Electronic Supplementary Material (ESI) for Analyst.

Supporting Information

An easily available endoplasmic reticulum-targeted near-infrared fluorescent probe for esterase imaging *in vitro* and *in vivo*

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Experiment section

Materials

Solvents and other common reagents were obtained from Energy Chemical. Phosphate buffer saline (pH=7.4) was used to prepare all aqueous solutions. lysozyme, lipase, trypsin, tyrosinase, lactoferrin and pepsine were purchased from Sigma-Aldrich. Esterase was purchased from Shanghai Myrell Chemical Technology Co., Ltd. AEBSF was purchased from Shanghai Bide Pharmaceutical Technology Co., Ltd. Anhydrous ethanol, piperidine, anhydrous dichloromethane, n-hexane, ethyl acetate and sodium chloride were purchased from Shanghai Lingfeng Chemical Reagent Co., Ltd. 2-(3-cyano-4,5,5-trimethyl-5H-furan-2-ylidene) malononitrile was purchased from Suzhou Nakai Technology Co., Ltd. Potassium chloride, calcium chloride and magnesium chloride were purchased from Tianjin Damao Chemical Reagent Factory. Ferric chloride was purchased from Xilong Science Co., Ltd. GSSH was purchased from Wuhan Biocar biomedical Co., Ltd. Potassium bromide, aqueous hydrogen sulfide and acetyl chloride were purchased from Shanghai Macleans Biochemical Technology Co., Ltd. Adenosine-5-triphosphate disodium salt was purchased from Shanghai Aladdin Biochemical Technology Co., Ltd. Phosphate buffer saline (pH=7.4) was purchased from Biological Industries. Dimethyl sulfoxide was purchased from Beijing Bailingwei Technology Co., Ltd. Sodium hypochlorite was purchased from Hebei Bailingwei Superfine Materials Co., Ltd. Hochest33342 was purchased from Beijing Biolab Technology Co., Ltd. Mitochondrial tracker, endoplasmic reticulum tracker and lysosomal tracker were purchased from Thermo Fisher Scientific Co., Ltd. GSH, Cys and HCy were purchased from Shanghai Jizhi Biochemical Technology Co., Ltd. Mice were purchased from Jiangsu ALF Biotechnology Co. Ltd (Jiangsu, China).

Instruments

UV-vis absorption spectra were recorded on a Rarian 50 Conc UV-Visible spectrophotometer. Fluorescence emission spectra were recorded on Edinburgh FS5 fluorescence spectrophotometer, the power of the Edinburgh FS5 fluorescence spectrophotometer is 150W, the parameter settings were Source light path: Xe900, Emission detector light path: R928, Iris setting: 100 and Dwell time(s) :0.100. The ^{1}H NMR and ^{13}C NMR spectra were measured on a Bruker ARX 400 MHz spectrometer. Record high-resolution mass spectra (HRMS) on the GCT Premier CAB 048 mass spectrometer running in MALDI-TOF mode. The CCK8 test was carried out on Thermo's microplate reader (Mutiskan GO). A confocal laser scanning microscope (Stellaris 5, Leica, Germany) was used for cell imaging experiments, the parameter settings were scan mode: xyt, scan speed: 600 Hz, zoom: 2, laser intensity: 5.0000%, format: 1024×1024 and speed: 400. In vivo imaging was performed on the VISQUE® In Vivo Smart-LF imaging system, the parameter settings were exposure time: 10 second, binning: medium, f/stop:1, field of view: C and sbuject height: 1.50 cm. The absolute quantum yield of ER-CE was obtained in PL Quantam Yieled Spectrometer C11347 (HAMAMATSU).

Synthesis of ERp

2-(3-cyano-4,5,5-trimethyl-5H-furan-2-ylidene) malononitrile (199mg, 1 mmol), 8-Hydroxyjulonidine-9-formaldehyde (217 mg, 1 mmol) and a drop of piperidine was mixed in 10 mL

ethanol, The reaction was refluxed overnight, cooled to room temperature, and purified by silica gel chromatography with the mixed solvent of PE/EA (4:1~1:3, v/v) to give ERp as a green solid (325 mg, 78 %). ^{1}H NMR (400MHz, DMSO-d6) δ 9.79 (s, 1H), 8.44 (s, 1H), 7.46 (s, 1H), 6.72 (d, J = 14.2Hz, 1H), 3.41–3.34 (m, 4H), 2.66 (t, J = 6.2Hz, 2H), 2.58 (d, J = 12.8Hz, 2H), 1.84 (p, J = 6.1Hz, 4H), 1.66 (s, 6H). ^{13}CNMR (101 MHz, DMSO - d6) δ 177.98, 173.70, 157.58, 151.49, 118.01, 115.12, 114.22, 114.12, 113.99, 106.62, 97.01, 50.76, 49.96, 27.08, 26.53, 21.43, 21.02, 20.40. HRMS (MALDI-TOF): m/z: $[M-H]^-$ calcd for $C_{24}H_{21}N_4O_2$: 397.16700; found: 397.16696.

Synthesis of ER-CE

ERp (100 mg, 0.24 mmol), acetyl chloride (100 mg, 1.27 mmol), and triethylamine (128 mg, 1.27 mmol) were dispersed in dichloromethane. The reaction was stirred at room temperature for 2h, and the crude product was purified by with the mixed solvent of PE/EA (4:1~1:1, v/v) to give ER-CE as a blue solid (144 mg, 81%). ^{1}H NMR (400MHz, DMSO-d6) δ 8.19 (d, J = 15.4Hz, 1H), 7.74 (s, 1H), 6.82 (d, J = 15.4Hz, 1H), 5.77 (s, 1H), 3.42 (s, 4H), 2.74 (d, J = 6.3Hz, 2H), 2.37 (s, 4H), 1.92 (d, J = 5.9Hz, 2H),1.84(s, 2H),1.65(s, 6H). ^{13}CNMR (101MHz, DMSO - d6) δ 178.37, 175.00, 168.91, 149.80, 149.47, 142.53, 127.25, 120.87, 114.41, 114.35, 114.23, 113.55, 113.43, 107.95, 98.44, 55.38, 50.39, 50.16, 49.81, 27.18, 25.62, 21.22, 20.91, 20.80, 20.07. HRMS(MALDI-TOF): m/z: $[M+H]^+$ calcd for $C_{26}H_{25}N_4O_3$: 441.19212; found: 441.19287.

Photostability

To investigate the photostability of the probe system, the PL intensities of ER-CE (654nm) in PBS solution were monitored by an Edinburgh FS5 fluorescence spectrophotometer, upon continuous irradiation with a 150 W Xe light (600 nm) of the fluorescence spectrophotometer, respectively. The photostability of ER-CE was demonstrated by plotting I/I_0 versus the irradiation time, where I is the PL intensity of ER-CE after the irradiation time of t, and I_0 is the PL intensity of ER-CE before light irradiation.

General procedures for the detection of esterase

Unless otherwise noted, all the spectral measurements were performed in 5 mM phosphate buffer (pH 7.4, containing 1% DMSO) according to the following procedure. Esterase was dissolved in Phosphate buffer saline (pH=7.4). The stock solution (1.0 mM) of probe ER-CE was first prepared in DMSO. 10 μL of ER-CE stock solution was added to 1 mL PBS followed by addition of different volume of esterase solution. The mixture was incubated 50min at 37 °C, and the reaction solution was transferred to a quartz cell with an optical length of 1 cm for measurement. The excitation and emission slit were set at 5.0 nm and 5.0 nm, respectively.

Determination of the detection limit of ER-CE toward addition of esterase

Based on the linear fitting in Figure3C, the detection limit (C) was estimated as follows:

$$C = 3\sigma/K$$

Where σ was the standard deviation obtained from three individual fluorescent intensity (654 nm) of ER-CE (10 μM) without any esterase and K was the slope obtained after linear fitting the titration curves in Figure3C.

Cell Culture and imaging

The Hela cells and 4T1 cells were cultured in DMEM (containing 10% heat-inactivated FBS, 100 $mg \cdot mL^{-1}$ penicillin and 100 $mg \cdot mL^{-1}$ streptomycin) at 37 °C in a humidified incubator with 5% CO_2. Before the experiments, the cells were pre-cultured until confluence was reached. The HeLa cells in the logarithmic growth phase were seeded in a confocal dish with a cell density of approximately 1×10^4 cells/well, and then the confocal dish (200 μL fresh medium) was placed in a 37° cell incubator containing 5% CO_2 for 24 h. Subsequently, the original medium was discarded, and ER-CE (200 μL, 1 μM) was diluted in fresh medium and other subcellular trackers (100 nM) were added, and incubated at 37°C for 10 min for colocalization imaging. For the colocalization imaging experiment with the treatment of the inhibitor, the ABESF (1 mM) was pre-incubated for 5 h and then the probe ER-CE (1 μM) and the endoplasmic reticulum tracker (100 nM) were added for imaging. After inhibitor treatment, the probe ER-CE fluorescence imaging channel was weak. In order to understand the location more clearly, we amplified the red channel signal.

Cell viability

HeLa cells in the logarithmic growth phase were seeded in 8 flat-bottomed 96-well plates (about 1×10^4 cells/well), and incubate them at 37°C under 5% CO_2 for 24 h. DMEM medium was used to dilute the probe ER-CE or ERp to the required concentration (0, 5, 10, 15, 20, and 25 μM), aspirate the original medium in the 96-well plate, and different concentrations of medium (100 μL) was added to dilute. Incubate the probe solution for 4, 8, 12, and 16 hours, respectively. After the incubation period, aspirated the original medium, washed each well with 100 μL PBS, add 100 μL CCK8 solution diluted in the medium, and incubated at 37°C under 5% CO_2 for 2 h. After the incubation completed, a microplate reader (Mutiskan GO) was used for determination. Under the same experimental conditions, the cells without the treatment of probe acted as the reference, and the cell survival rate was calculated based on the following formula:

Cell viability (%) = (Absorbance of treated cells /Absorbance of untreated cells) ×100.

All experiments were repeated three times under the same conditions.

Fluorescence Imaging in mice

We divided 8 C57BL6J mice (6 weeks old) into two groups. The experimental group was injected with 4T1 cells (2×10^6) subcutaneously in the lower right part of each mouse. The mice of normal group were not injected with tumor cells. During all injection and imaging procedures, mice were anesthetized with isoflurane gas. After 7 days of tumor growth, the two groups of mice were injected subcutaneously with 100 μL PBS containing 100 μM ER-CE, and the images were obtained by the small animal optical in vivo imaging system. The exposure time during imaging was 10 seconds.

Computational Methods Details

GaussView 5.0[1] and Gaussian 09[2] software were used for structural visualization and simulation respectively. The structure of the compound is optimized to obtain the minimum energy form. The B3LYP function of DFT is implemented in the current research. For orbitals description, Pople basis set' 6-31G(d) is used for carbon, hydrogen, nitrogen, and oxygen atoms. Generate the highest occupied molecular orbitals (HOMOs) and lowest unoccupied molecular orbitals (LUMOs) to understand the electron density distribution of all compounds.

Molecular docking program

a. imported the ER-CE small molecule file into ChemBio3D Ultra 14.0 to minimize the energy, then set the Minimum RMS Gradient to: 0.001, and then saved the small molecule in mol2 format. Finally, imported the optimized small molecules into AutodockTools-1.5.6 for hydrogenation, charge calculation, charge distribution, set rotatable keys, and saved as "pdbqt" format.

b. downloaded the key target protein (2BCE) from the PDB database; used Pymol 2.3.0 to removed protein crystal water, original ligands, etc., and imported the protein structure into AutoDocktools (v1.5.6) for hydrogenation, charge calculation, charge distribution, and atom assignment Type and saved as "pdbqt" format.

c. used AutoDock Vina 1.12 for docking, and 2BCE related parameters are set as: center_x = 23.6, center_y = 0.834, center_z = 29.443 as the center coordinates; the size of the grid box is set to 126×126×126 (the spacing of each grid point 0.375Å), and the rest of the parameters were the default settings.

d. used PyMOL2.3.0 and LigPlot V2.2.4 to analyze the interaction mode of the docking results.

Figures and tables

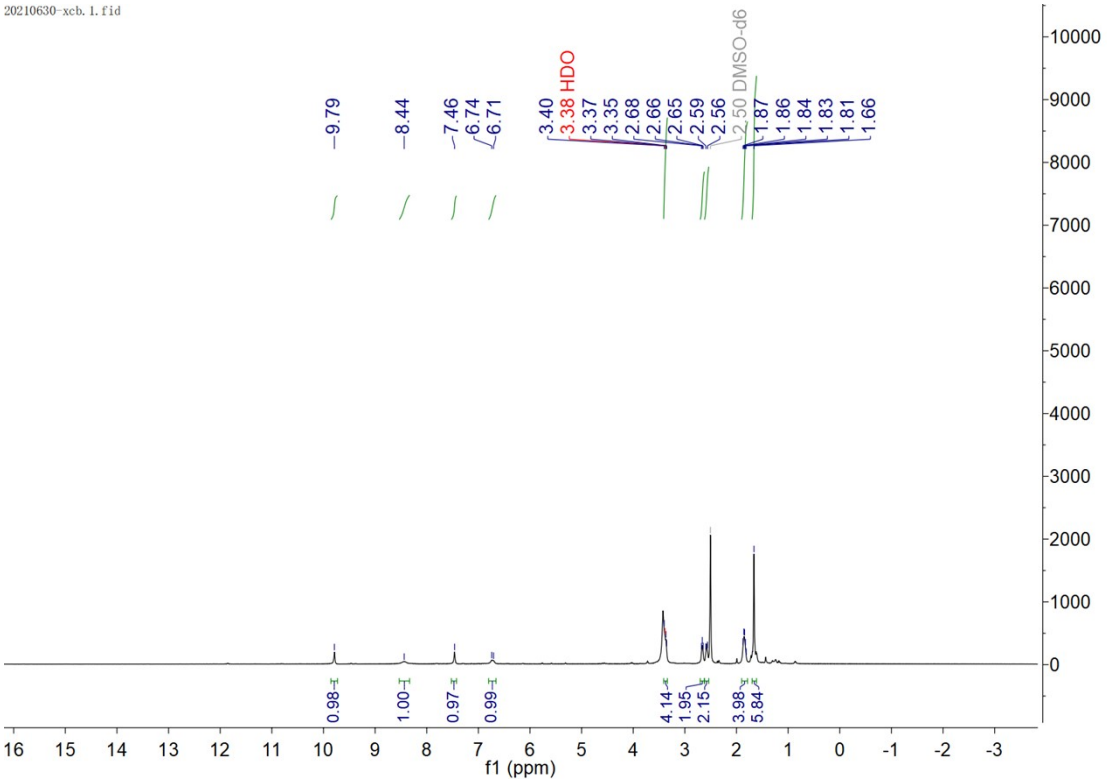

Figure S1. ^{1}HNMR spectrum of **ERp** in d_6-DMSO.

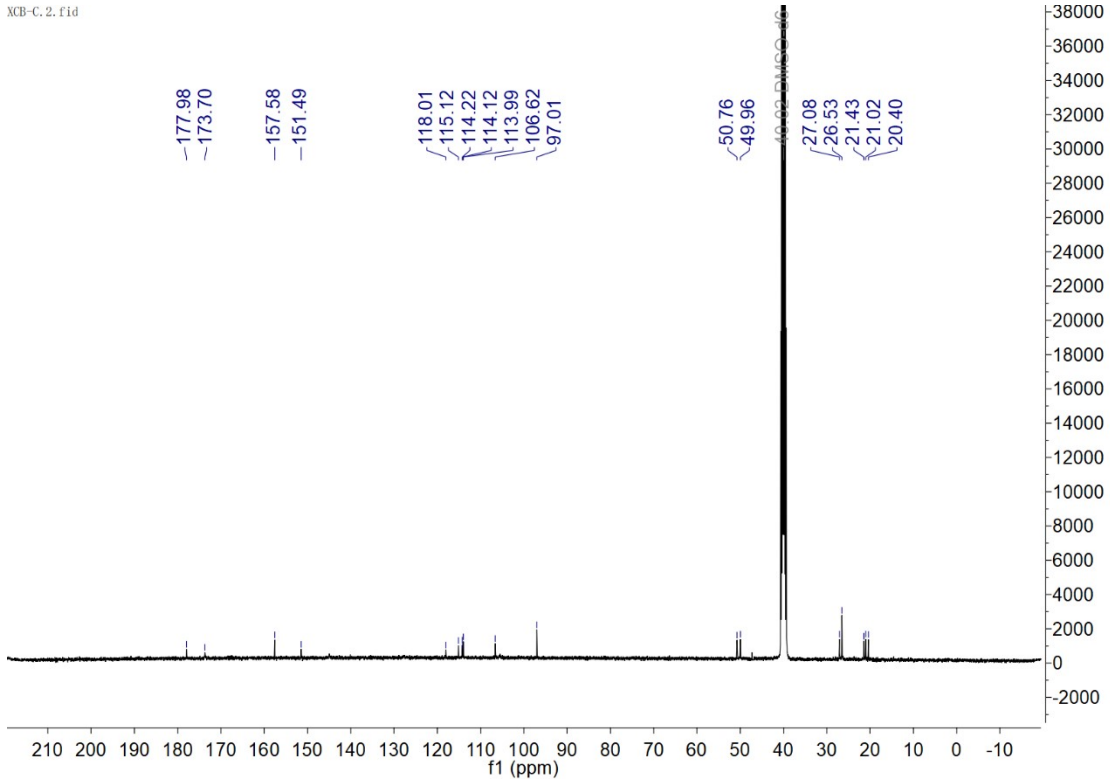

Figure S2. ^{13}CNMR spectrum of **ERp** in d_6-DMSO.

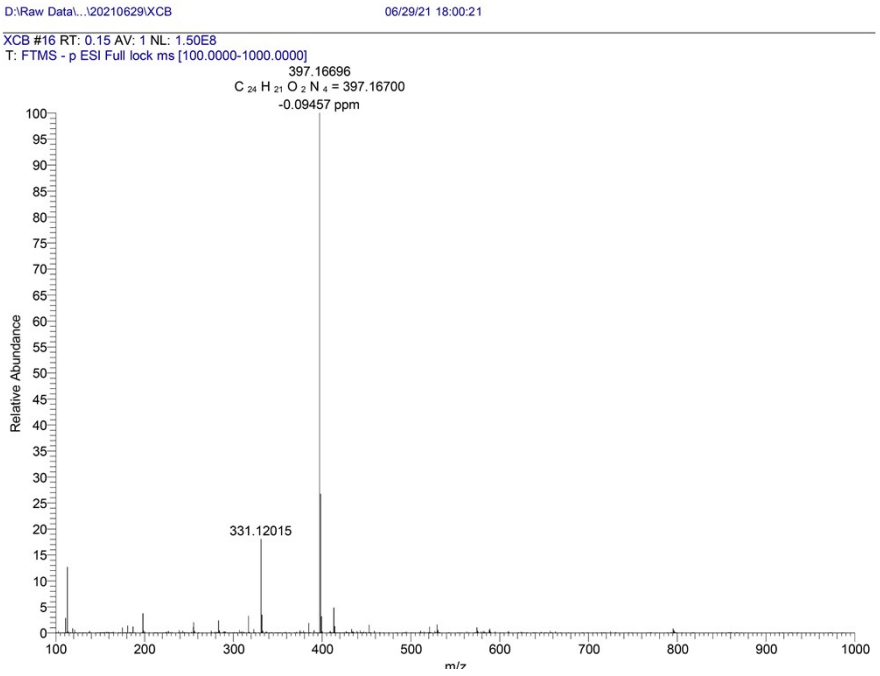

Figure S3. HRMS spectrum of **ERp**.

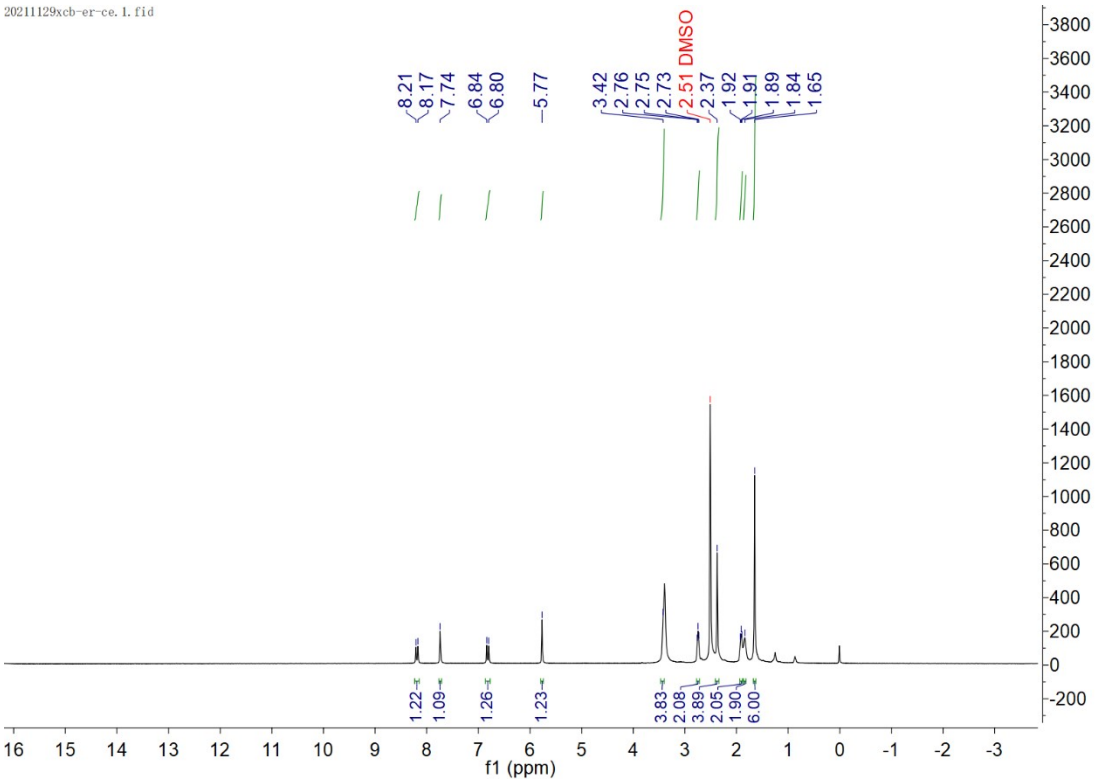

Figure S4. [1]HNMR spectrum of **ER-CE** in d_6-DMSO.

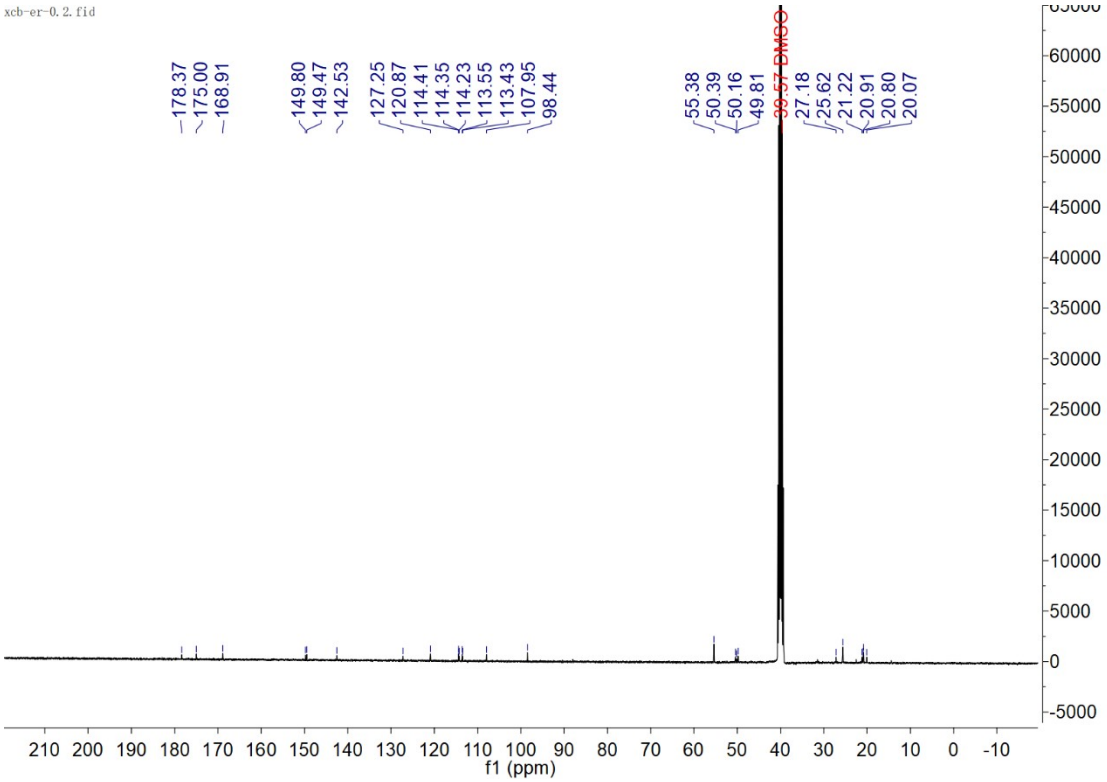

Figure S5. [13]CNMR spectrum of **ER-CE** in d_6-DMSO.

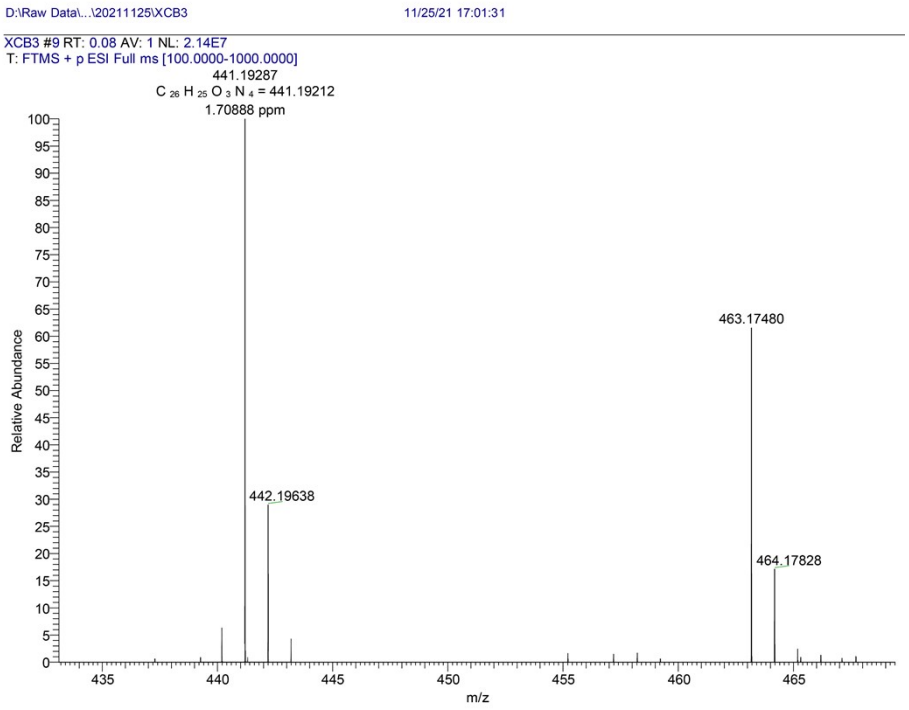

Figure S6. HRMS spectrum of **ER-CE**.

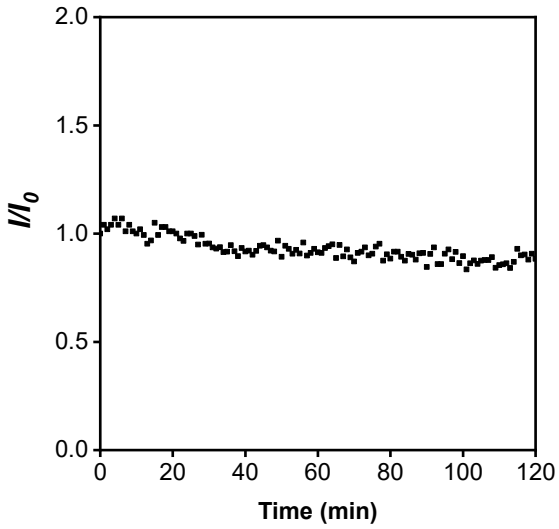

Figure S7. The light stability test of **ER-CE** in 120 min, **ER-CE** = 10 μM. λ_{ex} = 600 nm.

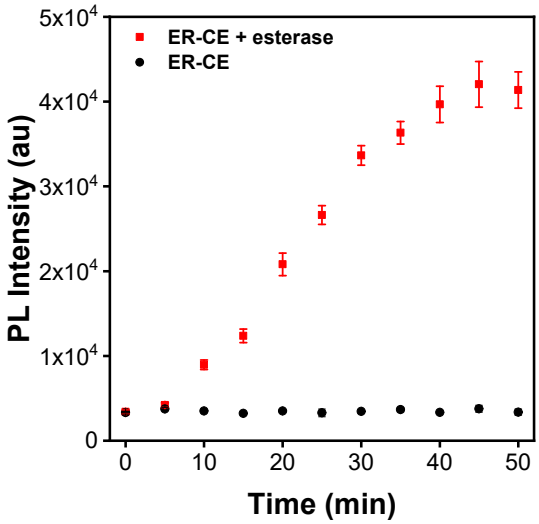

Figure S8. Time-dependent fluorescence intensity (654nm) of **ER-CE** (10 μM) with different concentrations (0 and 0.18 U/mL) of esterase. λ_{ex} = 600 nm.

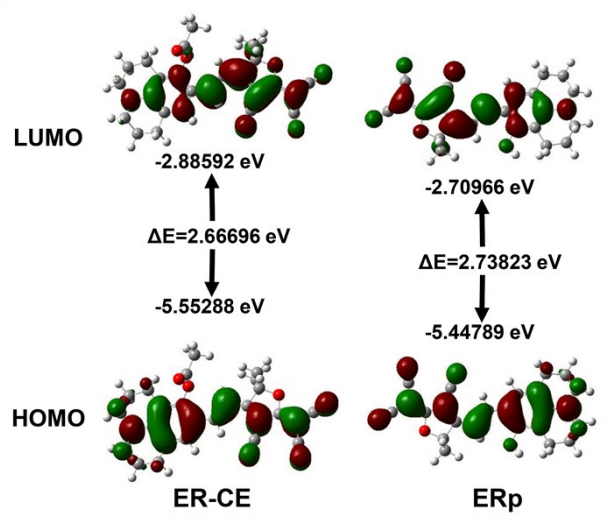

Figure S9. Frontier orbital pictures. ER-CE (LUMO and HOMO). ERp (LUMO and HOMO) calculated at B3LYP/6-31G(d) level.

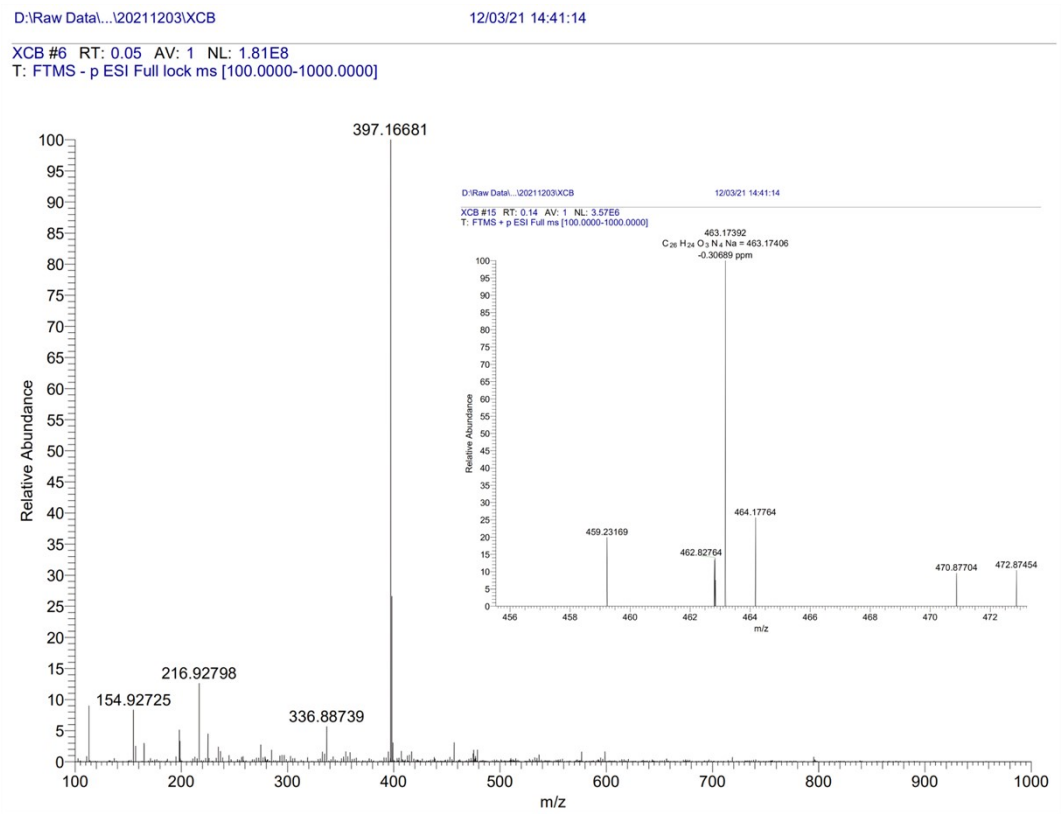

Figure S10. HRMS spectrum of ER-CE after incubation with esterase at 37°C for 1 h. ERp (calcd for $C_{24}H_{21}N_4O_2$: 397.16700, found: 397.16681). ER-CE (calcd for $C_{26}H_{24}N_4O_3Na$: 463.17406, found: 463.17392).

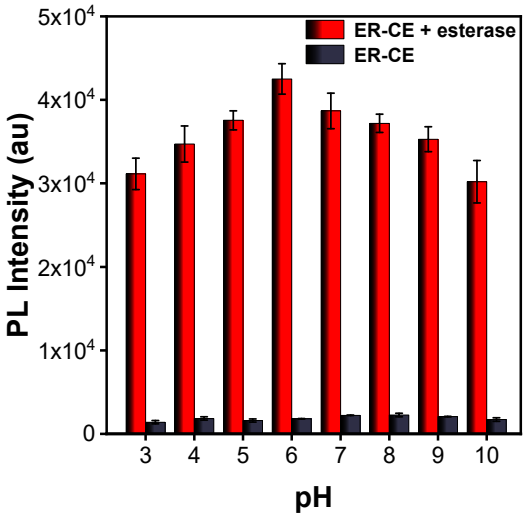

Figure S11. The PL intensity (at 654 nm) of **ER-CE** (10 μM, black bars) and **ER-CE** (10 μM) + esterase (0.18 U/mL, red bars) in different pH buffers. λ_{ex} = 600 nm.

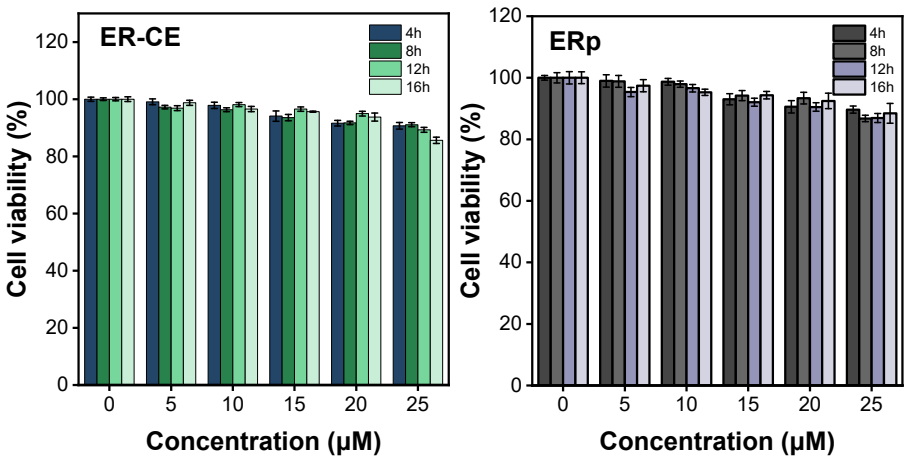

Figure S12. Cell viability of Hela cells at varied concentrations of **ER-CE** and **ERp** by using CCK8 method.

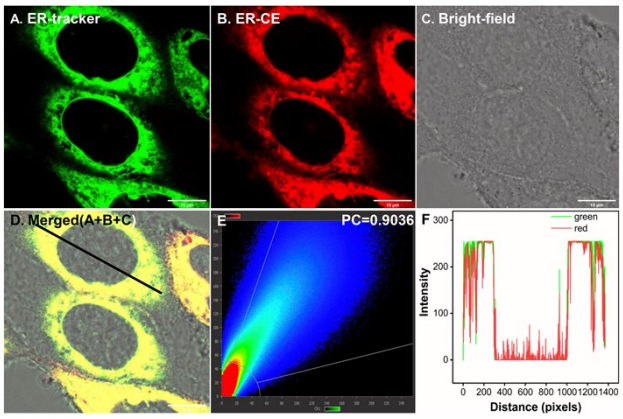

Figure S13. Colocation fluorescence image. HeLa cells were incubated with ERp (1 μM) and ER-tracker (100 nM) for 10 min. (A) Green channel of ER-tracker; (B) red channel of ERp; (C) Bright-field; (D) merged images of A, B and C. (E) Intensity scatter plot of two channels (A and B). (F) Intensity of interest regions of two channels (A and B). Scale bar = 10 μm.

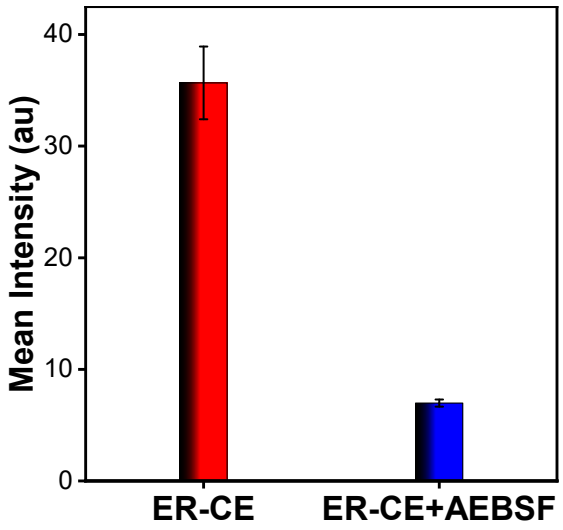

Figure S14. The mean intensity of the red channel in each row of cells in Figure 6.

Table S1. Comparison of reported fluorescent probes for esterase detection.

References	Targeting	Detection limit (U/mL)	$\lambda_{ex}/\lambda_{em}$ (nm)	Biological application
ACS Appl. Mater. Interfaces, 2018, **10,** 31088-31095	/	9.51×10^{-5}	490/551	Discrimination of Live and Dead Cells
J. Mater. Chem. B, 2014, **2**, 3438-3442.	Lysosomal	2.4×10^{-3}	356/532	lysosomal esterase imaging
Anal. Chem. 2017, **89**, 3162-3168	Mitochondria	5×10^{-3}	365/580	Cell imaging
Anal. Chem. 2019, **91**, 15840-15845	Endoplasmic Reticulum	/	380/414	Cells and acute liver injury model
ACS Med. Chem. Lett. 2014, **5**, 321-325	Endoplasmic Reticulum	/	571/584	Cell imaging
J. Mater. Chem. B, 2019, **7**, 2989-2996.	Lysosomal	1.2×10^{-4}	520/575	Serum, live cells and tissues imaging
Chem. Commun. 2016, **52**, 1835-1838.	/	30 pg	510/525	Cells imaging
Chem.-Eur. J. 2015, **21**, 9645-9649.	/	4.0×10^{-3}	388/678	Cells imaging
Analyst. 2020, **145**, 1408-1413.	/	4.73×10^{-5}	480/505	Discrimination of Live and Dead Cells
Anal. Chim. Acta, 2022, **1190**, 339248.	/	0.216×10^{-3}	455/560	Cells and zebrafish imaging
This work	**Endoplasmic Reticulum**	**1.46×10^{-3}**	**600/654**	**Cells and tumor mice imaging**

References:

1. J. Tirado-Rives and W. L. Jorgensen, *J Chem. Theory. Comput.* 2008, **4**, 297–306.

2. T. Mahmood, N. Kosar and K. Ayub, *Tetrahedron*. 2017, **73**, 3521-3528.