

## Electronic Supplementary Information (ESI)

# Observation of peroxynitrite overproduction in cells during 5-fluorouracil treatment via a ratiometric fluorescent probe

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## **Experimental Section**

### **General remarks for experimental**

<sup>1</sup>H NMR, <sup>13</sup>C NMR spectra were measured on a Bruker Avance 400 MHz spectrometer and a 600 MHz NMR spectrometer (JEOL ECZ600R/S3) equipped with a 14.09 T superconducting magnet and a 5.0 mm 600MHz broadband Z-gradient high resolution ROYAL probe (JEOL RESONANCE Inc., Japan). Proton Chemical shifts of NMR spectra were given in ppm relative to internal reference TMS (1H, 0.00 ppm). Mass spectra were measured on a HP-1100 LC-MS spectrometer. UV-vis spectra were recorded on UV-3900 spectrometer. Fluorescence spectra were recorded on FLS 1000 fluorimeter. Confocal microscopy fluorescence images were acquired on Leica TCS SP8. The solvents used for UV-vis and fluorescence measurements were of HPLC grade. Unless otherwise noted, materials were obtained from commercial suppliers and were used without further purification. All the solvents were dried according to the standard methods prior to use. All of the solvents were either HPLC or spectroscopic grade in the optical spectroscopic studies.

### **Fluorescence analysis.**

Stock solution of the probe (5 mM) was prepared in HPLC grade DMSO. Stock solutions of analytes were prepared in twice-distilled water. For spectral measurements, the probe was diluted to 10 μM with 10 mM PBS solution containing 1 mM Triton X-100. 3.0 mL probe solution was placed in a quartz cell of 1 cm optical path length each time. All spectroscopic experiments were carried out at room temperature.

### **Cell culture**

MCF-7 cells were cultured in 1640 medium and BI serum. Add 1% streptomycin, 10% fetal bovine serum (FBS), and 1% penicillin to the medium. The cells were then placed in a cell incubator containing 5% CO<sub>2</sub> and cultured at 37 °C. One day before imaging, the cells were digested into confocal dishes for subsequent experiments.

### **Cytotoxicity assay**

MCF-7 was prepared into a density of 10<sup>6</sup>cell / mL with culture medium, and then the cells were seeded into 96-well plates. 100ul of medium was added to each well and placed in a cell culture incubator (5% CO<sub>2</sub> / 95% Air). Medium culture, after attachment is completed, add a series of probe solutions (0uM, 2.5uM, 5uM, 10uM, 15uM, 20uM, 25uM, 30uM) to 96-well plates, and place them in the cell culture incubator (5% CO<sub>2</sub> / 95% Air) Continue incubating for 24h, aspirate the culture medium from the 96-well plate, add 100ul culture medium, and then add 10ul CCK-8 solution to each well, and continue in the cell incubator (5 % CO<sub>2</sub> / 95% Air) for 1-4 h, and then take out the absorbance at 450nm on a microplate reader.

### **Imaging of ONOO<sup>-</sup> in living cells.**

All cell imaging experiments were performed on MCF-7 cells. The excitation wavelength was set at 405 nm, and the blue channel and the red channel collection wavelength ranges were set as 450-510 nm and 610 - 670 nm, respectively.

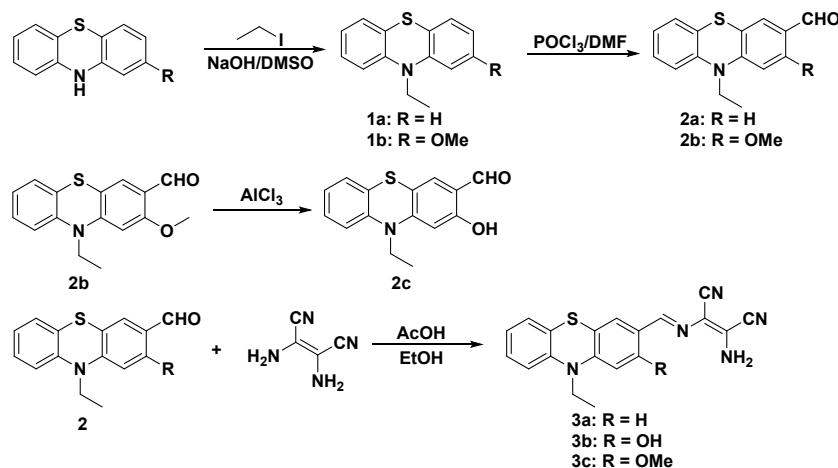
For detecting exogenous ONOO<sup>-</sup>, probe (10 μM) was first incubated with the cells for 30 min, and

then the cells were washed 3 times with a physiological PBS buffer solution. SIN-1 was then added (100  $\mu\text{M}$ ) for another 30 min.

For detecting endogenous  $\text{ONOO}^-$ , cells were first incubated with (1  $\mu\text{g}/\text{mL}$ ) LPS and (50  $\text{ng}/\text{mL}$ )  $\text{IFN-}\gamma$  for 24 h. Then probe (10  $\mu\text{M}$ ) was added. The cells were incubated for another 30 min and washed 3 times before imaging.

**Imaging of  $\text{ONOO}^-$  in cells treated by 5-FU.**

Cells were cultured with different amounts of 5-FU for 20 h. Then, probe (10  $\mu\text{M}$ ) was added for incubation for another 30 min. The cells were washed 3 times before confocal imaging.



**Scheme S1**

### Preparation and Characterization of 1

Phenothiazine (10 mmol) and crushed NaOH (20 mmol) were dissolved in DMSO (20 mL). Then iodoethane (3.9 g, 25 mmol) was added. The mixture was stirred at 65 °C for 9 h under Ar atmosphere. After the reaction was completed, the reaction was cooled down, and was poured into 300 mL water. CH<sub>2</sub>Cl<sub>2</sub> (100 mL × 2) was added for extraction. The combined organic phase was then washed with brine and dried over Na<sub>2</sub>SO<sub>4</sub>. After removal of the solvent under reduced pressure by evaporation, the crude product was purified by silica column chromatography with petroleum ether/CH<sub>2</sub>Cl<sub>2</sub> = 5/1 as the eluent to afford a white solid.

**1a:** 1.8 g, 79.3%. <sup>1</sup>H NMR (600 MHz, CDCl<sub>3</sub>) δ 7.24 – 7.10 (m, 4H), 6.90–6.89 (m, 4H), 3.93 (s, 2H), 1.41 (t, *J* = 7.0 Hz, 3H). ESI-MS: *m/z* 228.05 [M + H]<sup>+</sup>: (calcd 228.08).

**1b:** 2.3 g, 89.5%. <sup>1</sup>H NMR (600 MHz, DMSO-*d*<sub>6</sub>) δ 7.18 (ddd, *J* = 8.4, 7.2, 1.4 Hz, 1H), 7.12 (dd, *J* = 7.6, 1.7 Hz, 1H), 7.06 – 6.98 (m, 2H), 6.96 – 6.90 (m, 1H), 6.57 – 6.54 (m, 2H), 3.91 (q, *J* = 6.9 Hz, 2H), 3.74 (s, 3H), 1.29 (t, *J* = 6.9 Hz, 3H). ESI-MS: *m/z* 258.07 [M + H]<sup>+</sup>: (calcd 258.09).

### Preparation and Characterization of 2a and 2b

DMF (1.7 mL) was added dropwise to POCl<sub>3</sub> at 0 °C under Ar atmosphere. After stirring for 15 min, a solution of compound **1** (10 mmol) dissolved in DMF (7.5 mL) was added to the above mixture. The mixture was heated to 60 °C and stirred for another 4 h. Then the mixture was poured into ice water. Stirring for about 2 h gave orange precipitate. The crude product was obtained by filtration and was purified over silica column chromatography with hexane/ EtOAc = 5/1 as the eluent, giving **2a** and **2b** as a yellow solid.

**2a:** 997 mg, 39.1%. <sup>1</sup>H NMR (600 MHz, CDCl<sub>3</sub>) δ 9.79 (s, 1H), 7.63 (dd, *J* = 8.4, 1.9 Hz, 1H), 7.57 (d, *J* = 1.9 Hz, 1H), 7.20 – 7.13 (m, 1H), 7.10 (dd, *J* = 7.6, 1.5 Hz, 1H), 6.96 (d, *J* = 4.7 Hz, 1H), 6.90 (t, *J* = 7.7 Hz, 2H), 3.98 (s, 2H), 1.45 (t, *J* = 7.0 Hz, 3H). ESI-MS: *m/z* 256.02 [M + H]<sup>+</sup>: (calcd 256.08).

**2b:** 2.48 g, 87%. <sup>1</sup>H NMR (600 MHz, CDCl<sub>3</sub>) δ 10.19 (s, 1H), 7.51 (s, 1H), 7.13 (t, *J* = 7.6 Hz, 1H), 7.09 (dd, *J* = 7.6, 1.5 Hz, 1H), 6.93 (t, *J* = 7.3 Hz, 1H), 6.87 (d, *J* = 8.2 Hz, 1H), 6.37 (s, 1H), 3.97 (s, 2H), 3.90 (s, 3H), 1.45 (t, *J* = 7.0 Hz, 3H). ESI-MS: *m/z* 286.03 [M + H]<sup>+</sup>: (calcd 286.09).

### Preparation and Characterization of 2c

AlCl<sub>3</sub> (1.98 g, 15 mmol) was added into a 100 mL round bottom flask charged with 15 mL anhydrous CH<sub>2</sub>Cl<sub>2</sub>. A solution of compound **2b** (1.43 g, 5 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (5 mL) was added dropwise to the mixture at 20 °C under stirring. After 12 h, aqueous HCl (2 M, 6 mL) was added and the mixture was stirred for 0.5 h. Then most of CH<sub>2</sub>Cl<sub>2</sub> was removed under reduced pressure and EtOAc was added for extraction. The combined organic phase was washed with brine, and dried over Na<sub>2</sub>SO<sub>4</sub>. After removal of the solvent under reduced pressure by evaporation, the crude product was purified by column chromatography over silica gel petroleum ether/CH<sub>2</sub>Cl<sub>2</sub> = 3/1 as eluent to afford **2c** as a yellow viscous substance, which will be formed into solid after standing (1.26 g, 92.6%). <sup>1</sup>H NMR (600 MHz, CDCl<sub>3</sub>) δ 11.39 (s, 1H), 9.60 (s, 1H), 7.21 – 7.11 (m, 2H), 7.10 (dd, *J* = 7.7, 1.7 Hz, 1H), 6.97 (td, *J* = 7.5, 1.3 Hz, 1H), 6.92 (dd, *J* = 8.3, 1.3 Hz, 1H), 6.39 (s, 1H), 3.95 (q, *J* = 7.2 Hz, 2H), 1.45 (t, *J* = 7.1 Hz, 3H). ESI-MS: *m/z* 272.05 [M + H]<sup>+</sup>: (calcd 272.07).

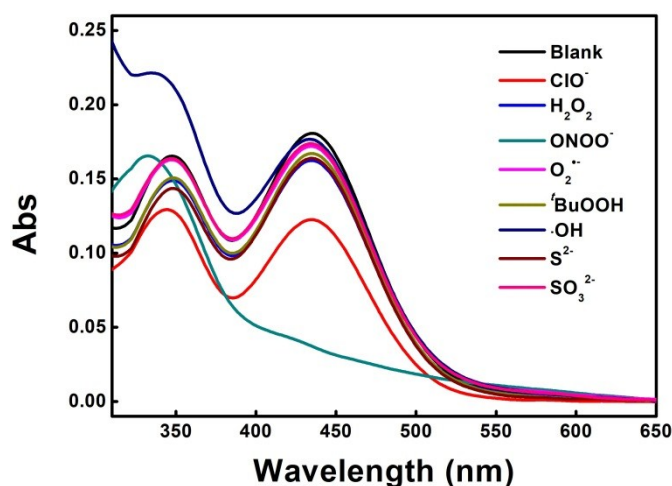
### Preparation and Characterization of **3**

To a solution of compound **2** (0.5 mmol) and 2,3-diaminobut-2-enedinitrile (66 mg, 0.6 mmol) in EtOH (10 mL) was added 2 drops of AcOH. The mixture was stirred at room temperature for 12 h. After that, the precipitate was filtered and washed with cold EtOH, afford **3** as a red solid.

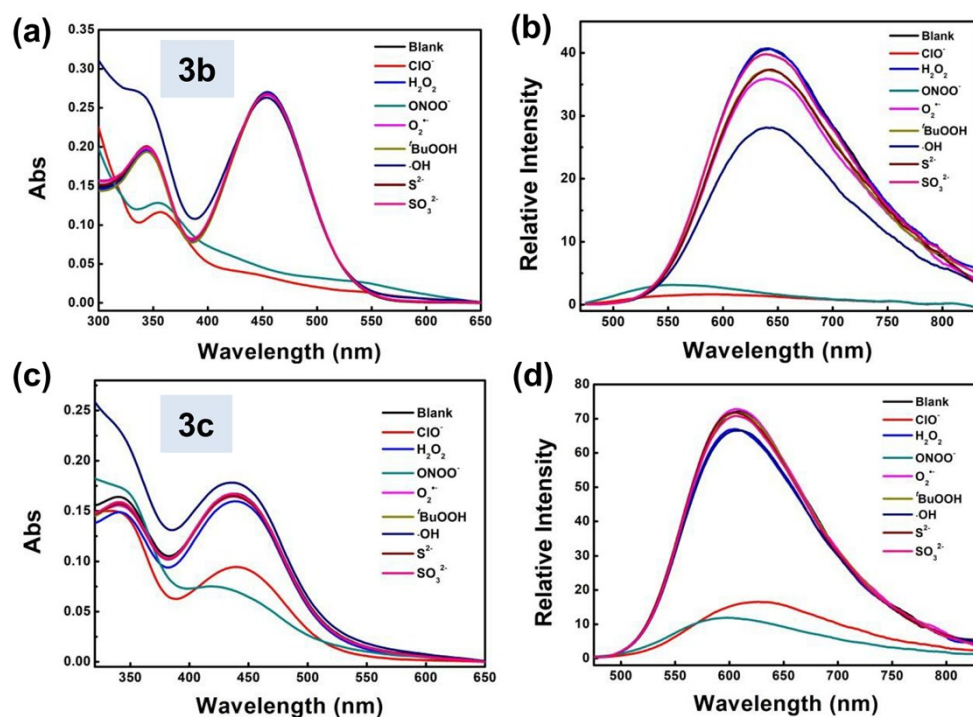
**3a**: 43 mg, 24.9%. <sup>1</sup>H NMR (600 MHz, DMSO-*d*<sub>6</sub>) δ 8.13 (s, 1H), 7.91 (d, *J* = 1.9 Hz, 1H), 7.83 (s, 2H), 7.73 (dd, *J* = 8.6, 1.9 Hz, 1H), 7.23 – 7.19 (m, 1H), 7.14 (dd, *J* = 7.6, 1.4 Hz, 1H), 7.06 – 7.03 (m, 2H), 6.99 – 6.95 (m, 1H), 3.97 (q, *J* = 6.9 Hz, 2H), 1.32 (t, *J* = 6.9 Hz, 3H). <sup>13</sup>C NMR (150 MHz, DMSO-*d*<sub>6</sub>) δ 154.20, 147.49, 143.54, 130.61, 130.46, 128.37, 127.60, 126.79, 126.54, 123.59, 123.56, 122.50, 116.36, 115.45, 115.14, 114.36, 103.56, 42.07, 13.05. ESI-MS: *m/z* 346.1127 [M + H]<sup>+</sup>: (calcd 346.1121).

**3b**: 67 mg, 37.1%. <sup>1</sup>H NMR (600 MHz, DMSO-*d*<sub>6</sub>) δ 10.51 (s, 1H), 8.42 (s, 1H), 7.82 (s, 1H), 7.71 (s, 2H), 7.24 – 7.17 (m, 1H), 7.13 (dd, *J* = 7.6, 1.5 Hz, 1H), 7.04 (d, *J* = 8.2 Hz, 1H), 6.96 (td, *J* = 7.5, 0.9 Hz, 1H), 6.56 (s, 1H), 3.91 (q, *J* = 6.9 Hz, 2H), 1.34 (t, *J* = 6.9 Hz, 3H). <sup>13</sup>C NMR (150 MHz, DMSO-*d*<sub>6</sub>) δ 159.36, 151.47, 149.02, 142.96, 128.10, 127.44, 126.52, 125.40, 123.47, 122.99, 116.57, 116.31, 115.37, 114.61, 113.13, 104.49, 103.23, 42.36, 12.90. ESI-MS: *m/z* 362.1057 [M + H]<sup>+</sup>: (calcd 362.1070).

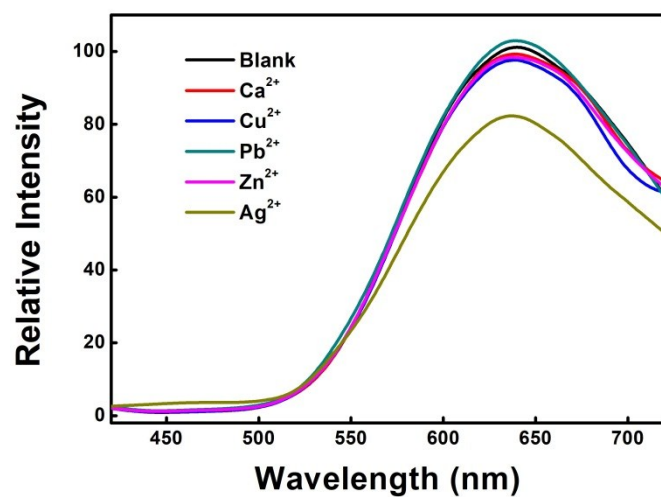
**3c**: 142 mg, 75.6%. <sup>1</sup>H NMR (600 MHz, DMSO-*d*<sub>6</sub>) δ 8.42 (s, 1H), 8.02 (s, 1H), 7.78 (s, 2H), 7.21 (ddd, *J* = 8.5, 7.4, 1.5 Hz, 1H), 7.15 (dd, *J* = 7.6, 1.5 Hz, 1H), 7.07 (d, *J* = 7.8 Hz, 1H), 6.98 (td, *J* = 7.5, 1.0 Hz, 1H), 6.63 (s, 1H), 4.04 (q, *J* = 6.9 Hz, 2H), 3.93 (s, 3H), 1.35 (t, *J* = 6.9 Hz, 3H). <sup>13</sup>C NMR (150 MHz, DMSO-*d*<sub>6</sub>) δ 160.22, 149.62, 149.12, 143.23, 128.15, 127.55, 125.91, 125.84, 123.66, 123.39, 118.34, 116.63, 115.28, 114.71, 114.54, 104.42, 99.95, 56.61, 42.23, 13.23. ESI-MS: *m/z* 376.1229 [M + H]<sup>+</sup>: (calcd 376.1227).



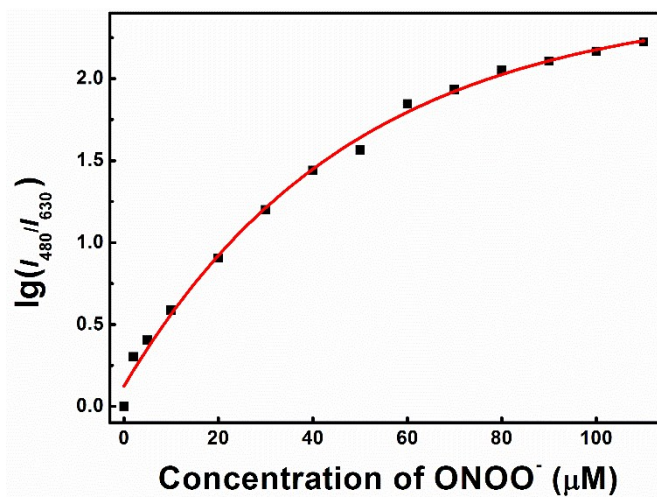
**Figure. S1** UV-vis absorption spectra of **3a** (10  $\mu\text{M}$ ) before and after addition of various reactive species (100  $\mu\text{M}$ ) in PBS solution (pH 7.2-7.4, 10 mM, containing 1 mM Triton X-100).



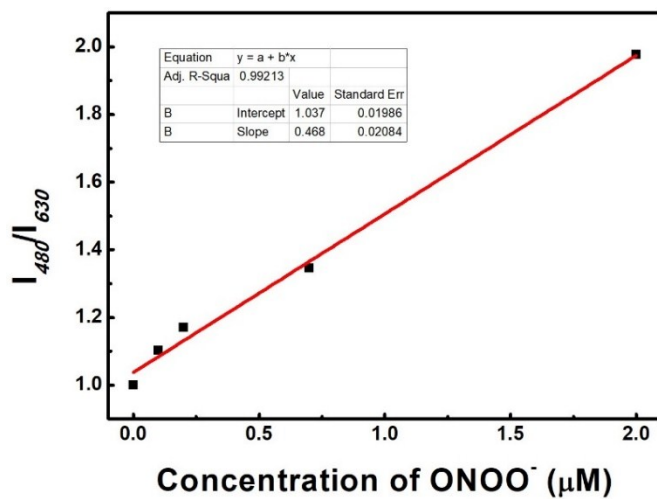
**Figure S2.** UV-vis absorption spectra and emission spectra of **3b** (a and b) and **3c** (c and d) (10  $\mu\text{M}$ ) before and after addition of various reactive species (100  $\mu\text{M}$ ) in PBS solution (pH 7.2-7.4, 10 mM, containing 1 mM Triton X-100). For **3b**:  $\lambda_{\text{ex}} = 455$  nm, slit width: 6 nm/6 nm. For **3c**:  $\lambda_{\text{ex}} = 440$  nm, slit width: 6 nm/6 nm.



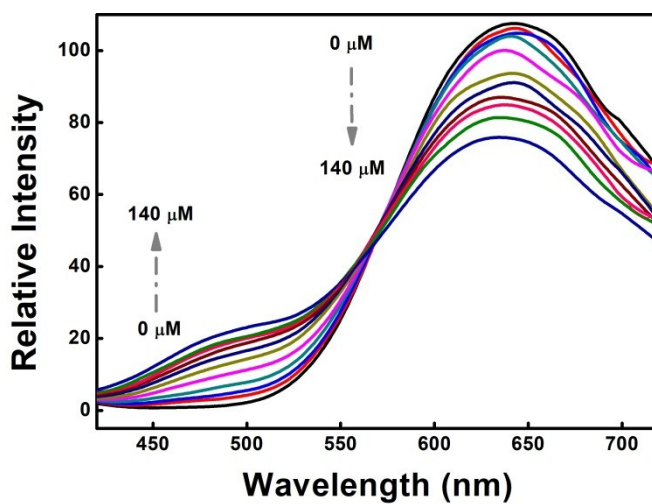
**Figure S3.** Emission spectra of **3a** before and after addition of various metal ions (100  $\mu\text{M}$ ) in PBS solution (pH 7.2-7.4, 10 mM, containing 1 mM Triton X-100).  $\lambda_{\text{ex}} = 370$  nm, slit width: 6 nm/6 nm.



**Figure S4.** Fluorescence titration plot of **3a** toward  $\text{ONOO}^-$ .  $\lambda_{\text{ex}} = 370$  nm, slit width: 6 nm/6 nm.

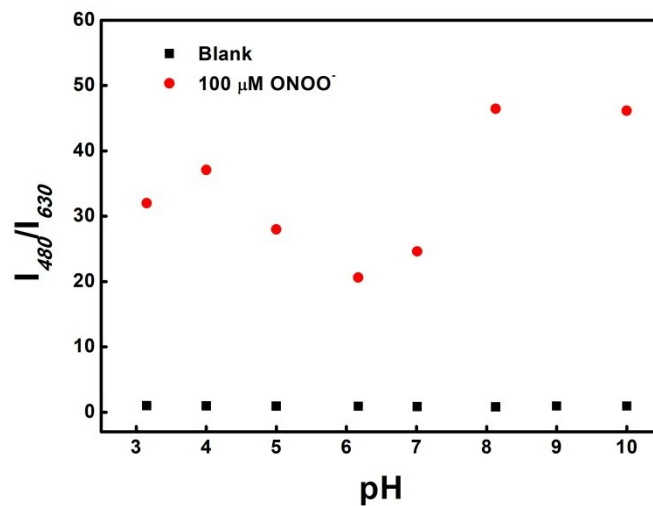


**Figure S5.** Linear relationship between fluorescence ratios ( $I_{480}/I_{630}$ ) of **3a** (10  $\mu\text{M}$ ) versus various concentrations of  $\text{ONOO}^-$ .

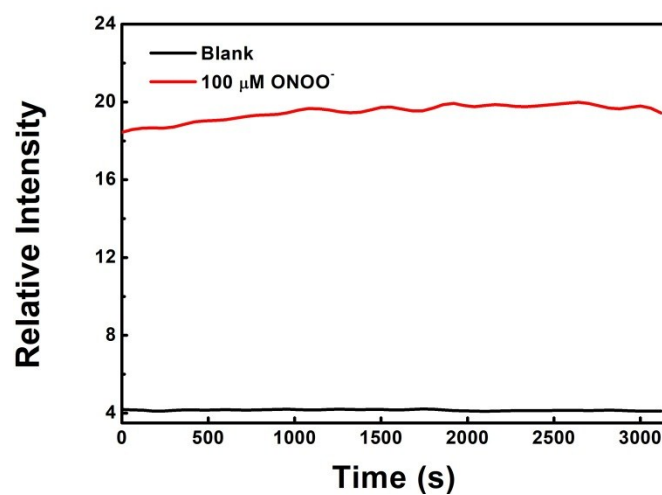


**Figure S6.** Fluorescence titration of **3a** toward  $\text{ClO}^-$ .  $\lambda_{\text{ex}} = 370 \text{ nm}$ , slit width: 6 nm/6 nm.

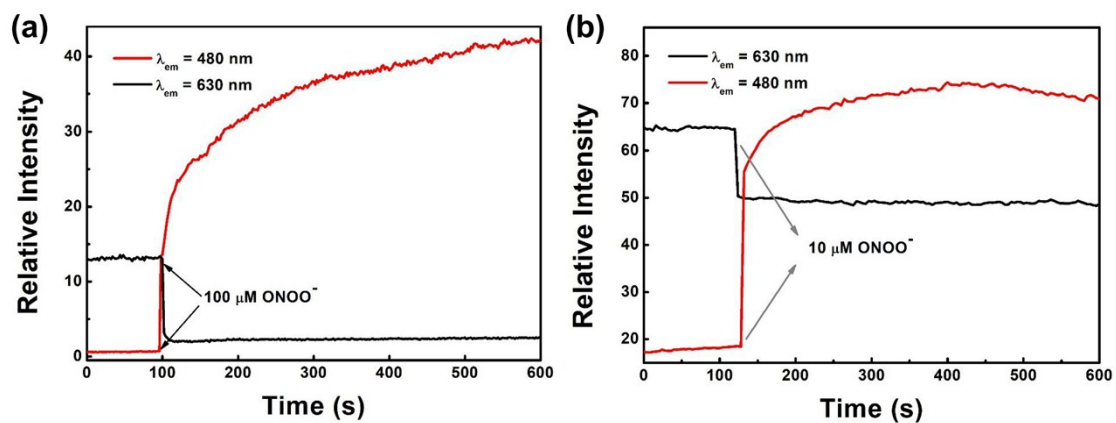




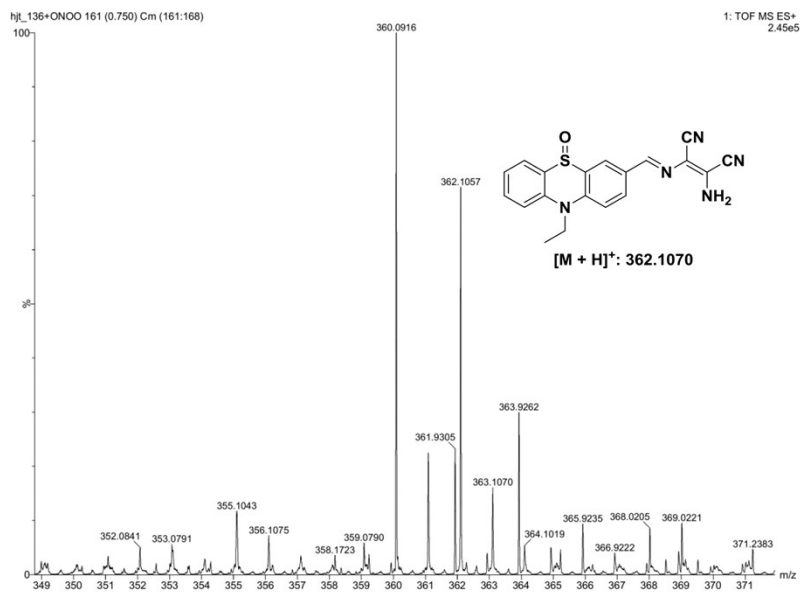
**Figure S7.** Fluorescence emission ratios ( $I_{480}/I_{630}$ ) of **3a** (10  $\mu\text{M}$ ) in the presence or absence of  $\text{ONOO}^-$  (100  $\mu\text{M}$ ) in B-R buffer solutions with different pH values.



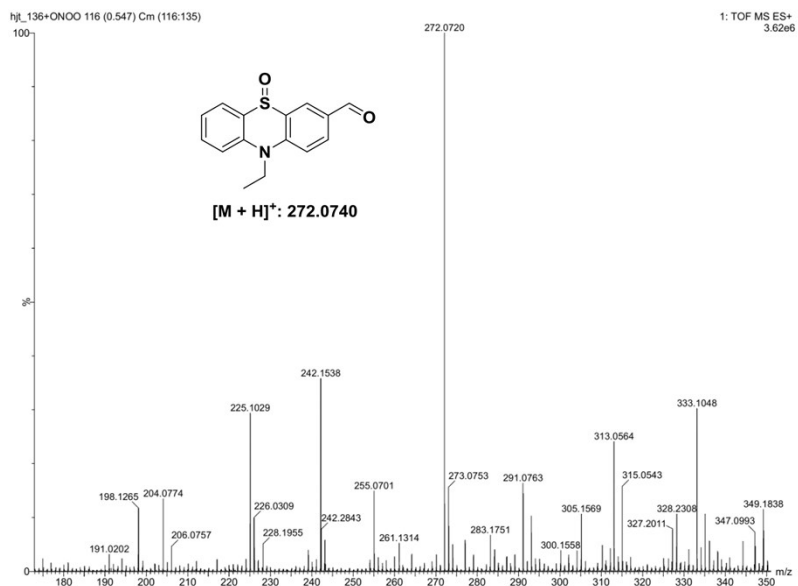
**Figure S8.** Dynamic emission intensity of **3a** (10  $\mu\text{M}$ ) with or without  $\text{ONOO}^-$  (100  $\mu\text{M}$ ). For **3a**,  $\lambda_{\text{em}} = 630 \text{ nm}$ ; for **3a** with  $\text{ONOO}^-$ ,  $\lambda_{\text{em}} = 480 \text{ nm}$ .  $\lambda_{\text{ex}} = 370 \text{ nm}$ .



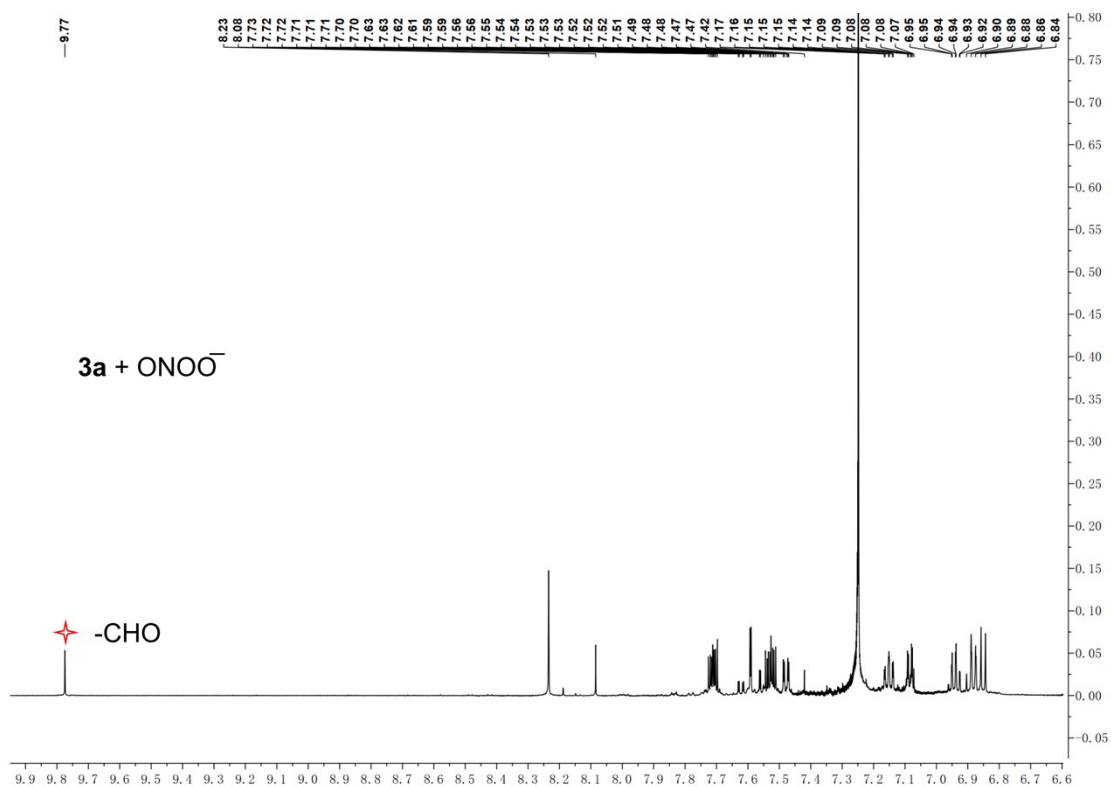
**Figure S9.** Time dependent fluorescence emission of **3a** (10 μM) to (a) ONOO<sup>-</sup> (10 μM) and (b) ONOO<sup>-</sup> (100 μM).



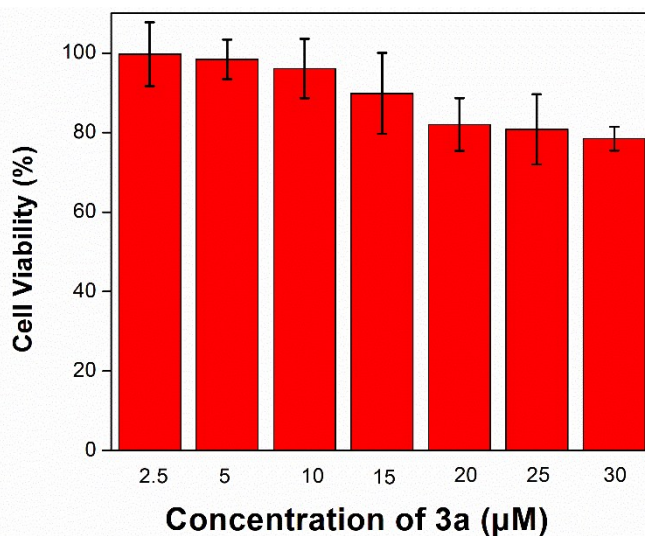
**Figure S10.** ESI spectra of **3a** upon addition of 10 equiv ONOO<sup>-</sup> (100 μM).



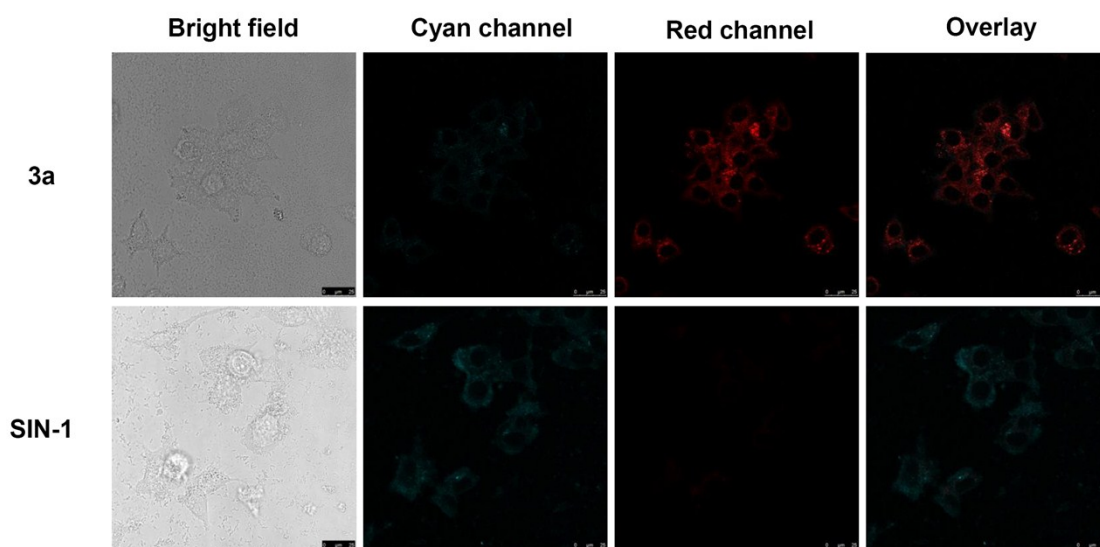
**Figure S11.** ESI spectra of **3a** upon addition of 10 equiv ONOO<sup>-</sup> (100 μM).



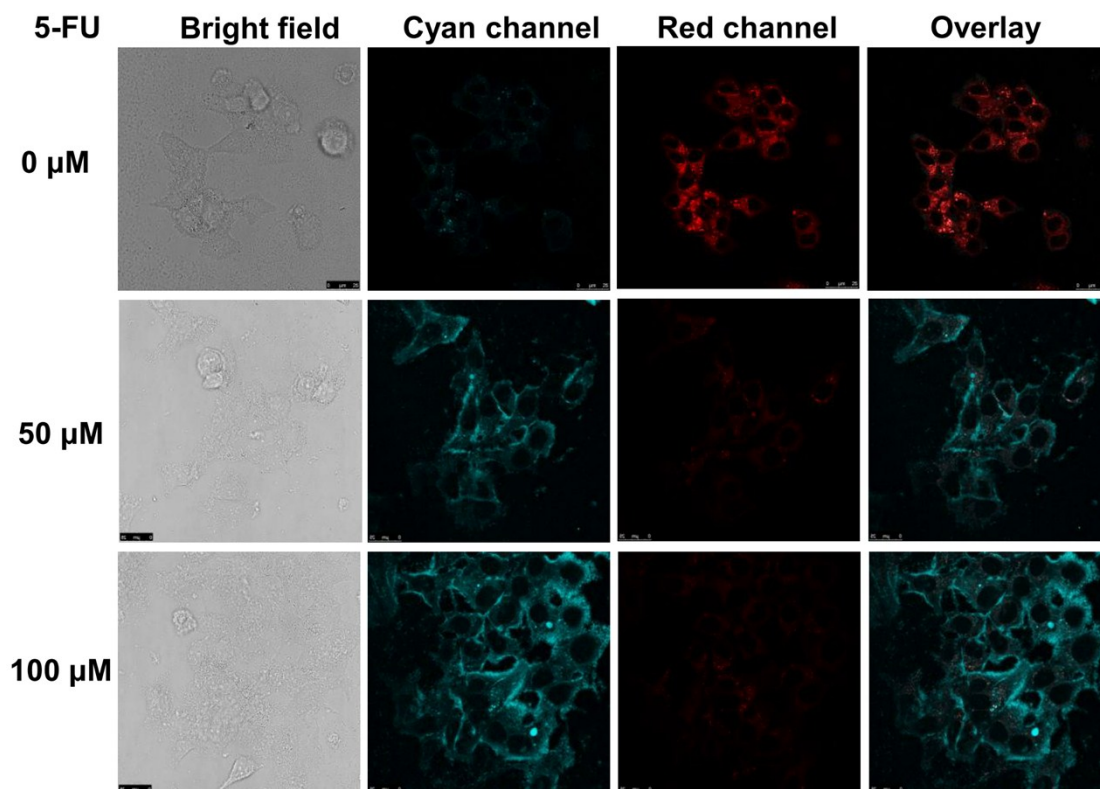
**Figure S12.** <sup>1</sup>H NMR spectrum of **3a** upon addition of 10 equiv ONOO<sup>-</sup> (100 μM) in CDCl<sub>3</sub>.



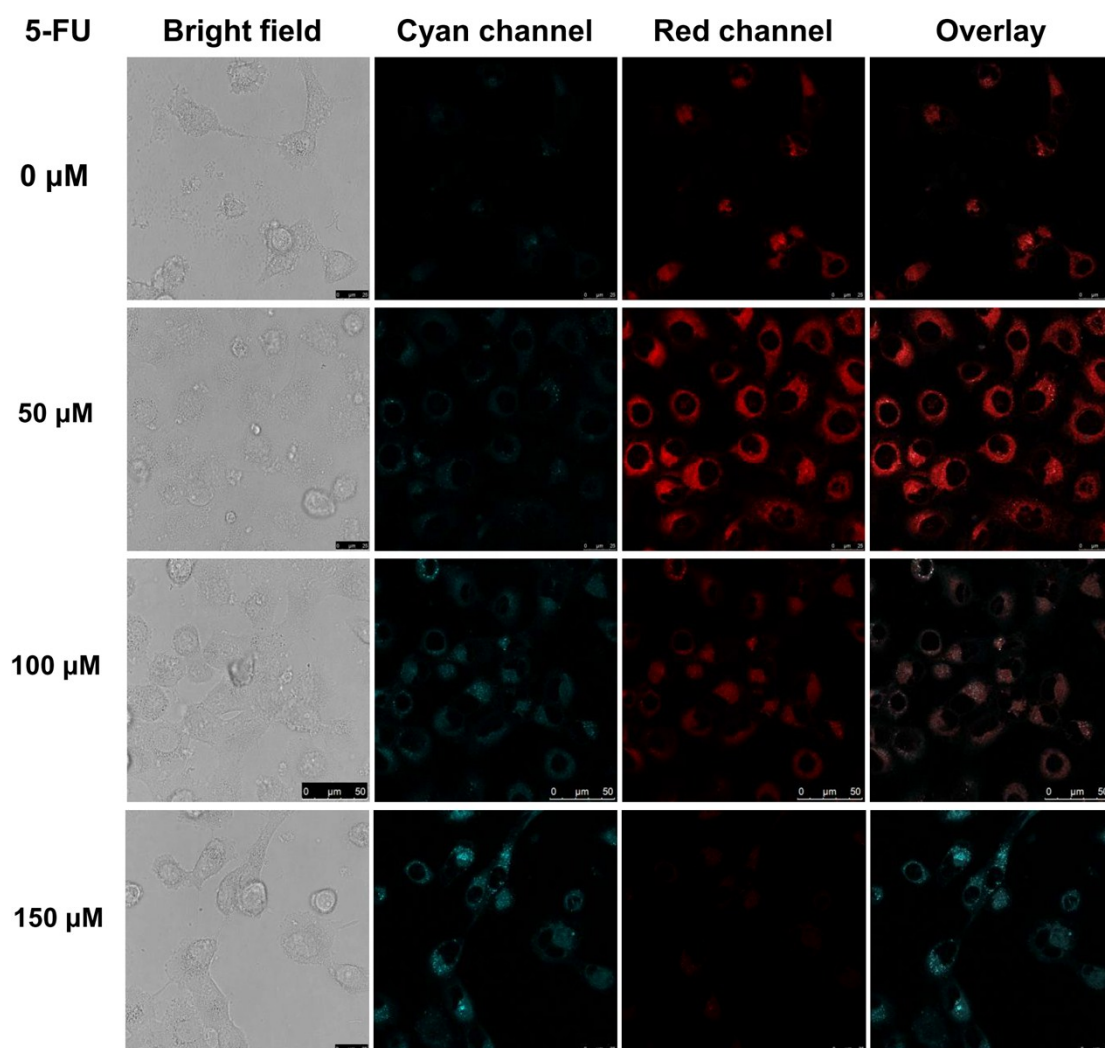
**Figure S13** Cytotoxicity of **3a**. MCF-7 cells were incubated with **3a** (0–30 µM) for 24 h. Results are mean ± SD, n = 5.



**Figure S14.** Confocal fluorescence images of exogenous  $\text{ONOO}^-$  in MCF-7 cells. MCF-7 cells were stained with 10 µM **3a** for 30 min (top), and incubated with 100 µM SIN-1 for another 30 min (down). Scale bar: 25 µm. Cyan channel:  $\lambda_{\text{ex}} = 405 \text{ nm}$ ,  $\lambda_{\text{em}} = 450 \text{ nm} - 510 \text{ nm}$ ; red channel:  $\lambda_{\text{ex}} = 405 \text{ nm}$ ,  $\lambda_{\text{em}} = 610 \text{ nm} - 670 \text{ nm}$ .

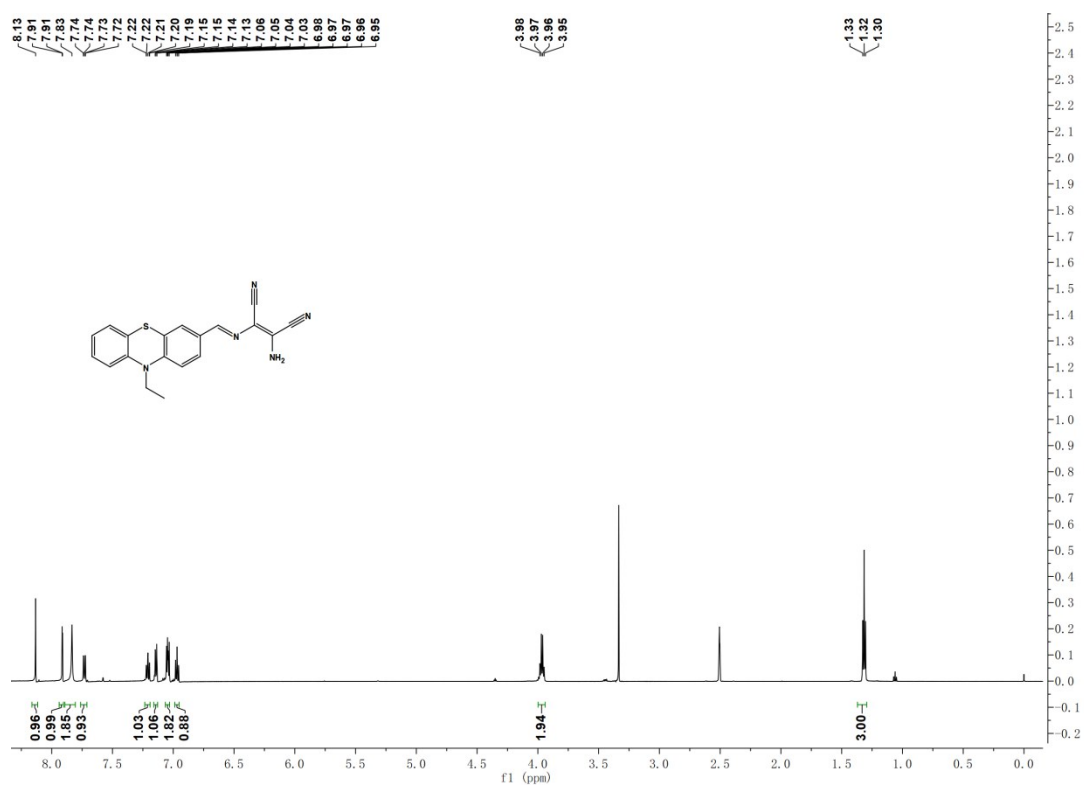


**Figure S15.** Detection of ONOO<sup>-</sup> generation in 5-FU-treated MCF-7 cells. (a) MCF-7 cells were stained with different amounts of 5-FU (0  $\mu\text{M}$ , 50  $\mu\text{M}$ , 100  $\mu\text{M}$ ) for 20 h. Then, probe **3a** (10  $\mu\text{M}$ ) was added for incubation for another 30 min before confocal imaging. (b) Intensity ratio (cyan/red) changes *versus* different concentrations of 5-FU. Error bars are  $\pm$  SEM. Scale bar: 25  $\mu\text{m}$ . Cyan channel:  $\lambda_{\text{ex}} = 405 \text{ nm}$ ,  $\lambda_{\text{em}} = 450 \text{ nm} - 510 \text{ nm}$ ; red channel:  $\lambda_{\text{ex}} = 405 \text{ nm}$ ,  $\lambda_{\text{em}} = 610 \text{ nm} - 670 \text{ nm}$ .

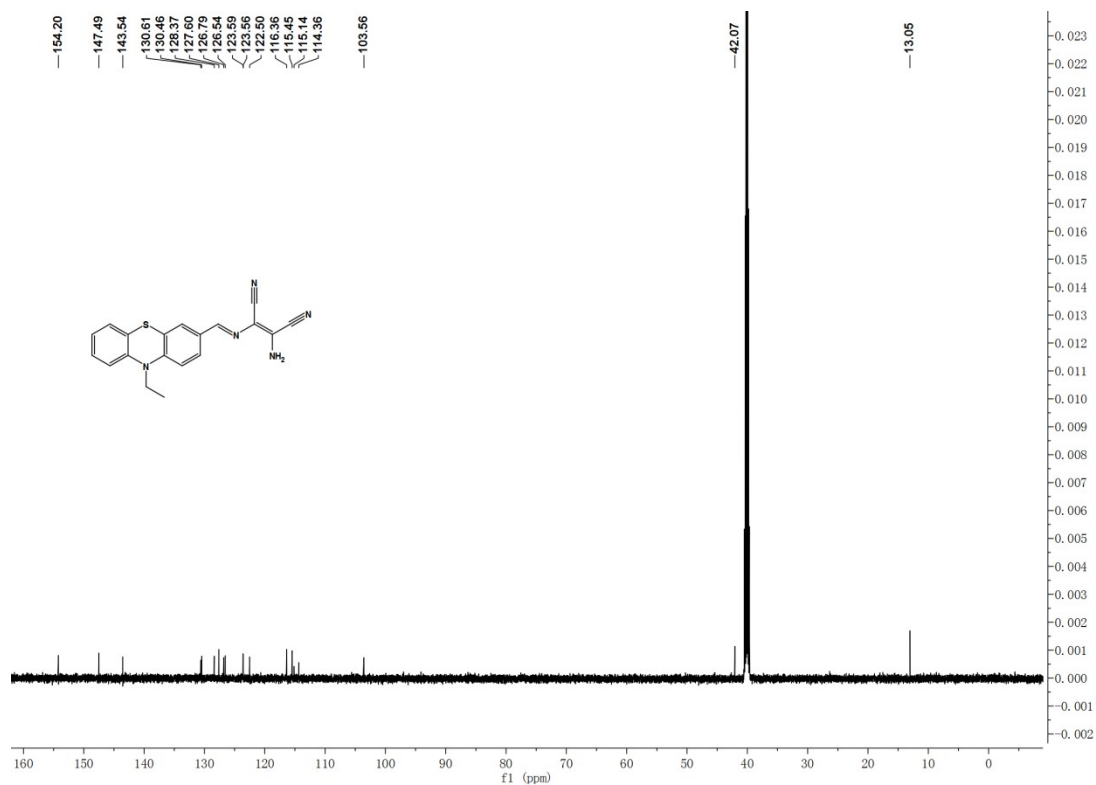


**Figure S16.** Detection of ONOO<sup>-</sup> generation in 5-FU-treated Het-1A cells. (a) Het-1A cells were stained with different amounts of 5-FU (0  $\mu\text{M}$ , 50  $\mu\text{M}$ , 100  $\mu\text{M}$ , 150  $\mu\text{M}$ ) for 20 h. Then, probe **3a** (10  $\mu\text{M}$ ) was added for incubation for another 30 min before confocal imaging. (b) Intensity ratio (cyan/red) changes *versus* different concentrations of 5-FU. Error bars are  $\pm$  SEM. Cyan channel:  $\lambda_{\text{ex}} = 405 \text{ nm}$ ,  $\lambda_{\text{em}} = 450 \text{ nm} - 510 \text{ nm}$ ; red channel:  $\lambda_{\text{ex}} = 405 \text{ nm}$ ,  $\lambda_{\text{em}} = 610 \text{ nm} - 670 \text{ nm}$ .

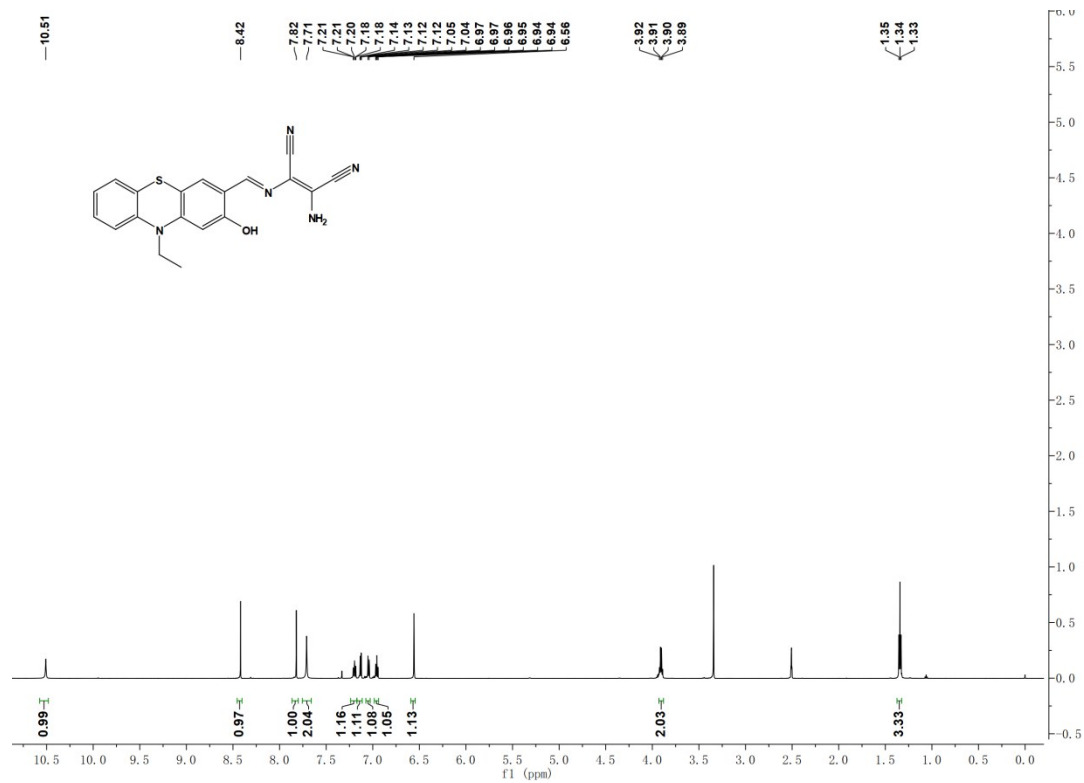
$^1\text{H}$  NMR Spectrum of **3a** in  $\text{DMSO-}d_6$  (600 MHz):



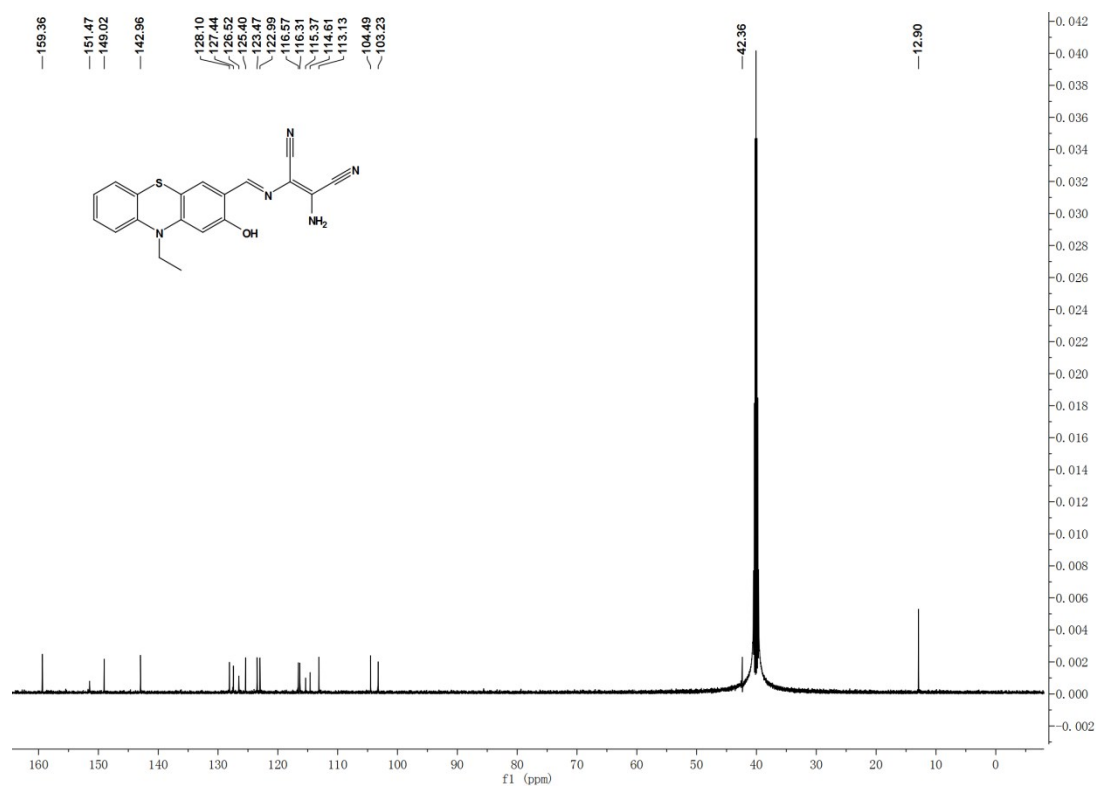
$^{13}\text{C}$  NMR Spectrum of **3a** in  $\text{DMSO-}d_6$  (150 MHz):



$^1\text{H}$  NMR Spectrum of **3b** in  $\text{DMSO-}d_6$  (600 MHz):

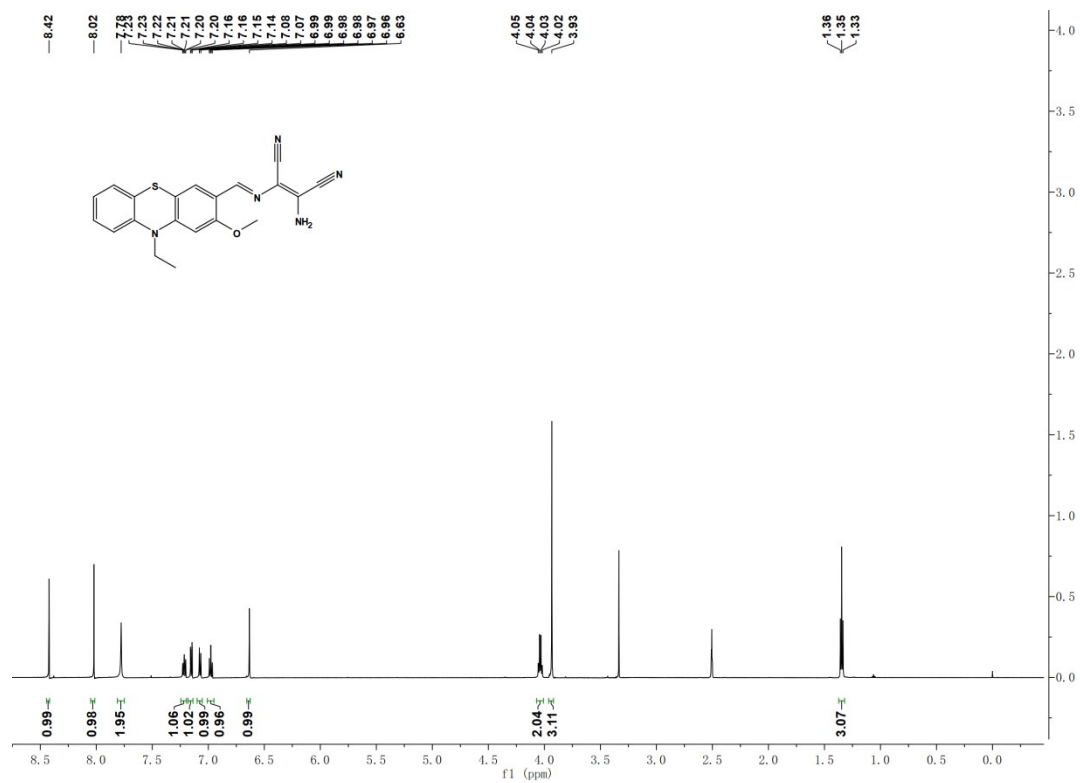


<sup>13</sup>C NMR Spectrum of **3b** in DMSO-*d*<sub>6</sub> (150 MHz):

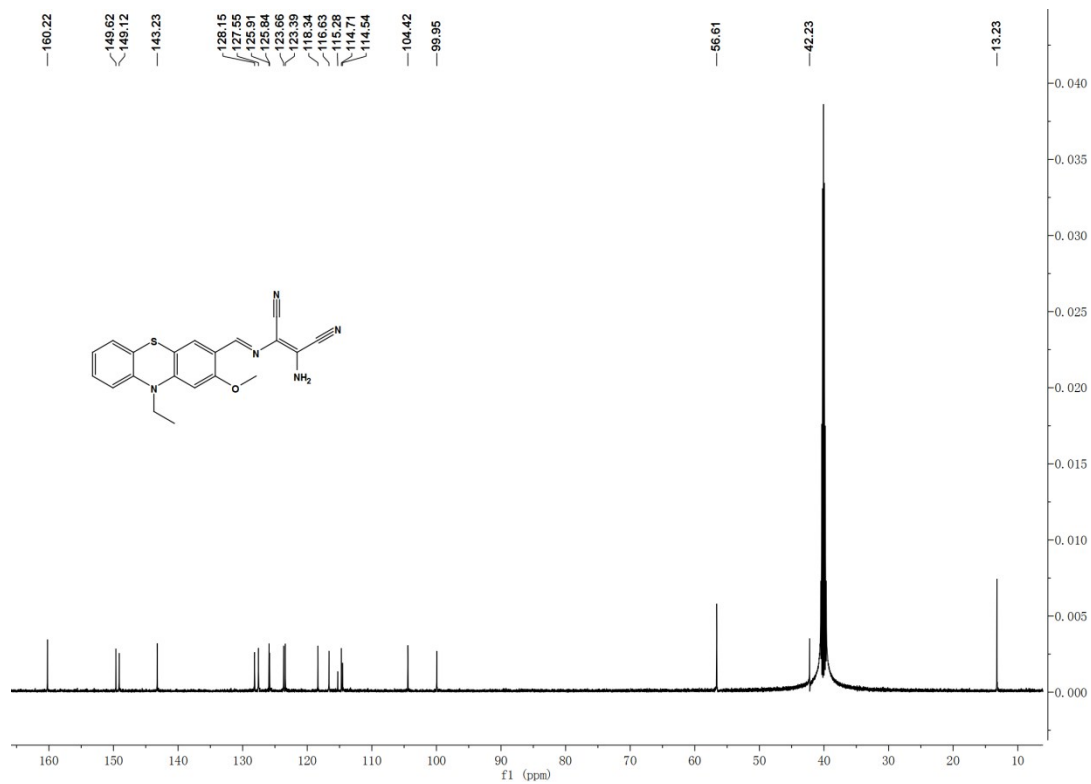


<sup>1</sup>H NMR Spectrum of **3c** in DMSO-*d*<sub>6</sub> (600 MHz):

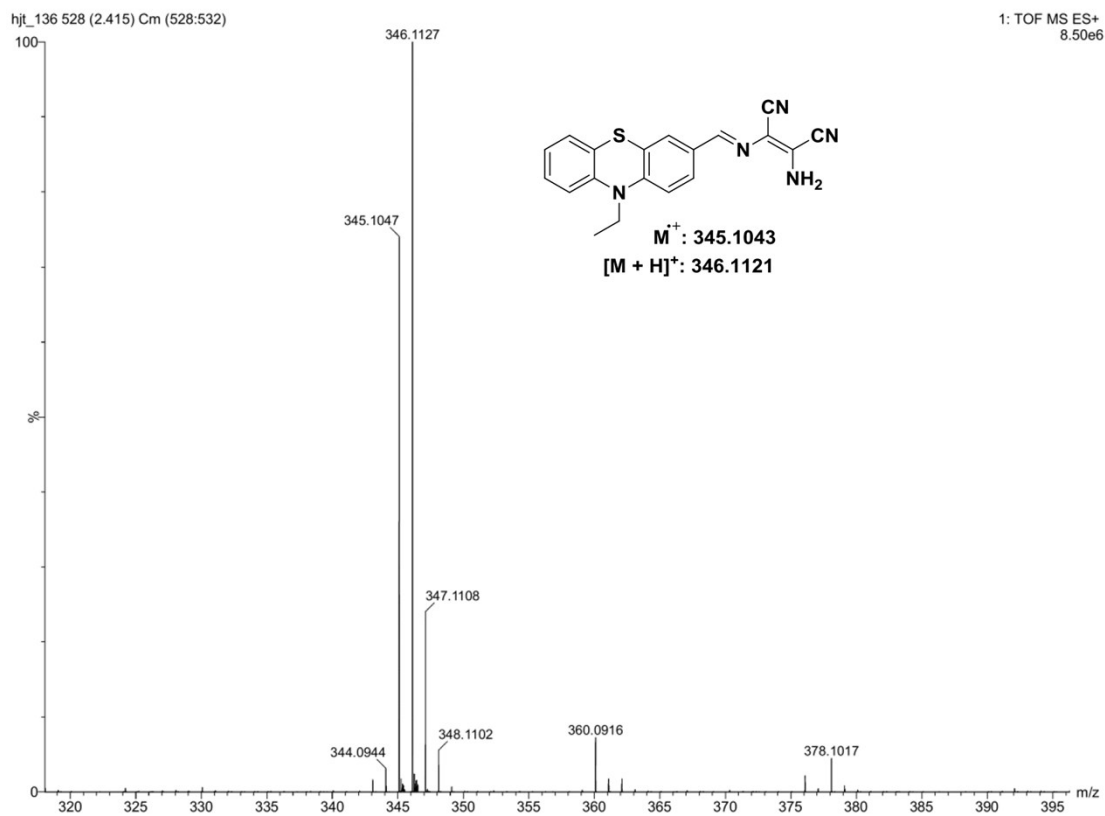




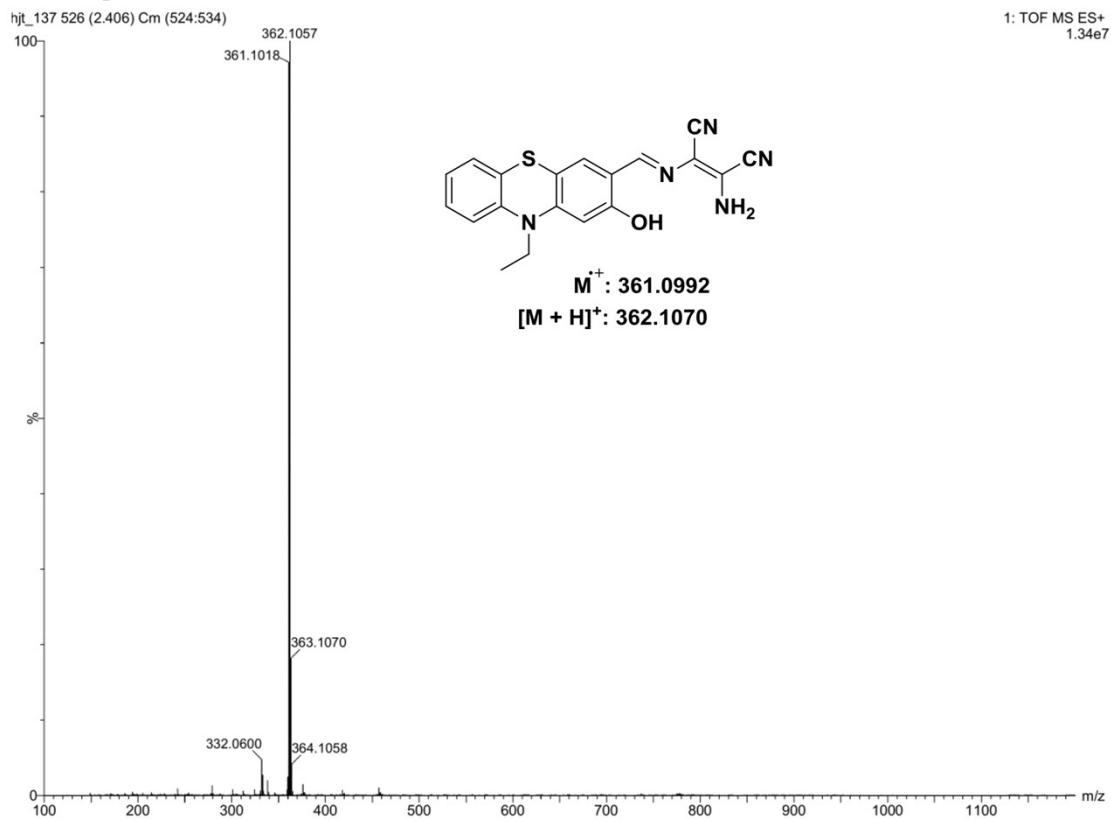
**<sup>13</sup>C NMR Spectrum of 3c in DMSO-*d*<sub>6</sub> (150 MHz):**



**HRMS spectra of 3a:**



HRMS spectra of **3b**:



HRMS spectra of **3c**:

hjt\_138 537 (2.454) Cm (537:552)

1: TOF MS ES+  
1.19e7

