Supporting Information

A near-infrared GPX4 fluorescent probe for non- small cell lung cancer imaging

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Experimental section

1. Materials and instruments

The general chemicals used in the report were purchased from Energy Chemical Co., and J&K Scientific Ltd., and all of the solvents were of analytic grade. All reactions were monitored by layer chromatography (TLC) using 0.25 mm silica gel plates with UV indicator (GF-254).

^{1}H NMR and ^{13}C NMR spectra were detected by Vaian DLG 400 spectrometer and Bruker Avance III 400 spectrometer. Mass spectrometric (MS) data were carried out using LTQ Orbit rap XL instruments and TOF LC/MS instruments. Absorption spectra were measured on a PerkinElmer Lambda 35 UV/vis spectrophotometer (PerkinElmer). Fluorescence spectra were obtained with a VAEIAN CARY Eclipse fluorescence spectrophotometer (Serial No. FL0812: M018). Flow cytometry was measured by Acoustic Focusing Flow Cytometer. Confocal laser scanning microscope (CLSM) images were performed on Olympus FV3000 confocal laser.

2. Synthesis of ENBO-ML210

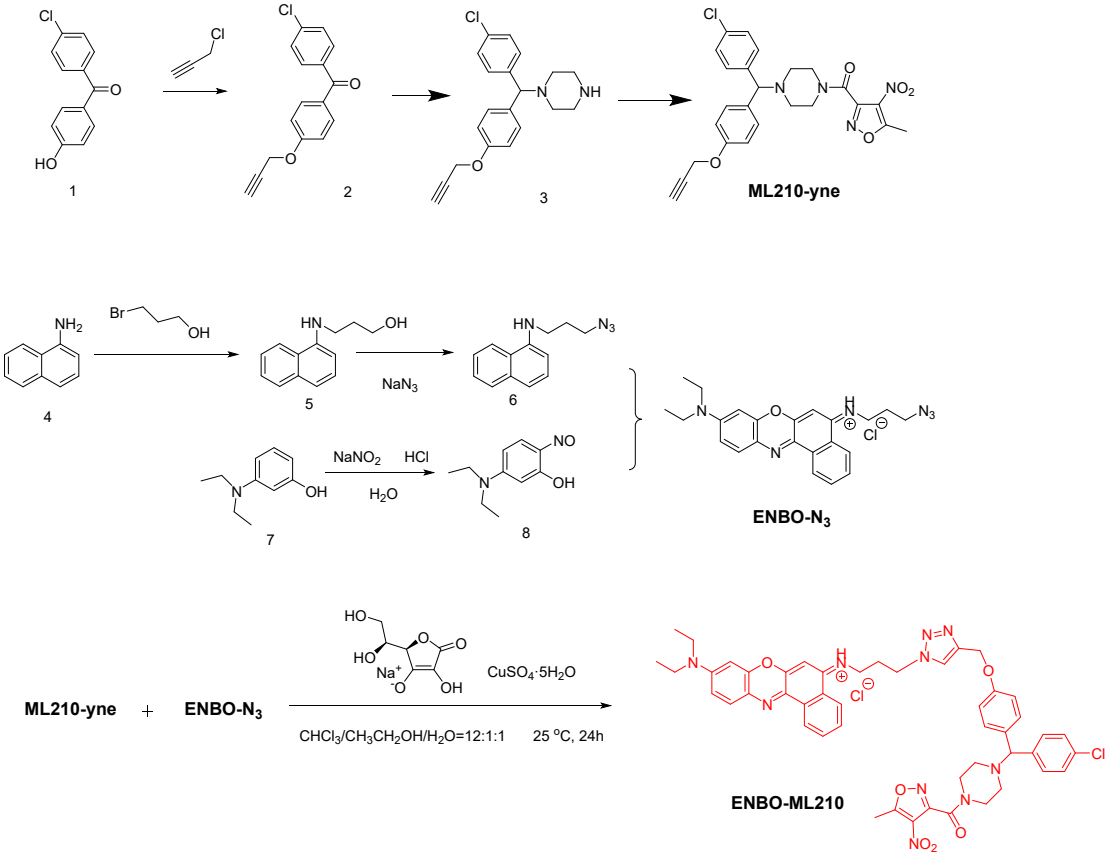

Fig. S1 Synthetic route of **ENBO-ML210**.

The GPX4 inhibitor **ML210-yne** was synthesized according to reference with a yield of 30%.[1] Fluorophore **ENBO-N_3** was synthesized according to reference,[2] get dark blue solid 0.23 g, yield: 47.4%, ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.42 – 9.25 (m, 1H), 8.84 – 8.72 (m, 1H), 7.90 – 7.67 (m, 3H), 6.99 (dd, *J* = 9.4, 2.7 Hz, 1H), 6.68 (s, 1H), 6.59 (d, *J* = 2.7 Hz, 1H), 3.94 (t, *J* = 7.1 Hz, 2H), 3.67 – 3.55 (m, 6H), 2.26 (p, *J* = 6.7 Hz, 2H), 1.35 (t, *J* = 7.1 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 158.82, 153.13, 151.54, 147.52, 135.22, 132.59, 131.96, 130.70, 130.56, 128.80, 126.23, 124.34, 124.04, 114.05, 95.74, 93.22, 53.47, 49.12, 45.98, 42.22, 28.35, 18.44, 12.71. HRMS: m/z ([$C_{23}H_{25}N_6O^+$]) calculated for 401.2084; found: 401.2081.

Synthesis of fluorescence probe **ENBO-ML210: ENBO-N_3** (100 mg, 0.229 mmol), **ML210-yne** (136 mg, 0.275 mmol), sodium ascorbate (54 mg, 0.275 mmol), $CuSO_4$•$5H_2O$ (37 mg, 0.151 mmol) were added to a two-neck round bottom flask. The mixture was dissolved in 14 mL of mixed solvents (chloroform/ethanol/water = 12/1/1), and reacted at room temperature for 24 h under nitrogen atmosphere and dark conditions. After the reaction, the solution was distilled under reduced pressure to remove the solvent, and the product was separated by column chromatography (eluent: CH_2Cl_2/MeOH=20/1) to obtain 35 mg of a dark blue solid, yield: 11.9%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.19 (s, 1H), 8.83 (d, *J* = 8.1 Hz, 1H), 8.62 (d, *J* = 8.2 Hz, 1H), 8.30 (s, 1H), 7.98 (t, *J* = 7.6 Hz, 1H), 7.93 – 7.85 (m, 2H), 7.44 (d, J = 8.2 Hz, 2H), 7.39 – 7.26 (m, 5H), 7.05 (s, 1H), 6.94 (dd, *J* = 12.3, 5.4 Hz, 3H), 5.06 (s, 2H), 4.59 (t, *J* = 6.9 Hz, 2H), 4.39 (s, 1H), 3.78 (q, *J* = 6.9 Hz, 2H), 3.69 (q, *J* = 7.0 Hz, 6H), 2.81 (s, 3H), 2.37 (q, *J* = 6.8 Hz, 4H), 2.21 (s, 2H), 1.24 (t, *J* = 6.9 Hz, 8H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 173.86, 158.03, 157.69, 156.60, 154.06, 153.41, 152.07, 148.45, 143.21, 142.13, 134.37, 133.96, 133.13, 132.42, 131.84, 131.21, 130.22, 130.05, 129.7….9, 129.29, 129.01, 128.73, 125.22, 124.53, 124.35, 123.80, 115.96, 115.30, 96.07, 94.03, 73.14, 61.65, 56.50, 51.81, 51.06, 47.68, 45.94, 42.06, 29.15, 19.02, 14.00, 13.01. HRMS: m/z calculated for ([$C_{48}H_{48}ClN_{10}O_6^+$]): 895.3435; found: 895.3432.

3. Cell confocal imaging experiments

In this experiment, NCI-H1299 (human non-small cell lung cancer) cells were used as the research object, and MCF-7 (human breast cancer cells), 4T1 (mouse breast cancer cells) and LO2 (human normal liver cells) were as control. H1299 cells were cultured in RPMI-1640 medium containing 10% imported fetal bovine serum and 1%

penicillin/streptomycin; MCF-7, 4T1 and LO2 cells were cultured in DMEM medium containing 10% fetal bovine serum and 1% penicillin/streptomycin. All cell lines were grown to logarithmic growth phase at 37°C in a 5% CO_2 incubator. After trypsinization, a cell suspension of 1×10^5 cells/mL was prepared for subsequent experiments.

Cellular uptake imaging. Take 100 μL of NCI-H1299 cell suspension (concentration of $1x10^5$ cells/mL), put it into a co-polymerization petri dish containing 2 mL of medium, and incubate at 37 °C in a 5% CO_2 incubator for 24 h. Rinse twice with PBS buffer and add 0.5 μL of 2 mM **ENBO-ML210** stock solution. The fluorescence intensity of the intracellular fluorescent inhibitor in different time periods were observed under a confocal microscope. Excitation wavelength: 640 nm, fluorescence emission wavelength: 650-750 nm.

Cell competitive imaging. 100 μL of NCI-H1299 cell suspension (concentration of $1x10^5$ cells/mL) was put into a co-polymerization dish containing 2 mL of fresh medium, and cultured at 37 °C in a 5% CO_2 incubator for 24 h. The first group was added with 0.5 μL of 2 mM **ENBO-ML210** stock solution; the second group was first added with 2 μM inhibitor ML210yne and incubated for 1 h. then, 0.5 μL of 2 mM **ENBO-ML210** stock solution was added. The fluorescence intensity of the red channel was observed after 30 min and 90 min under a confocal microscope, respectively. Excitation wavelength: 640 nm, fluorescence emission wavelength: 650-750 nm.[3]

Cytotoxicity experiments (MTT). Mitochondrial dehydrogenases in living cells can reduce MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to formazan crystals. NCI-H1299 cells were prepared into a single-cell suspension with a concentration of 5×10^4 cells/mL, and 100 μL per well was seeded into a 96-well plate, and the surrounding 32 wells were filled with 100 μL PBS buffer, 37 °C, after 24 h incubation, different concentrations **ENBO-ML210** were added. In the experimental group, 100 μL **ENBO-ML210** and ML210yne with different concentrations were added, respectively, so that the respective concentrations were 0-10 μM, 6 duplicate wells were set per group, and were incubated for another 24 h. Replace the original culture medium with 100 μL of medium solution containing 0.5 mg/mL MTT. After the cells were incubated for 4 h, the culture medium was carefully removed, the supernatant was aspirated, and 100 μL of DMSO was added to each well and shaken on a shaker for 10 min to dissolve the blue formazan. Finally, absorbance (OD value) was measured with a UV-Vis microplate reader at wavelengths of 490 nm and 570 nm (630 nm).

$$\text{Cell viability\%} = (OD_{dye} - OD_{blank})/(OD_{control} - OD_{blank}) \times 100\%$$

4. Flow cytometry

H1299 cells and LO2 cells were prepared using 6-well plates and were seeded at 1 $\times 10^4$ cells/well. After 24 h incubation at 37 °C, different concentrations of **ENBO-ML210** (0.1 μM, 0.2 μM, 0.3 μM, 0.5 μM) were incubated for 2 h. Then, cells were washed with cold PBS for three times, detached by trypsin-EDTA (Gibco) and centrifuged. Resuspend the cell pellet in 0.5 mL of PBS and detected through a flow cytometer. Data were analyzed by using FlowJo software.

5. *In vivo* fluorescence imaging

All animal experiments involved in this study were conducted in accordance with the "Guidelines for the Care and Use of Laboratory Animals" published by the National Institutes of Health (8th edition, 2011) and approved by the Animal Ethics Review Committee of Dalian University of Technology 5-6 weeks Balb/c female nude mice were used in the experiment. The preparation concentration is 1×10^7 cells/mL of NCI-H1299 cell suspension in PBS, 100 μL was injected subcutaneously into the right thigh of mice with a 1 mL syringe. After the tumor volume reached about 100 mm^3 (about 14 days), it was used for in vivo tumor-targeted fluorescence imaging. The above tumor-bearing mice were taken and injected with 100 μL **ENBO-ML210** (2 μM) from the tail vein, and then the distribution of **ENBO-ML210** at different time points (1, 3, 6, and 12 h) was observed with a small animal imager. After imaging, the test mice were euthanized, tumor and organs such as heart, liver, spleen, lung and kidney were dissected and collected, and corresponding fluorescent images were collected.

6. Intracellular immunofluorescence imaging

H1299 cells were cultured for 24 h, 1 μM **ENBO-ML210** was added to the cells and incubated for 2 h. Then the cells were fixed with 1 mL immunostaining fixing solution for 10-15 min and washed twice with immunostaining wash solution for 5 min. After removing the wash solution, add 1 mL immunostaining blocking solution and block it for 60 min, while shake gently on a shaker. Incubation of primary antibody: for GPX4 detection, cells were incubated with GPX4 rabbit polyclonal antibody diluted 1:100 in immunostaining primary antibody diluent overnight at 4 °C. Incubation of secondary antibody: Alexa fluor 488-labeled goat anti-rabbit IgG(H+L) (1:1000) was incubated

for 60 min at room temperature. After washing three times, DAPI (Nuclear dye) in PBS was added and incubated for 5-10 min. It was possible to observe directly by CLSM.

7. Molecular docking

The molecular **ENBO-ML210** structure file was drawn and saved in pdb format as a covalent molecular docking ligand. The X-ray crystal structure of GPX4 was downloaded from PDB protein database, PDB code: 6HN3. ADFR software suite was used for covalent molecular docking, and the covalent binding site was Sec 46. Docking box center coordinates and dimensions: center x = -8.936, y = 2.775, z = -9.270; size: x = 30, y = 30, z = 30.

8. *In vivo* biosafety evaluation

After imaging, the mice were euthanized, and the tumors and major organs (heart, liver, spleen, lung, and kidney) of the mice were dissected and collected, and were fully fixed and preserved with 4% formalin. After paraffin-embedding, the tissues were sectioned, observed by H&E staining, and histological analysis was carried out.

9. Supporting figures

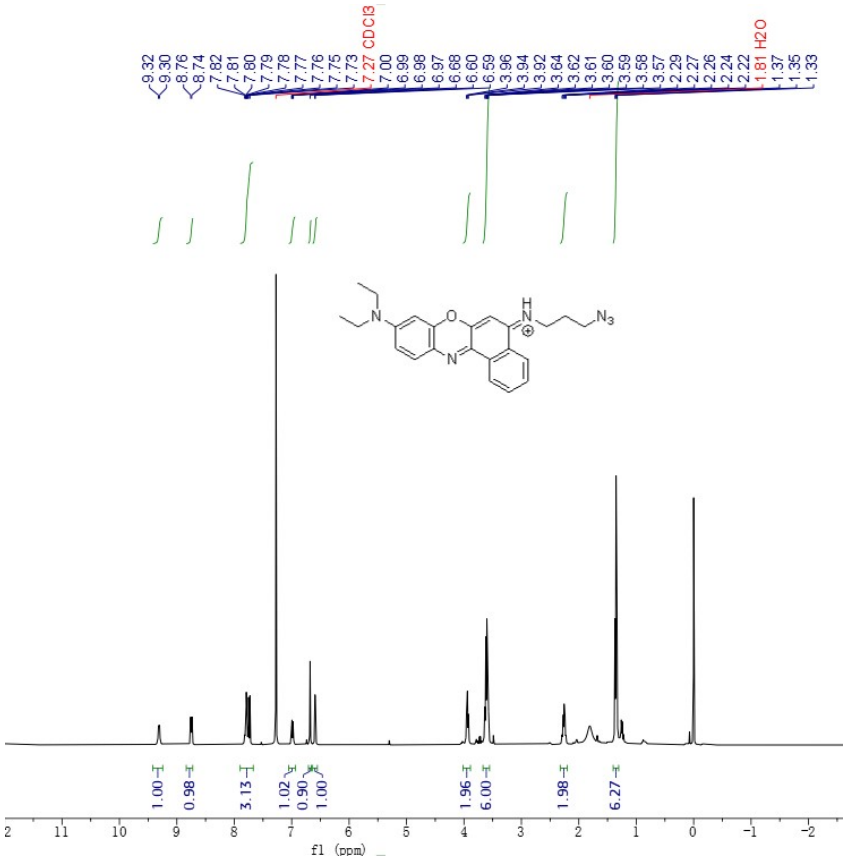

Fig. S2 ^{1}H NMR spectrum of **ENBO-N$_3$** in $CDCl_3$ (400 MHZ).

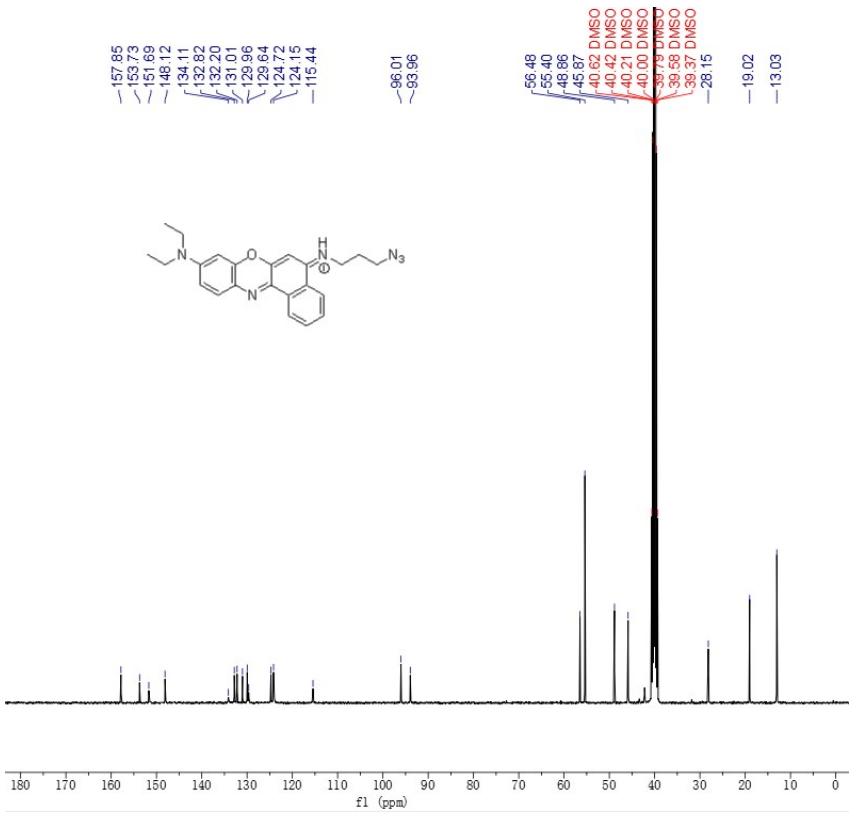

Fig. S3 ^{13}C NMR spectrum of **ENBO-N_3** in $CDCl_3$ (101 MHZ).

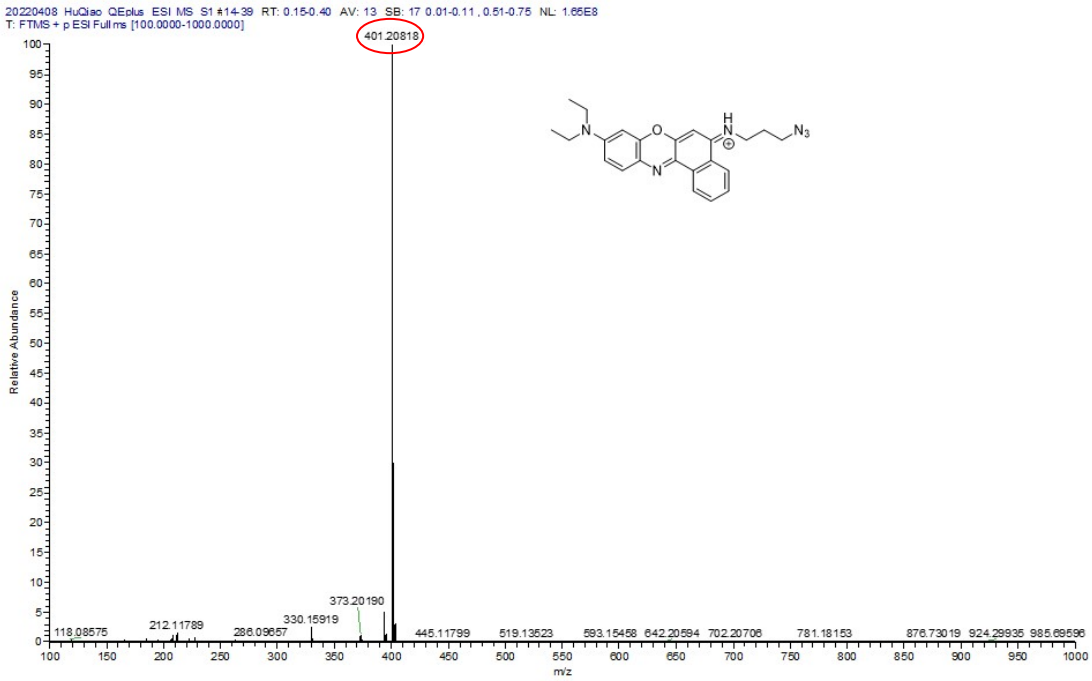

Fig. S4 HRMS (ESI) spectrum of **ENBO-N_3**.

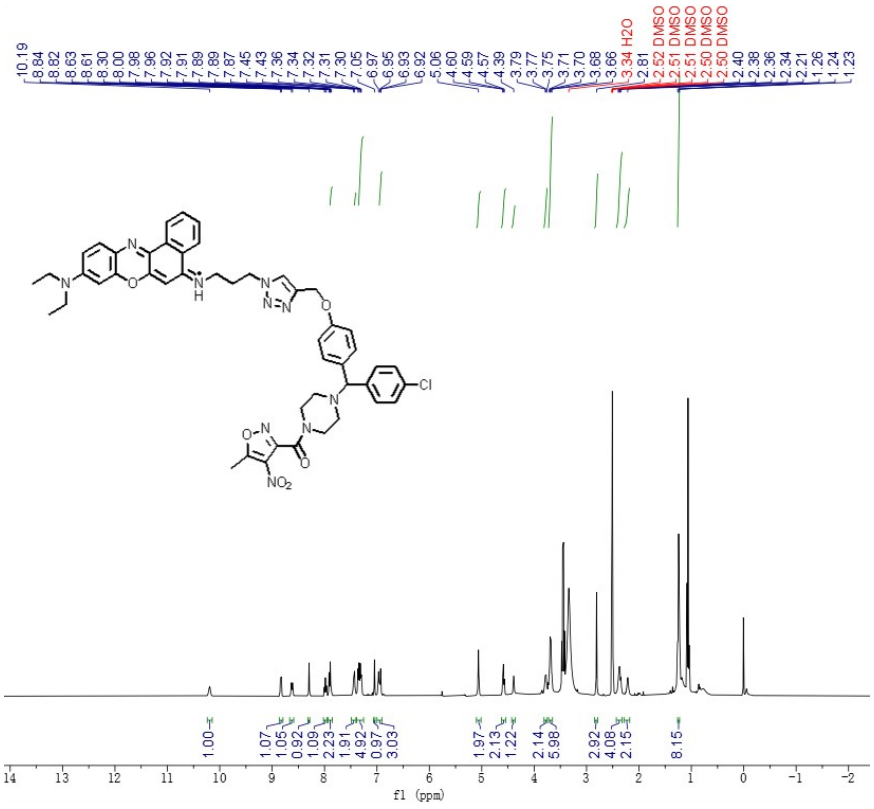

Fig. S5 ^{1}H NMR spectrum of **ENBO-ML210** in DMSO- d_6 (400 MHZ)

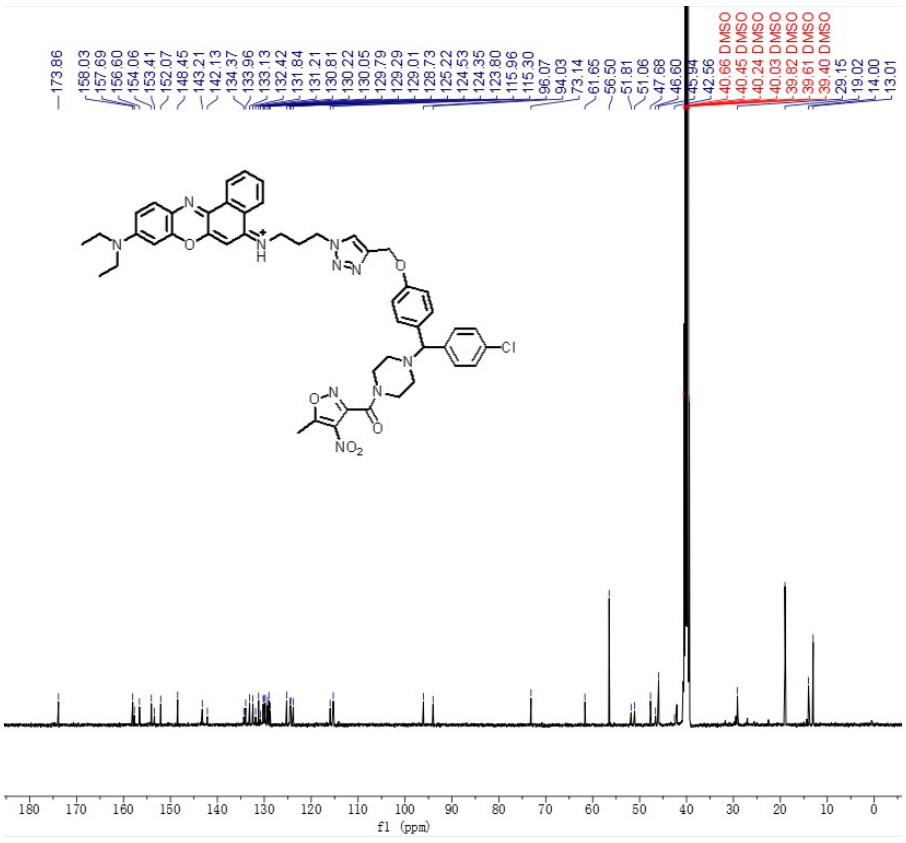

Fig. S6 ^{13}C NMR spectrum of **ENBO- ML210** in DMSO- d_6 (101 MHZ).

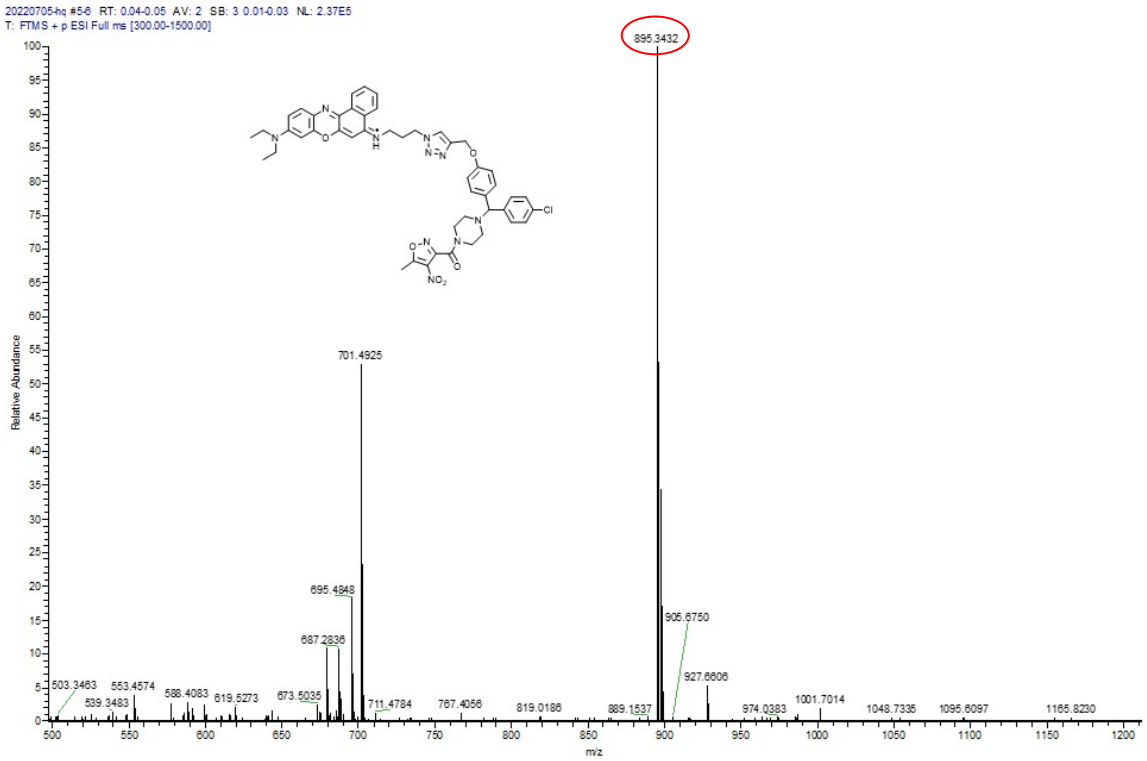

Fig. S7 HRMS (ESI) spectrum of **ENBO-ML210**.

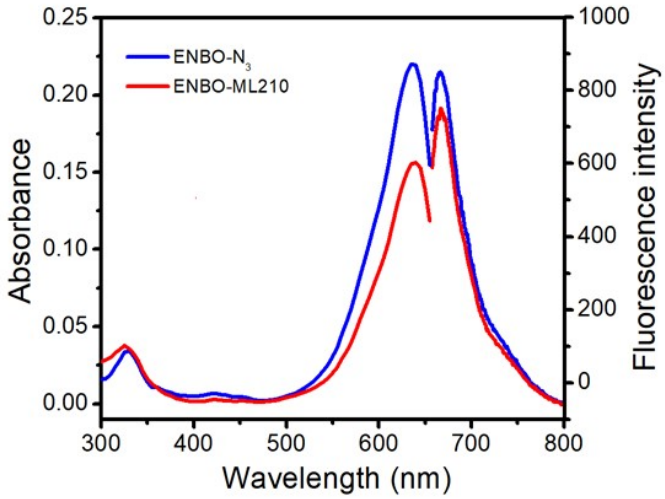

Fig. S8 UV-vis absorption spectra and fluorescence spectra of **ENBO-ML210** and **ENBO-N_3**.

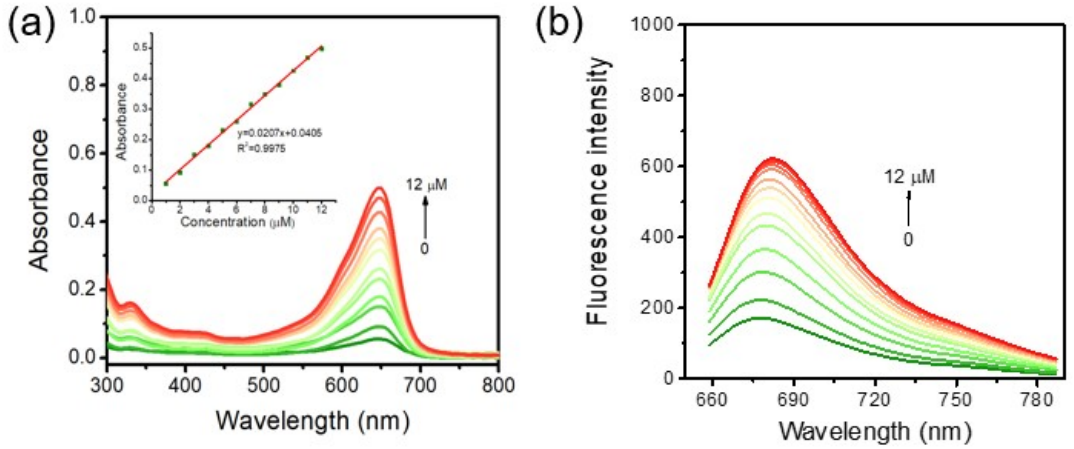

Fig. S9 (a) The absorbance and (b) fluorescence spectra of **ENBO-ML210** *versus* concentrations in MeOH/PBS solution (v/v=3/7, pH=7.4, 0.01 M) (inset: linear relationships).

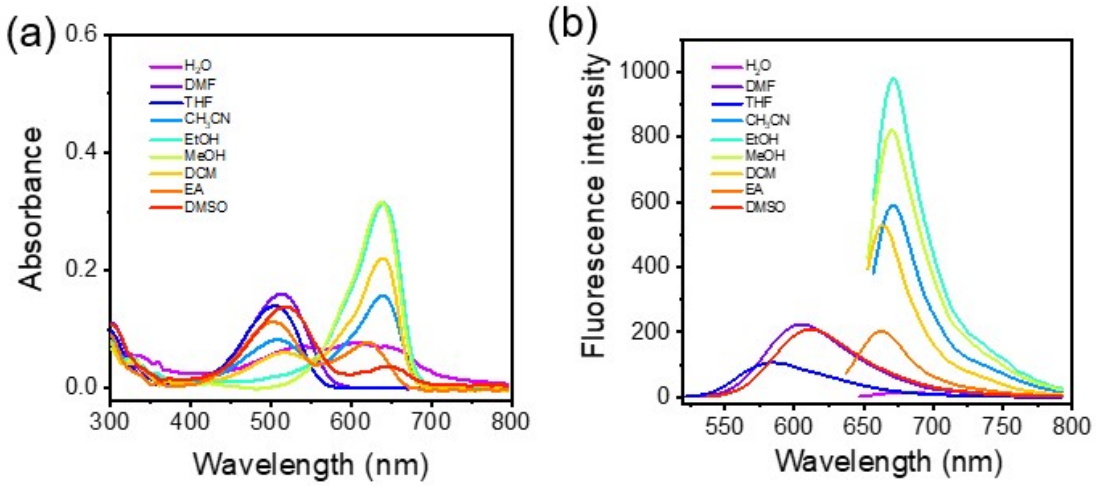

Fig. S10 (a) UV-vis absorption and (b) fluorescence spectra of **ENBO-ML210** in different solvents.

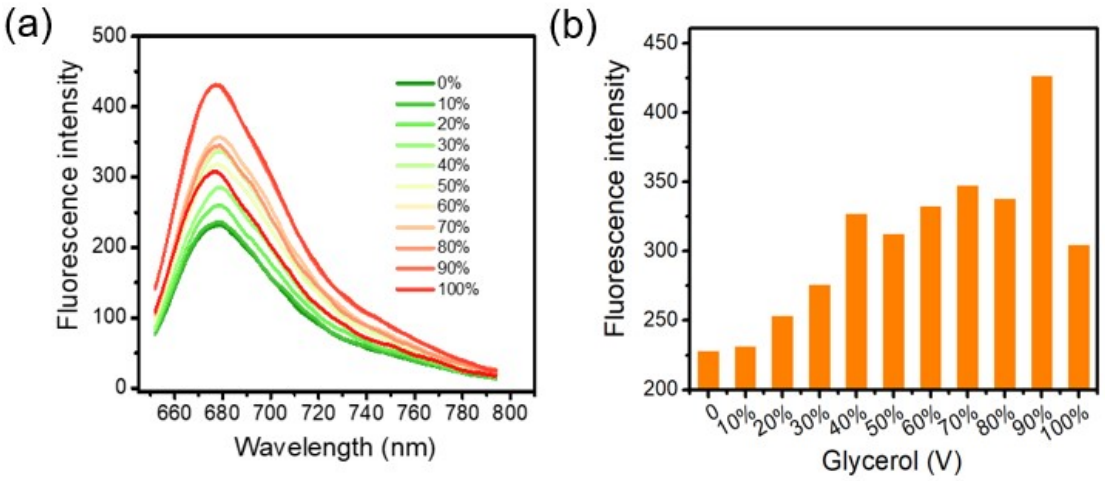

Fig. S11 Fluorescence spectra (a) and the fluorescence intensity changes (b) of **ENBO-ML210** in different ratios of glycerol/ methanol mixtures.

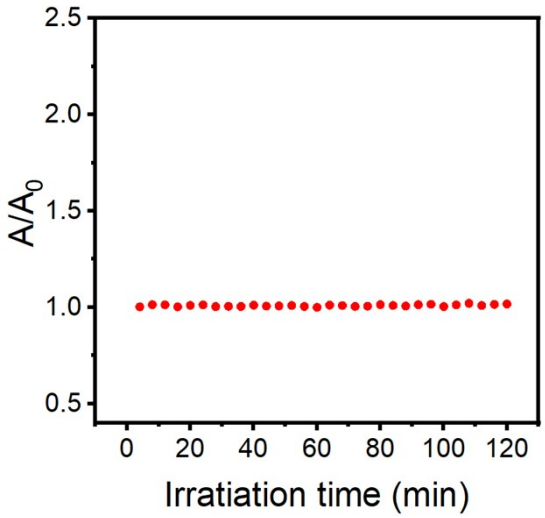

Fig. S12 Effect of 150 W Xe lamp irradiated time on the absorbance of **ENBO-ML210** in MeOH/PBS solution (v/v=3/7, pH=7.4, 0.01 M).

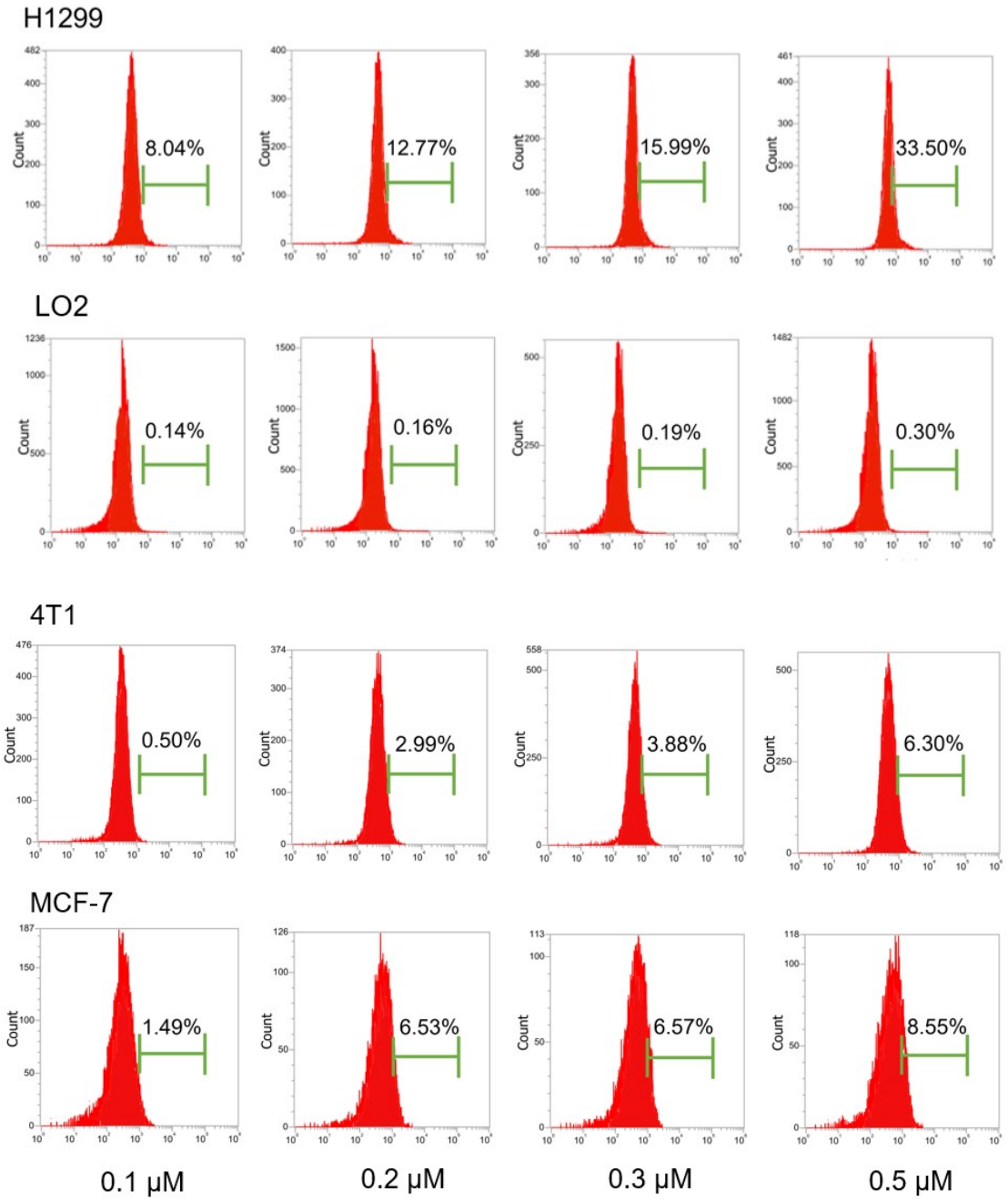

Fig. S13 The uptake of different concentrations of **ENBO-ML210** in H1299, LO2, 4T1 and MCF-7 cells was measured by flow cytometry (Numbers indicate the percentage of **ENBO-ML210** red fluorescence).

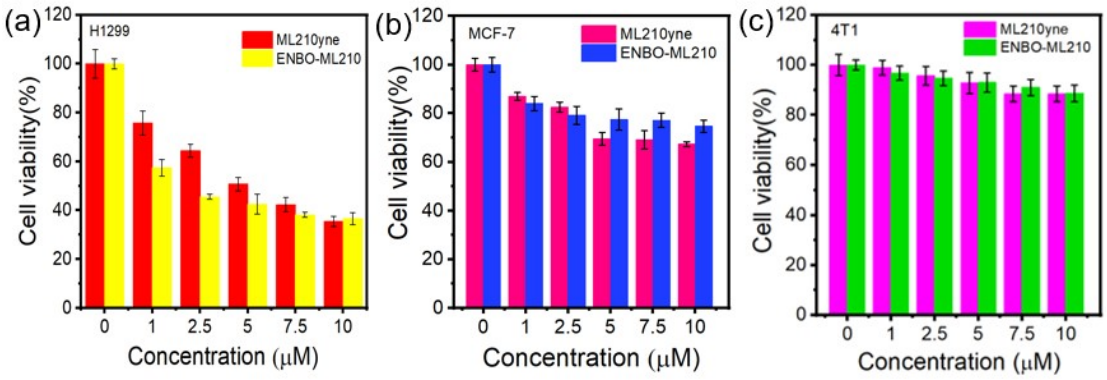

Fig. S14 Cytotoxicity of ML210yne and **ENBO-ML210** in (a) H1299, (b) MCF-7 and (c) 4T1 cells.

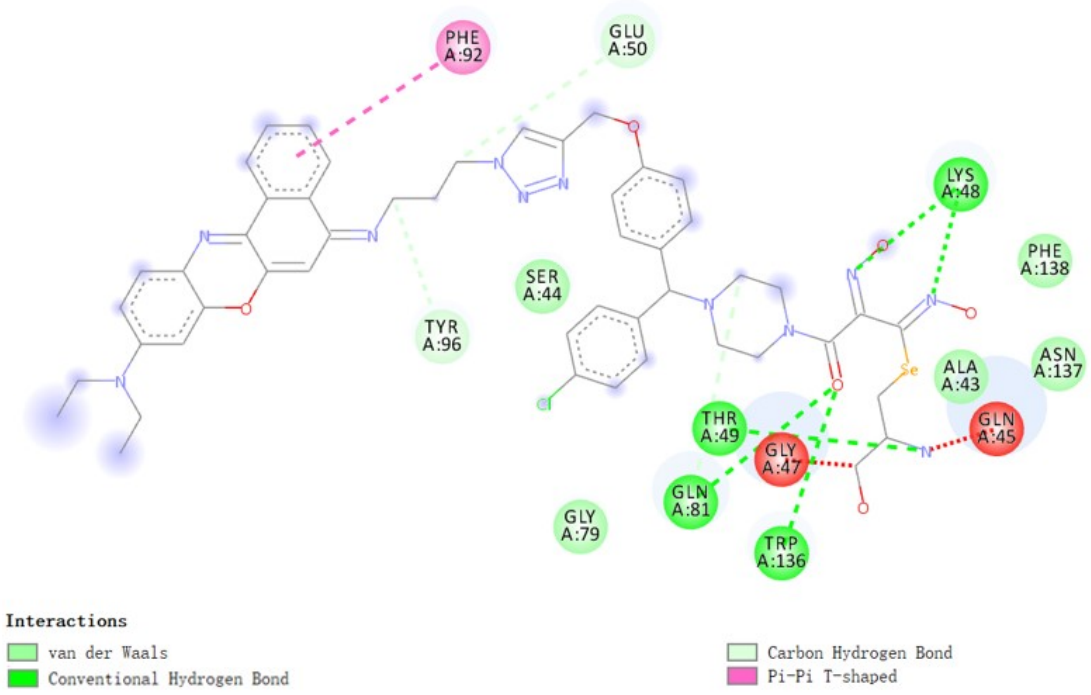

Fig. S15 Schematic diagram of 2D interaction between **ENBO-ML210** and GPX4 in molecular docking.

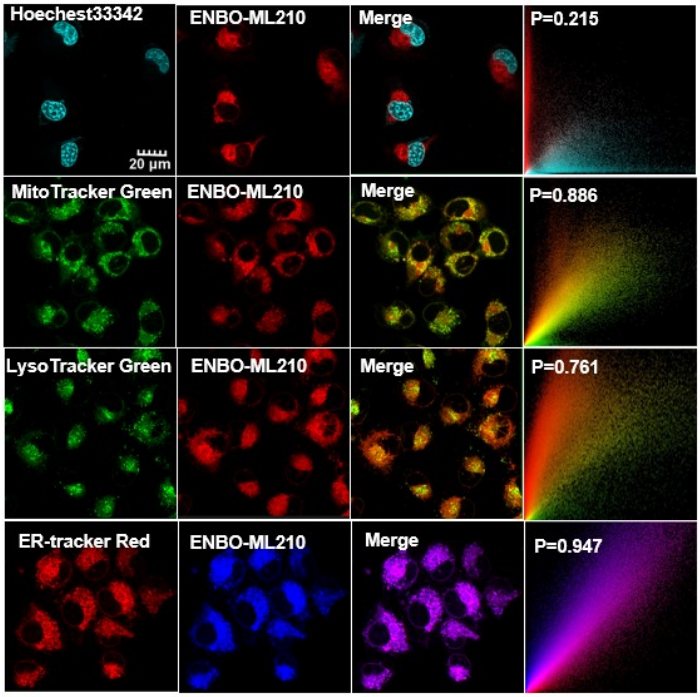

Fig. S16 Subcellular localization imaging of **ENBO-ML210** in H1299 cells. The excitation/emission wavelengths are provided as follows: **ENBO-ML210** (λex = 640 nm, λem = 650–750 nm), Hoechst 33342 (λex = 405 nm, λem = 440–480 nm), Mito Tracker Green (λex = 488 nm, λem = 500–550 nm), Lyso Tracker Green (λex = 488 nm, λem = 500–550 nm), and ER-tracker Red (λex = 561 nm, λem = 600-700 nm), scale bar = 20 μm.

References:

1. P. Kele, X. Li, M. Link, K. Nagy, A. Herner, K. Lorincz, S. Beni and O. S. Wolfbeis, *Org Biomol Chem*, 2009, **7**, 3486-3490.
2. J. K. Eaton, L. Furst, R. A. Ruberto, D. Moosmayer, A. Hilpmann, M. J. Ryan, K. Zimmermann, L. L. Cai, M. Niehues, V. Badock, A. Kramm, S. Chen, R. C. Hillig, P. A. Clemons, S. Gradl, C. Montagnon, K. E. Lazarski, S. Christian, B. Bajrami, R. Neuhaus, A. L. Eheim, V. S. Viswanathan and S. L. Schreiber, *Nat Chem Biol*, 2020, **16**, 497-506.
3. M. Maiorino, M. Conrad and F. Ursini, *Antioxid Redox Signal*, 2018, **29**, 61-74.