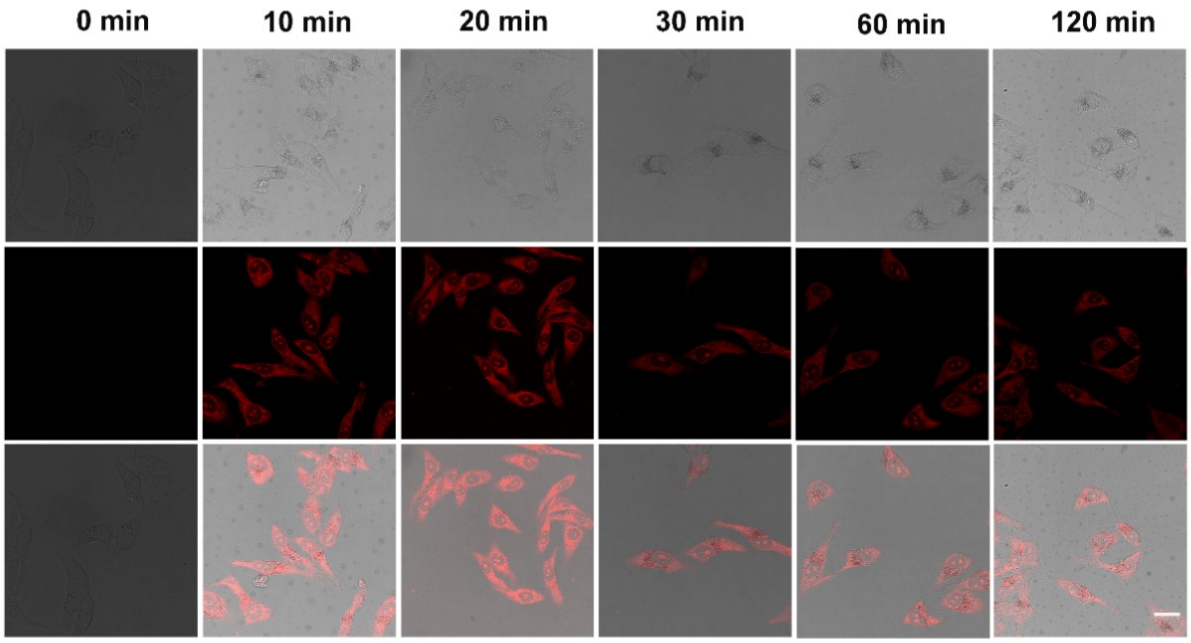


Fig. S16. Fluorescence images of HepG2 cells incubated with **MOR-A** (10 μM) for different time. Scale bar = 25 μM.

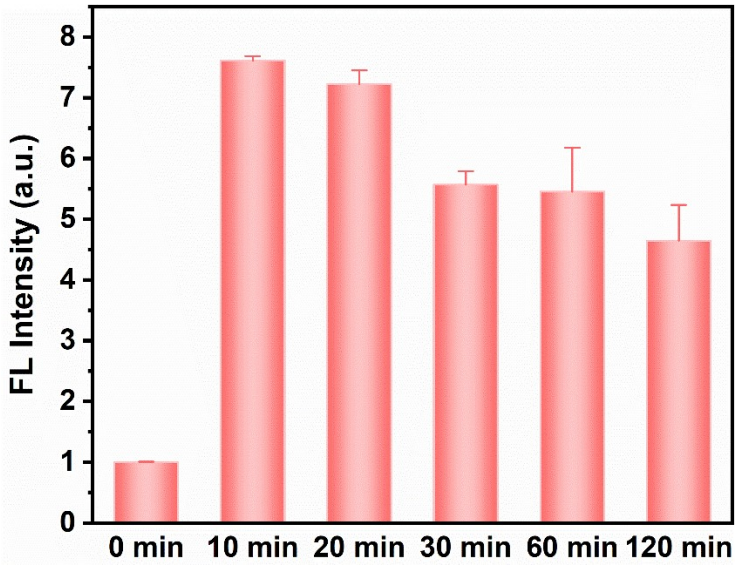


Fig. S17. Quantification of fluorescence intensities of Fig. S16 (each point represents Mean ± SD, n = 3).

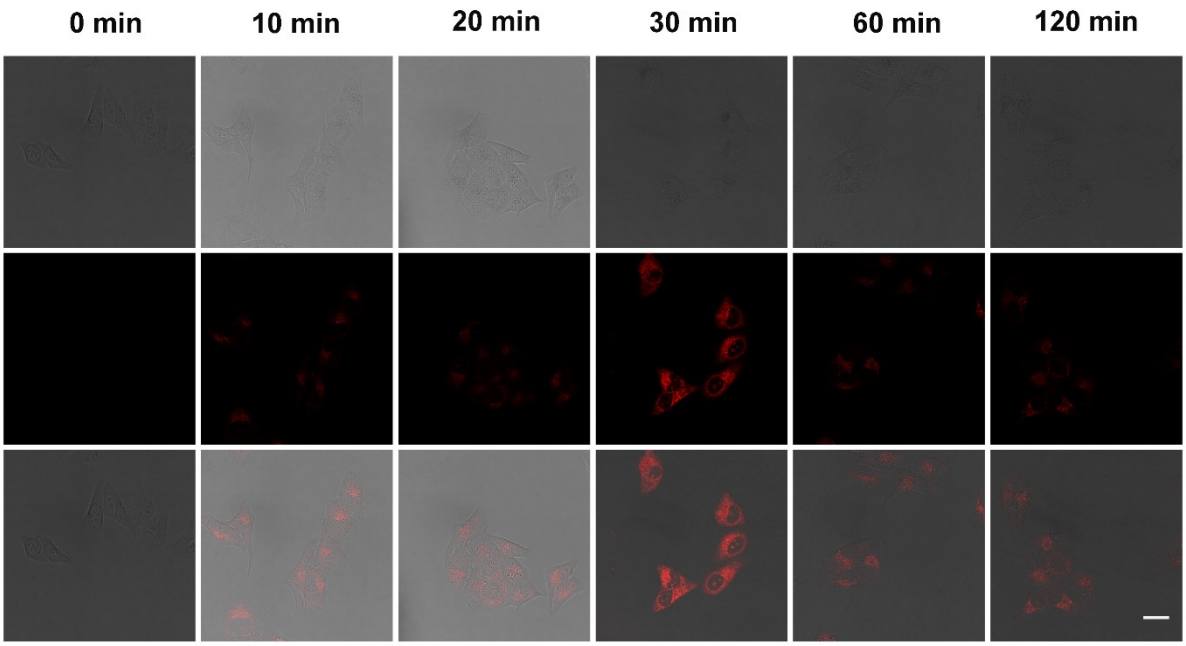


Fig. S18 Fluorescence images of HepG2 cells incubated with **MOR-F** (10 μM) for different time. Scale bar = 25 μM.

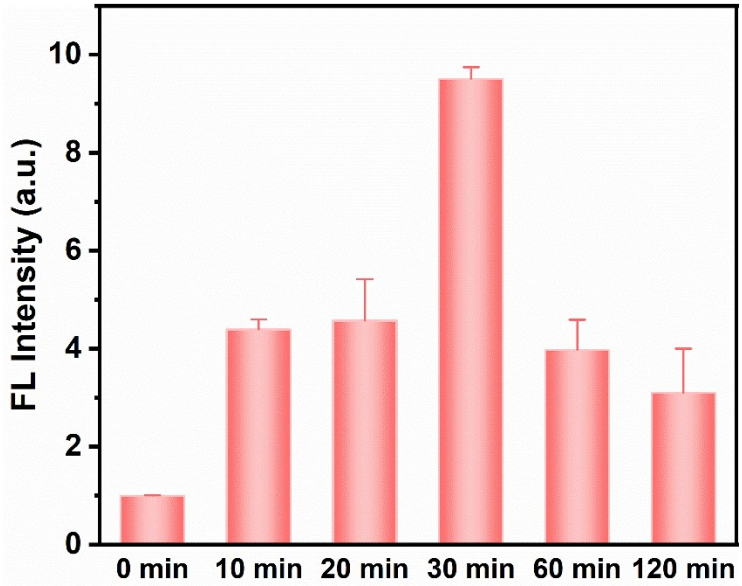


Fig. S19. Quantification of fluorescence intensities of Fig. S18 (each point represents Mean ± SD, n = 3).

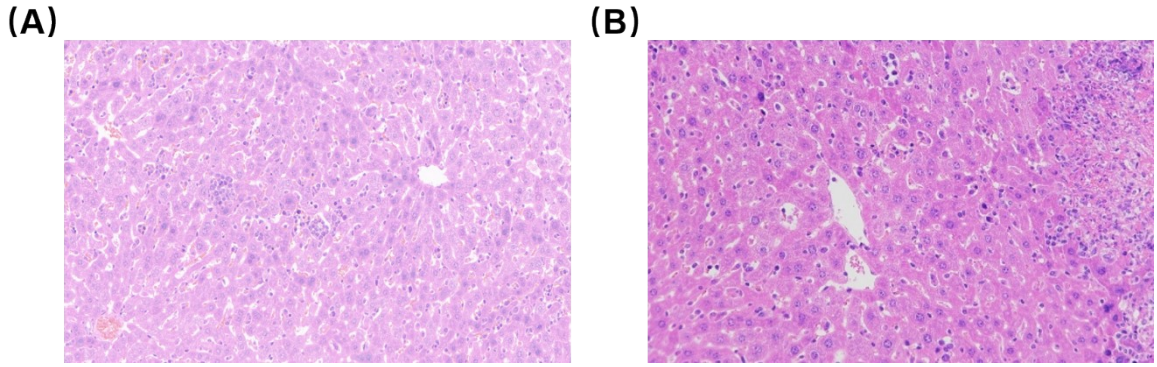


Fig. S20. H&E staining images of normal liver (A), and orthotopic liver tumor (B). Scale bar =100 μm.

Table S1. Comparison of other reported Cys-responsive fluorescent probes.

Reference	Limit of detection (μM)
Xiao Z. et al., **2019**[1]	0.83
Kang X. et al., **2019**[2]	2.96
Lu J. et al., **2020**[3]	2.29
Ying L. et al., **2020**[4]	16.67
Yang Y. et al., **2021**[5]	2.50
Wei L. et al., **2021**[6]	0.86
Gui F. et al., **2023**[7]	2.78
This work (**MOR-A**)	0.57
This work (**MOR-F**)	0.14

4. Reference

1 X. Zhang, H. Liu, Y. Ma, W. Qu, H. He, X. Zhang, S. Wang, Q. Sun and F. Yu, Dyes and Pigments, 2019, **171**, 107722.

2 K. Xiong, F. Huo, J. Chao, Y. Zhang and C. Yin, Anal. Chem., 2018, **91**, 1472-1478.

3 L. Jia, L.-Y. Niu and Q.-Z. Yang, Anal. Chem., 2020, **92**, 10800-10806.

4 Y. Liu, Y.-X. Wu, D. Zhang, H. Zhong, D. Li, K. He, W.-T. Wei and S. Yu, Talanta, 2020, **220**, 121364.

5 Y. Yang, Y. Feng, H. Li, R. Shen, Y. Wang, X. Song, C. Cao, G. Zhang and W. Liu, Sensors and Actuators B: Chemical, 2021, **333**, 129189.

6 W. Luo, S. Zhang, Q. Meng, J. Zhou, R. Jin, X. Long, Y.-P. Tang and H. Guo, Talanta, 2021, **224**, 121833.

7 G.-Q. Fu, Q. Song, Z.-Q. Wang, J.-J. Chao, H. Zhang, G.-J. Mao, D.-H. Chen and C.-Y. Li, Anal. Chem., 2023, **95**, 17559-17567.