Electronic supplementary information

Ratiometric two-photon fluorescent probe for in situ imaging of carboxylesterase (CE)-mediated mitochondrial acidification during medication

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1. Experimental section

1.1 Materials and Reagents

Benorilate was purchased from J&K Chemical Co. (Beijing, China), vinyl acetate, 2,4-Dihydroxybenzaldehyde, 4-picoline and 1,4-bis(chloromethyl)benzene were purchased from Aladdin Chemistry Co. (Shanghai, China). Solutions for metal ions (K^+, Na^+, Ba^{2+}, Cr^{3+}, Hg^{2+}, Li^+, Mn^{2+}, Sr^{2+}, Ca^{2+}, Co^{2+}, Ni^{2+}, Mg^{2+}, Al^{3+}, Zn^{2+}, Cu^{2+}, Fe^{2+}, Fe^{3+}) and anion (F^-, Cl^-, Br^-, I^-, SO_4^{2-}, SO_3^{2-}, HCO_3^-, NO_2^-, NO_3^-, OAc^-, ClO^-, N_3^-, PO_4^{3-}, S^{2-}) were prepared by dissolving the corresponding salts in deionized water. Diethyl glutaconate, GSH, Cys, Hcy, Gly, Glu, Val, Thr, Tyr, Trp, Ser, Phe, Met, Leu, Ile, Asp, Lys, a-CT, Trypsin, Pepsin, BSA, HAS, AChE, BChE, PON-1, PON-2, FAP, TBHP, Glucose, Human plasma and Carboxylesterase were purchased from Sigma-Aldrich Co. (Steinheim, Germany). B-R buffer solution (Britton-Robinson) was used to modify the pH. All reagents were analytical grade, and used without further purification. Double-distilled water was used in the experiments.

1.2 Instrumentation

Fluorescence spectra were recorded on a Hitachi F-7000 fluorescence spectrophotometer (HITACHI, Japan). UV-visible spectra were collected on a Cary 300 Bio UV-vis spectrophotometer (VARIAN, USA). ^{1}HNMR and ^{13}CNMR spectra were measured in the given solvent at RT on a Bruker ascend 500 (500.1 MHz, ^{1}H; 125.8 MHz, ^{13}C) instrument operated at the denoted spectrometer frequency given in mega Hertz (MHz). Chemical shifts are given in parts per million (ppm) relative to tetramethylsilane (TMS) as an external standard for ^{1}H and ^{13}C-NMR spectra and calibrated against the solvent residual peak. HR-MS date were recorded on a maxis ultra-high resolution-TOF MS system (Bruker Co., Ltd., Germany). Colocalization experiments images were obtained using a Leica DMI3000B (Germany). LSM 880 confocal laser scanning microscopy were used for the two-photon fluorescence images. (Zeiss Co., Ltd. Germany). All pH measurements were performed using a pH-3c digital pH-meter with glass-calcium electrode (Shanghai Lei Ci Device Works, Shanghai, China). Thin layer chromatography separation was performed on silica gel plates and column chromatography was conducted over silica gel (mesh 300-400), both of which were purchased from the JiangYou Chemical Company (Yan Tai, China).

1.3 Fluorometric analysis

Fluorescent spectra were recorded at the excitation wavelength of 410 nm and 530 nm, with the excitation / emission slit width of 10 nm/10 nm. Optical properties were evaluated under the physiological conditions using 10 μM of **CEMT** in B-R buffer solution (H_2O; pH = 7.42, 40 mM), while optical properties of pH were evaluated under the physiological conditions using 10 μM of **HMT** in DMF/B-R buffer solution (v/v: 1:100; pH = 7.42, 40 mM)

1.4 Cytotoxicity assay

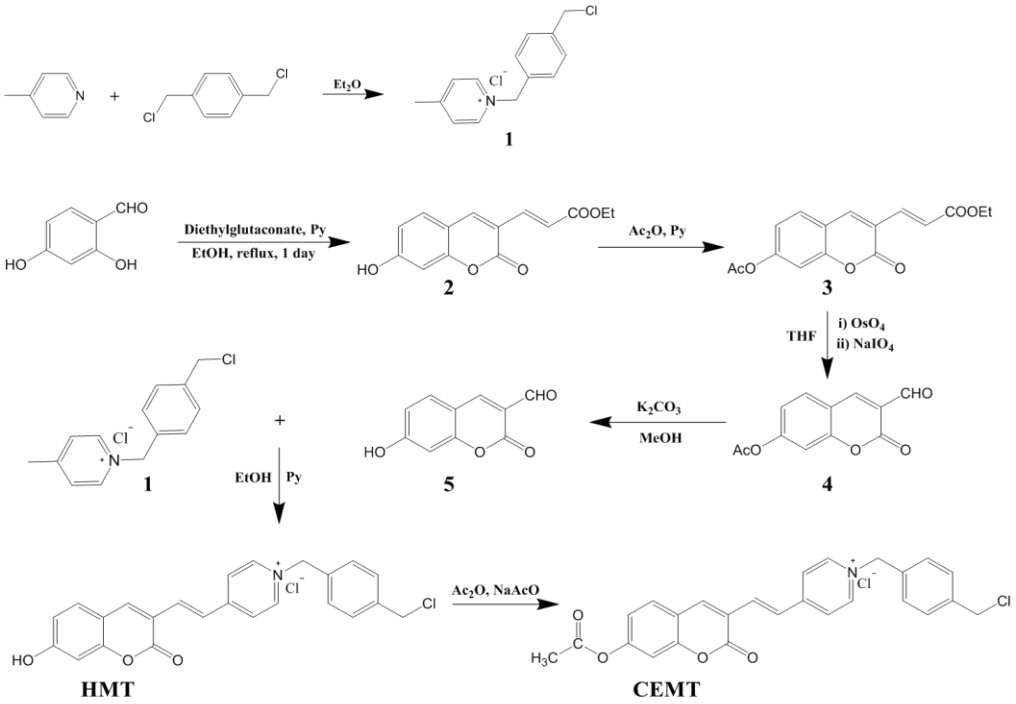
MTT assay was carried out to evaluate the cytotoxicity of **CEMT** and **HMT**. The HepG2 cells (School of Medicine of Shandong University) were first incubated with the 10% Fetal Bovine Serum (FBS, Invitrogen) in a

humidified incubator containing 5% CO_2 gas at 37℃ for 24 h. Then, the cells were respectivly treated by various concentrations of **CEMT** and **HMT** (1 μM, 10 μM, 50 μM, 0.1 mM, 0.5 mM and 1 mM) for 24 h. Next, 25 μL of methylthiazolyl tetrazolium (MTT) (5 mg mL^{-1}) was added to each well and was incubated for 4 h. The cytotoxicity tests were performed using MTT (n=7 replicates). The cell viability was expressed by the mean values ± standard deviation (SD).

1.5 The detection limit of CEMT to carboxylesterase

Detection limits for carboxylesterase were calculated using the following formula: detection limit = 3 SD/ *k*, where *k* is the slope of the curve equation and SD represents the standard deviation for the fluorescence intensity responses of probe to carboxylesterase. $I_{550}/I_{460}(4)$= 0.3876 × [CE] +1.1472 (R^2=0.9932), LOD = 3×0.01551/0.3876=0.12 μg/mL.

1.6 Figure S1: Synthesis steps of HMT and CEMT



1.6.1 Synthesis of 1-(4-(chloromethyl) benzyl)-4-methylpyridin-1-ium chloride (1):

4-picoline (4.65 g, 50 mmol) and 1,4-bis(chloromethyl)benzene (9.65 g, 55 mmol) were dissolved in toluene. The mixture was stirred for 4 h, then refluxed 30 min. After cooling and filtrating, the resulting solid was washed with anhydrous ether to give a white solid with a yield of 86%. ^{1}H NMR (CD_3OD-d_4, 500 MHz): δ 8.92-8.90(m, 2H), 7.95(d, J=6.4 Hz, 2H), 7.61-7.39(m, 4H), 5.83(s, 1H), 5.80 (s, 1H), 4.83(s, 2H), 2.67(s, 3H), ^{13}C NMR (CD_3OD-d_4, 125 MHz): δ 160.66, 143.65, 135.17, 133.55, 129.79, 129.52, 129.00, 128.84, 128.68, 128.00, 62.73, 45.06, 44.68, 20.68 ppm.

1.6.2 Synthesis of (E)-ethyl 3-(7-hydroxy-2-oxo-2H-chromen-3-yl)acrylate(2):

2,4-Dihydroxybenzaldehyde (0.74 g, 5.36 mmol) and diethyl glutaconate (1.0 mL, 5.65 mmol) were dissolved in EtOH (30 mL), then 3 drops of dry piperidine were added, and the mixture was reflux for 24 h. The reaction mixture was then kept below -10 °C, for 12 h. After filtering the resulting solid was washed twice with EtOH. Purification by flash chromatography (CH_3CN:CH_2Cl_2 1:20) gave product **2** as yellow solid, with a yield of 76%. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 8.41(s, 1H), 7.55(d, J=8.6 Hz, 1H), 7.49(d, J=10.0 Hz, 1H), 6.84(dd, J=5.0, 10.0 Hz, 2H), 6.73(s, 1H), 4.16(q, J=5.1 Hz, 2H), 1.24(t, J=5.1Hz, 3H), ^{13}C NMR (DMSO-d_6, 125 MHz): δ 166.73, 163.35, 159.67, 155.83, 145.97, 139.37, 131.33, 120.19, 116.73, 114.49, 111.92, 102.41, 60.53, 14.63 ppm.

1.6.3 Synthesis of (E)-ethyl 3-(7-acetoxy-2-oxo-2H-chromen-3-yl)acrylate(3):

Product **2** (0.2 g, 0.77 mmol) was dissolved in pyridine (4 mL) and acetic anhydride (4 mL). The mixture was stirred at room temperature for 1 h, and then poured into ice for another 0.5 h. After filtrating the crude product was dissolved in DCM. The organic solution was washed with water (3×100 mL) and NaCl saturated solution (3×200 mL). Drying over Na_2SO_4 and removal of the solvent under reduced pressure afforded the crude product as an off-white solid. Purification by flash chromatography (CH_3CN: CH_2Cl_2=1:5) gave product **3** as white solid, with a yield of 82%. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 7.84(s, 1H), 7.53(t, J=10.0Hz, 2H), 7.12(d, J=5.2 Hz, 1H), 7.09-7.05(m, 2H), 4.25(q, J=5.0 Hz, 2H), 2.34(s, 3H), 1.32(t, J=5.1 Hz, 3H), ^{13}C NMR (DMSO-d_6, 125 MHz): δ 168.56, 166.87, 158.71, 154.13, 142.80, 137.62, 129.25, 123.83, 121.75, 118.90, 116.76, 110.16, 60.78, 20.14, 14.27 ppm.

1.6.4 Synthesis of 3-formyl-2-oxo-2H-chromen-7-yl acetate(4):

Product **3** (2.2 g, 7.28 mmol) was dissolved in THF (200 mL), OsO_4 was added (0.1 g dissolved in 2 mL water) to the mixture and stirred for 0.5 h. then $NaIO_4$ (3.42 g, 16 mmol) added. The mixture was stirred at room temperature for 6 days. The resultant product was removed from the organic phase under vacumn. The resulting solid was partitioned between water and CH_2Cl_2. Drying over Na_2SO_4 and removal of the solvent under reduced pressure afforded the crude product as an off-white solid. Purification by chromatography (silica-solvent gradient from CH_2Cl_2 to CH_3CN: CH_2Cl_2 1:5). Then gave product **4** as a white solid, with a yield of 43%. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 10.02(s, 1H), 8.69 (s, 1H), 8.03(d, J=10.1 Hz, 1H), 7.38-7.24(m, 2H), 2.32(s, 3H), ^{13}C NMR (DMSO-d_6, 125 MHz): δ 188.48, 169.06, 159.44, 156.08, 155.79, 146.79, 132.91, 121.48, 119.54, 116.59, 110.77, 21.38 ppm.

1.6.5 Synthesis of 7-hydroxy-2-oxo-2H-chromene-3-carbaldehyde(5):

Product **4** (0.5 g, 2.15 mmol) and K_2CO_3 (0.595 g, 4.31 mmol) was dissolved in 40 mL of MeOH solvent. The mixture was stirred at room temperature for 1h. The pH of the reaction mixture was adjusted to 3~4 with 1N HCl solution. After filtrating and washing twice with water, the solid was dissolved with DCM and dried over Na_2SO_4, the solvent was removed under vacuum. Purification by chromatography (CH_3OH: CH_2Cl_2 1:10) gave product **5** as yellow solid, with a yield of 67%. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 9.95(s, 1H), 8.56 (s, 1H), 7.80(d, J=10.0 Hz, 1H), 6.88-6.77(m, 2H), ^{13}C NMR (DMSO-d_6, 125 MHz): δ 188.22, 165.41, 160.20, 158.02, 147.68, 133.80, 117.48, 114.99, 111.30, 102.72 ppm.

1.6.6 Synthesis of (E)-1-(4-(chloromethyl)benzyl)-4-(2-(7-hydroxy-2-oxo-2H-chromen-3-yl)vinyl) pyridin-1-ium （HMT）:

Product **5** (0.095 g, 0.5 mmol) and product **1** (0.134 g, 0.5 mmol) were dissolved in EtOH(50 mL), followed by dry piperidine(0.05 mL) and the solution was refluxed for 12h. The reaction mixture was then allowed to slowly cool to room temperature and then chilled to -20 °C. The crude purplish red solid were filtered off and washed twice with EtOH. Purification by flash chromatography (CH_3OH:CH_2Cl_2 1:2) gave **HMT** as red crystalline solid, with a yield of 39%. ^{1}H NMR (CD_3OD-d_4, 500 MHz): δ 8.87(d, J=4.9 Hz, 2H), 8.59-8.56(m, 2H), 8.18(d, J=8.0 Hz, 2H), 8.01-7.96(m,4 H), 7.54-7.48(m, 2H), 6.81(dd, J=2.3, 2.3 Hz, 1H), 6.71(d, J=2.2 Hz, 1H), 5.33(s, 2H), 4.11(s, 2H), ^{13}C NMR (CD_3OD-d_4, 125 MHz): δ 173.61, 163.56, 162.52, 157.05, 156.81, 154.21, 153.36, 148.59, 143.06, 142.74, 141.12, 131.54, 128.85, 127.77, 126.27, 123.88, 121.34, 114.92, 112.86, 103.35, 100.45, 100.04, 60.95, 43.57 ppm. HRMS：calculated for $C_{24}H_{19}Cl_2NO_3$ $[M]^+$=439.0737. Found 439.0876. (Figure S3)

1.6.7 Synthesis of (E)-4-(2-(7-acetoxy-2-oxo-2H-chromen-3-yl)vinyl)-1-(4-(chloromethyl)benzyl) pyridin-1-ium chloride (CEMT):

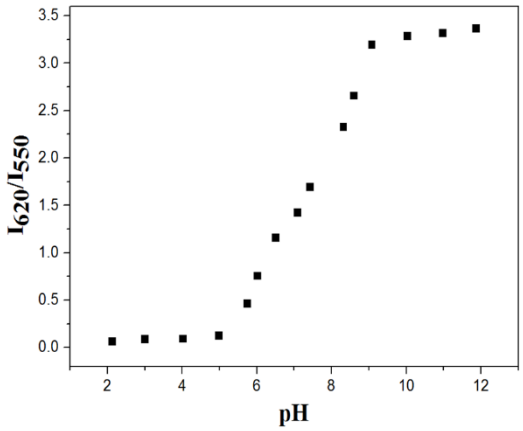
HMT(0.263 g, 0.6 mmol) and NaOAc (0.167 g, 2.04 mmol) was dissolved in 6 mL Ac_2O. The reaction mixture was stirred for 7 h at 80°C under an Ar atmosphere. The solvent was then evaporated under reduced pressure, and the crude product was dissolved in DCM, filtered and concentrated to give **CEMT** as a pink crystalline solid, with a yield of 23%. ^{1}H NMR (CD_3OD-d_4, 500 MHz): δ 8.75(d, J=6.9 Hz, 2H), 8.14 (s, 2H), 8.06(d, J=6.9 Hz, 2H), 7.77(q, J=15.9 Hz, 2H), 7.49-7.46(m, 4H), 6.81(dd, J=2.4, 2.4 Hz, 1H), 6.56 (d, J=2.15 Hz, 1H), 5.86(s, 2H), 4.52(s, 2H), 2.33(s, 3H), ^{13}C NMR (CD_3OD-d_4, 125 MHz): δ 174.50, 172.02, 162.00, 160.96, 155.49, 154.98, 152.50, 151.49, 147.05, 141.52, 141.19, 140.32, 129.96, 127.32, 126.22, 125.03, 122.79, 119.76, 113.35, 111.29, 103.82, 101.76, 98.87, 59.09, 41.72, 19.13 ppm. HRMS：calculated for $C_{26}H_{21}Cl_2NO_4$ $[M]^+$=481.0842. Found 481.0921. (Figure S2)

1.7 Optimization of sensing conditions

Before applying **CEMT** and **HMT** as a probe, sensing conditions were optimized. Time-dependent tests showed that **CEMT** responded to CE in 10 min and **HMT** responded rapidly to pH. (Figure S4), ensuring the in situ monitoring in biological systems. The selectivity of **CEMT** toward CE against other possible interference was tested. Various biologically relevant species, including anions (ClO^-, Cl^-, $ONOO^-$, O_2^-), metal ions (K^+, Na^+, Mn^{2+}, Ca^{2+}, Co^{2+}, Mg^{2+}, Al^{3+}, Zn^{2+}, Cu^{2+}, Fe^{2+}), and RSS (GSH, Cys, Hcy, Gly, Glu, Val, Thr, Tyr, Trp, Ser, Phe, Met, Leu, Ile, Asp, Lys, a-CT, Trypsin, Pepsin, BSA, HAS, AChE, BChE, PON-1, PON-2, FAP, TBHP, Glucose, Human plasma) were evaluated. The selectivity of **HMT** in various solutions of B-R (pH=5.31) containing tested compounds. Various biologically relevant species, including anions (Br^-, Cl^-, F^-, I^-, SO_4^{2-}, SO_3^{2-}, HCO_3^-, NO_2^-, NO_3^-, OAc^-, ClO^-, N_3^-, PO_4^{3-}, S^{2-}), metal ions (K^+, Na^+, Ba^{2+}, Cr^{3+}, Hg^{2+}, Li^+, Mn^{2+}, Sr^{2+}, Ca^{2+}, Co^{2+}, Ni^{2+}, Mg^{2+}, Al^{3+}, Zn^{2+}, Cu^{2+}, Fe^{2+}, Fe^{3+}), and RSS (Cys, GSH, Hcy) were evaluated. Relative fluorescence intensity recorded in Figure S5 indicated that anions, metal ions and RSS, resulted in minimal interference. As cellular pH values are in dynamic equilibrium, the reversibility for detecting H^+ is a very important parameter for a pH probe. Therefore, pH values were switched between 4.98 and 9.07 using aqueous solutions of hydrochloric acid and sodium hydroxide (Figure S6). On this basis, it can be observed that the probe is able to report in situ imaging of the carboxylesterase (CE)-mediated mitochondria acidification during medication in a biological environment.

1.8 The pKa value of HMT

On the basis of the Henderson-Hasselbalch equation ($\log[(I_{max}-I)/(I-I_{min})] = pH - pKa$). I_{max}, I_{min}, and I are the maximum, minimum, and observed fluorescence ratios at a given pH value, respectively. The calculated pKa value from the y-intercepts is 7.97.



1.9 Measurement of two-photon absorption cross-section (δ)

The **CEMT** and **HMT** were dissolved in water and then the two-photon fluorescence intensity was measured at 810nm using using fluorescein as reference. The two-photon absorption cross-section (δ) of **CEMT** and **HMT** were determined. The **CEMT** and **HMT** cross section were calculated by using following equation:

$$\delta_s = \frac{\delta_r(C_r n_r F_s \Phi_r)}{(C_s n_s F_r \Phi_s)}$$

The subscripts s and r refer to the sample and the reference molecules, respectively. δ is the two-photon absorption cross sectional value, the compounds concentration in solution was denoted as C, n is there refractive index of the solution, F is two-photon excited fluorescence integral intensity and Φ is the fluorescence quantum yield. The fluorescence quantum yield of **CEMT** and **HMT** in water solution were measured as 0.076 and 0.698, respectively. The δmax values of **CEMT** and **HMT** were acquired at 810 nm and were 75 GM (1 GM $=10^{-50}$ m^4s per photon) and 165 GM, respectively. On this basis, it can be concluded that the probe is able to be applied in two-photon fluorescence microscopic imaging.

2.0 Cell culture and imaging

HepG2 cells were seeded into 18 mm glass-bottom dishes with high-glucose DMEM medium containing 10% fetal bovine serum, 1% penicillin and 1% streptomycin. Cultures were maintained in an incubator (37°C, 5% CO_2/95% air). The cells were excited at 810 nm with a two-photon laser and the emission was collected green channel (400–500 nm), yellow channel (500–570 nm) and red channel (570–710 nm).

2.1 Colocalization experiments for HMT with MTDR

To confirm the mitochondria-targeting capability of **HMT** in various pH, we carried out the co-staining experiments in HepG2 cells using **HMT** with MTDR. Figure 3 showed the good correlation. Pearson's co-localization coefficient that described the correlation of intensity distribution between two channels, was determined to be 0.91 (pH=7.42) and 0.89 (pH=4.98), indicating that **HMT** predominantly accumulated in the mitochondria. Hence, the probe **HMT** was able to report the *in situ* imaging of carboxylesterase (CE)-mediated mitochondria acidification without suffering from leakage from the mitochondria during the acidification..

2.2 Photostability

To estimate the feasibility for detecting intracellular CE and pH, penetration and photostability of **CEMT** in HepG2 cell were investigated. Dynamic fluorescence imaging (Figure S8) indicated that **CEMT** in HepG2 cells could exhibit the rapid and strong fluorescence. Then, the fluorescence intensity remained almost unchanged for 20 min, displaying the satisfactory photostability of **CEMT**. Before the practical application, cytotoxicity of **CEMT** to living HepG2 cells was evaluated by MTT assay. Figure S9 showed that more than 92% cells still remained alive when 1 mM **CEMT** and **HMT** was added for 24 h, displaying the low toxicity of **CEMT** and **HMT** to the cultured HepG2 cells under experimental conditions. These experiments demonstrated that **CEMT** and **HMT** was suitable for living cell imaging.

2.3 Cardiac perfusion for two-photon fluorescence confocal imaging in tissues

Male Kunming mice (20 g) were fasted for 12 h to avoid the fluorescence interference from possible food. The mice was injected with 100 mL PBS buffer solution containing 20 μM **CEMT**, or/and 1 mM vinyl acetate or 1 mM benorilate, through the cardiovascular to ensure the perfusion to liver. The flow rate averages two drops per second (lasted 10 minutes). Ten minutes later, mice were dissected to isolate the livers, which were cut into slices for confocal imaging. The tissue imaging was excited at 810 nm with a two-photon laser and the emission was collected green channel (400–500 nm), yellow channel (500–570 nm) and red channel (570–710 nm).

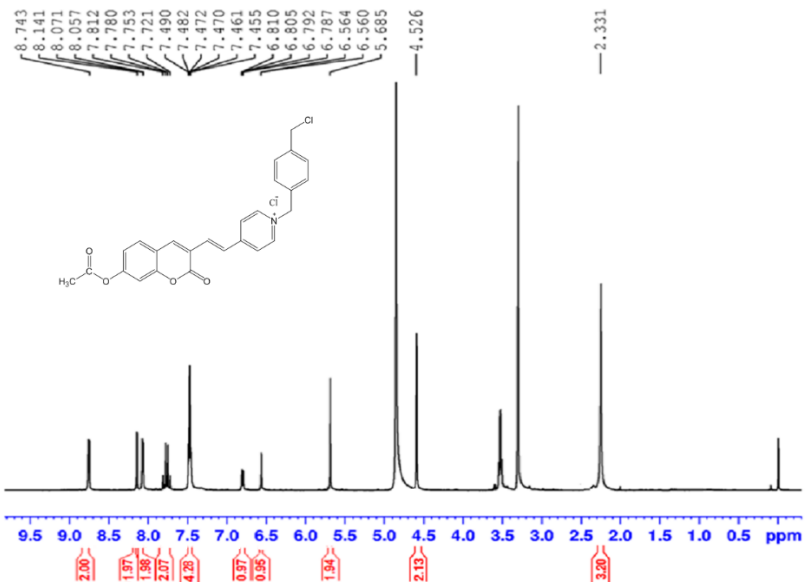
2.4 Live subject statement

Kunming mice were purchased from Changzhou Cavens Lab Animal Co. Ltd. All experiments were performed in compliance with Chinese national standard Laboratory Animal-Guideline for ethical review of animal welfare (GB/T 35892-2018) and all experiments followed institutional guidelines. All protocols were approved by the Institutional Animal Care and Use Committee in Binzhou Medical University, Yantai, China. Approval number: no. BZ2014-102R.

2. Supplementary Figures

2.1 Figure S2 ^{1}H, ^{13}C-NMR and HRMS for CEMT

A



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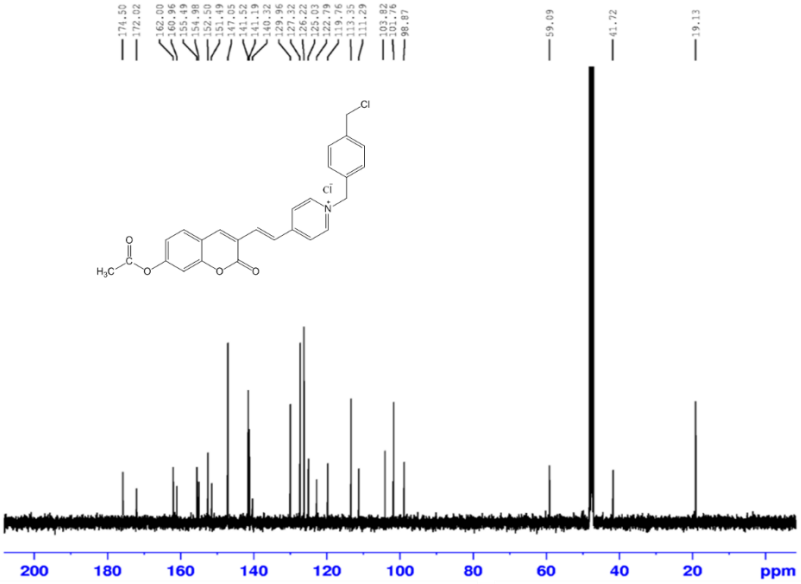

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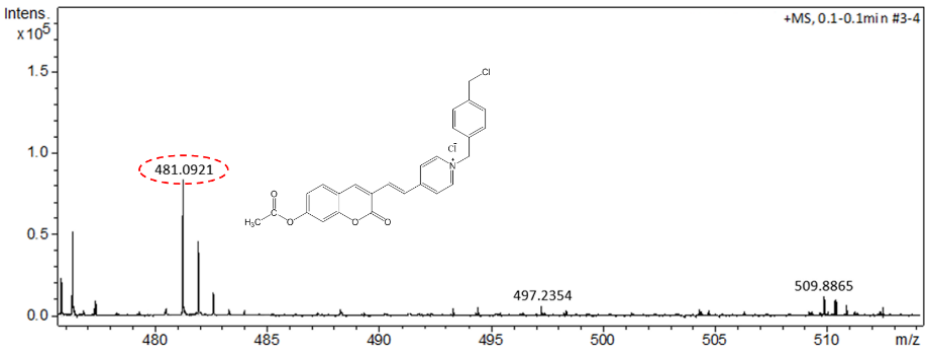
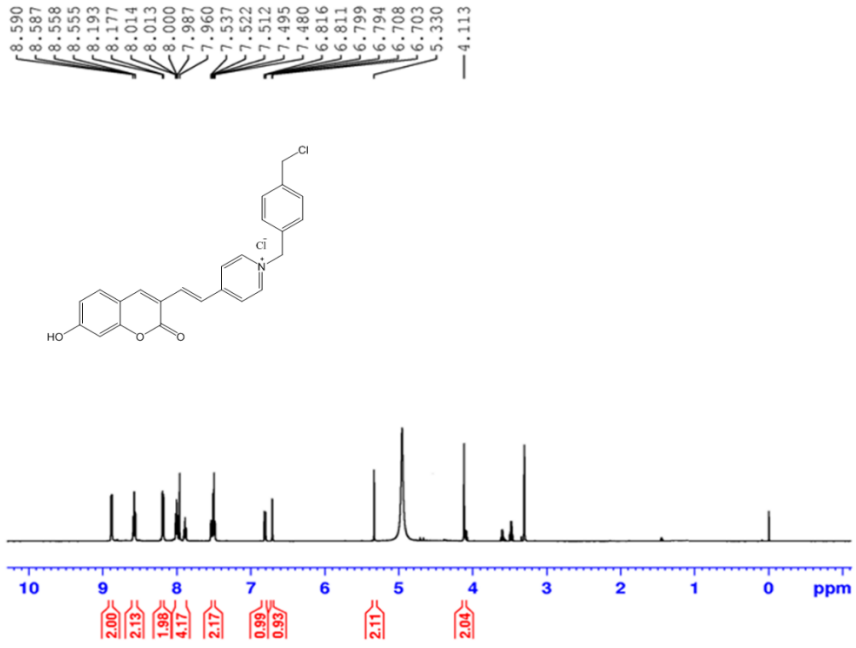


Figure S2 Characterization of **CEMT**: (A) ^{1}H NMR spectrum (CD_3OD-d_4); (B) ^{13}C NMR spectrum (CD_3OD-d_4) and (C) HRMS: $[M]^+$=481.0921.

2.2 Figure S3 ^{1}H, ^{13}C-NMR and HRMS for HMT

A



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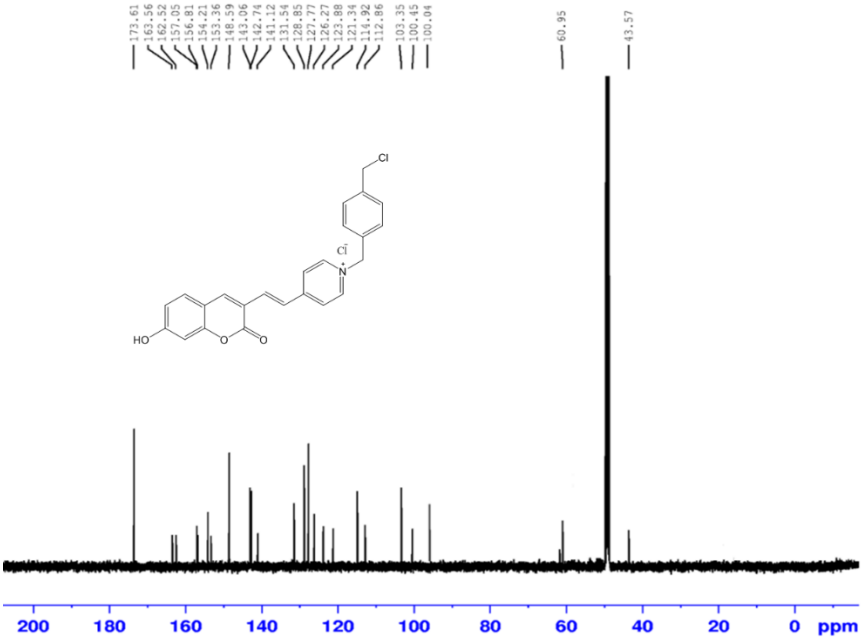

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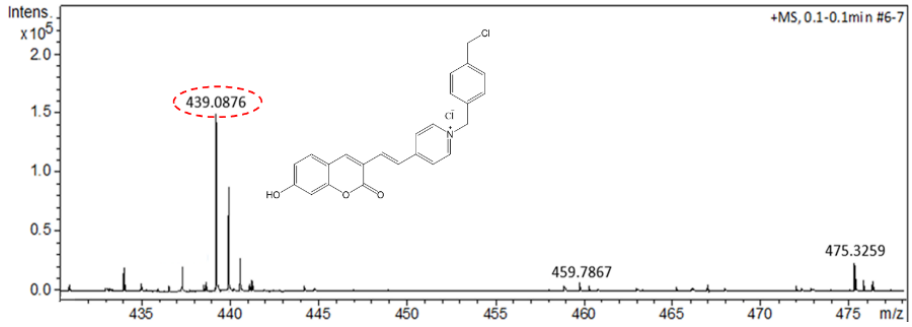

Figure S3 Characterization of **HMT**: (A) ^{1}H NMR spectrum (CD_3OD-d_4); (B) ^{13}C NMR spectrum (CD_3OD-d_4) and (C) HRMS: $[M]^+$=439.0876.

2.3 Figure S4 UV-vis spectra of CEMT and HMT

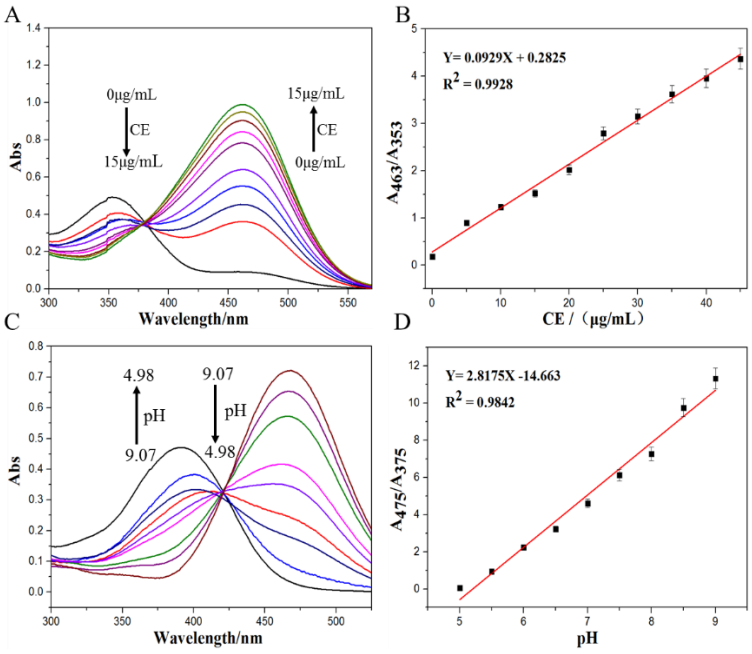

Figure S4 (A) Change of absorption of **CEMT** (10 μM) to CE (0-15 μg/mL) (B) Absorption ratio(A_{463}/A_{353}) of **CEMT** (10 μM) in the presence of CE (0~15 μg/mL) in B-R buffer solution (pH=7.42, 40 mM) at 37℃. (C) Change of absorption spectra of **HMT** (10 μM) for pH from 4.98 to 9.07. (D) Absorption ratio(A_{475}/A_{375}) of **HMT** (10 μM) in the presence of 40 mM B-R buffer solution at various pH from 4.98 to 9.07.

2.4 Figure S5 Time-dependent fluorescence of CEMT and HMT

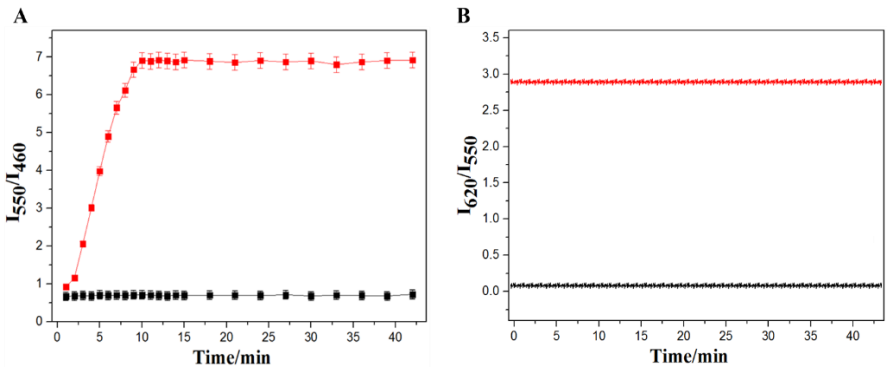

Figure S5 (A) Fluorescence ratio with the reaction time for probe **CEMT** (10 μM) with CE (15 μg/mL) in B-R buffer solution (pH=7.42, 40 mM) (Red signal: **CEMT**+CE. Black signal: **CEMT** only). (B) Fluorescence ratio with the reaction time for probe **HMT** (10 μM) with H^+(Red signal: B-R buffer, pH=9.07. Black signal: B-R buffer, pH=4.98.).

2.5 Figure S6 Selectivity of CEMT and HMT

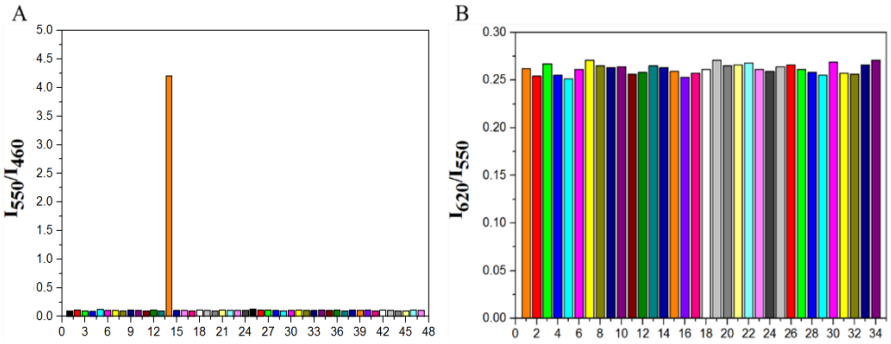

Figure S6 (A) Fluorescence ratio (I_{550}/I_{460}) of probe **CEMT** (10.0 μM) in B-R buffer solution (pH=7.42, 40 mM) containing tested compounds, including 1. a-CT(10 μg/mL); 2. Trypsin(10 μg/mL); 3. Pepsin(10 μg/mL); 4. BSA(50 μg/mL); 5. PBS(50 mM); 6. HAS(50 μg/mL); 7. AChE(10 μg/mL); 8. BChE(20 U/L); 9. PON-1(10 μg/mL); 10. PON-2(10 μg/mL); 11. FAP(10 μg/mL); 12. Ca^{2+}(100 mM); 13. Zn^{2+}(100 mM); 14. Carboxylesterase(10 μg/mL); 15. Mn^{2+}(100 mM); 16. Co^{2+}(100 mM); 17. Mg^{2+}(100 mM); 18. Fe^{2+}(100 mM); 19. Cu^{2+}(100 mM); 20. K^{+}(100 mM); 21. Al^{3+}(100 mM); 22. Na^{+} (100 mM); 23. Cl^{-}(100 mM); 24. human plasma(10 mM); 25. glucose(10 mM); 26. H_2O_2 (10 mM); 27. TBHP (10 mM); 28. OCl^{-} (10 mM); 29. O_2^{-} (10 mM); 30. ·OtBu (10 mM); 31. ·OH (10 mM); 32. $ONOO^{-}$ (10 mM); 33. Cys (1 mM); 34. Glu (1 mM); 35. Gly (1 mM); 36. Val (1 mM); 37. Thr (1 mM); 38. Tyr (1 mM); 39. Trp (1 mM); 40. Ser (1 mM); 41. Phe (1 mM); 42. Met (1 mM); 43. Leu (1 mM); 44. Ile (1 mM); 45. Asp (1 mM); 46. Lys (1 mM); 47. Carbonic anhydrase (10 μg/mL). (B) Fluorescence ratio (I_{620}/I_{550}) of probe **HMT** (10.0 μM) in various solutions of B-R (pH=5.31) containing tested compounds, including 1. blank (only B-R:); 2. K^{+}(20 mM); 3. Ca^{2+}(20 mM) ; 4. Na^{+}(20 mM) ; 5. Mg^{2+} (20 mM) 6. Li^{+}(20 mM); 7. Sr^{2+}(20 mM); 8. Ba^{2+}(20 mM); 9. Fe^{2+}(1 mM) ; 10. Fe^{3+}(0.1 mM); 11. Co^{2+}(1 mM) ;12. Ni^{2+}(20 mM) ; 13. Cu^{2+}(1 mM) ; 14. Zn^{2+}(20 mM); 15. Hg^{2+} (20 mM);16. Mn^{2+}(20 mM) ;17. Cr^{3+}(0.1 mM); 18. F^{-}(20 mM) ; 19. Cl^{-}(20 mM) ; 20. Br^{-}(20 mM) ; 21. I^{-}(20 mM) ; 22. SO_3^{2-}(20 mM); 23. SO_4^{2-}(20 mM); 24. S^{2-}(20 mM); 25. PO_4^{3-}(20 mM); 26. N_3^{-}(1 mM) ; 27. OAC^{-}(1 mM); 28. NO_3^{-}(1 mM); 29. NO_2^{-}(1 mM); 30. HCO_3^{-}(20 mM); 31. H_2O_2(0.1 mM), 32. Cys(1 mM) , 33. Hcy(1 mM) , 34. GSH(1 mM) .

2.6 Figure S7 pH reversibility study of HMT

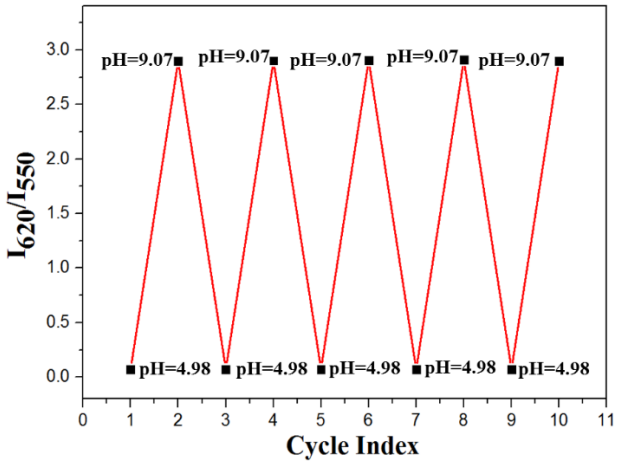

Figure S7 pH reversibility study of **HMT** (10.0 μM) between pH 4.98 and 9.07 in 40 mM B-R buffer solution.

2.7 Figure S8 Dynamic fluorescence imaging for penetration and photostability

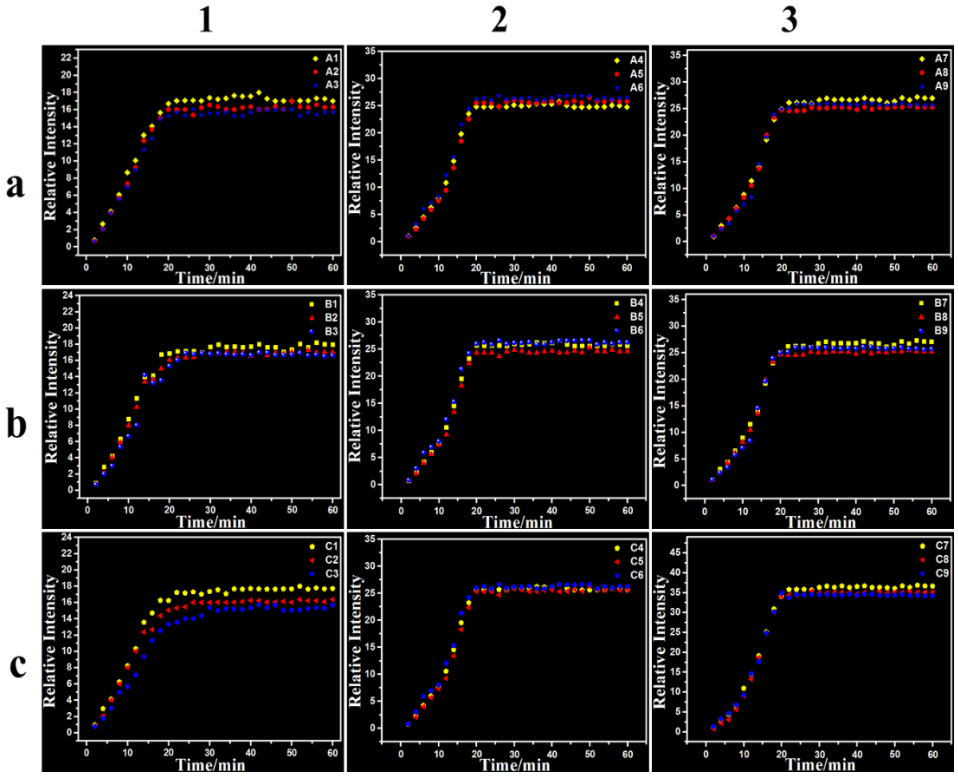

Figure S8 Dynamic fluorescence imaging of HepG2 cells. Intact cells which were incubated with **CEMT** (10 μM) in B-R buffer medium (a: pH=4.98, b: pH=7.42, c: pH=9.07, 37 ℃). Images were captured using 810 nm for two-photon excitation. Two-photon fluorescence emission window: (1) Green channel (400–500 nm). (2) Yellow channel (500–570 nm). (3) Red channel (570–710 nm).

2.8 Figure S9 Cell viability assay

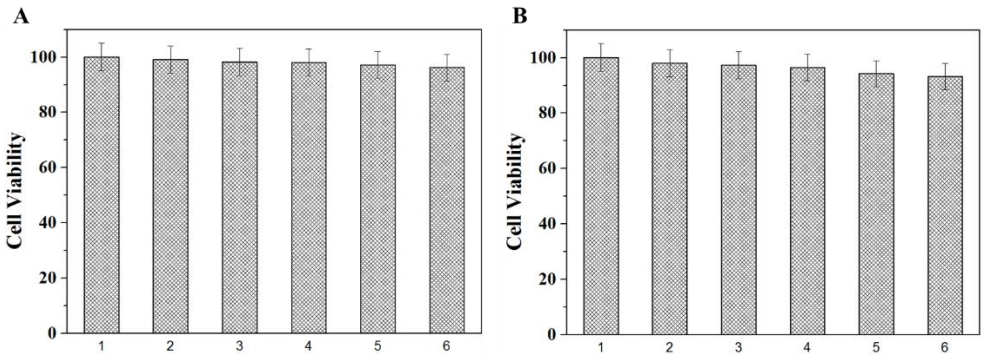

Figure S9 Cell viability of HepG2 cells treated with different concentrations of probe (A) **CEMT** and (B) **HMT** (1 μM, 10 μM, 50 μM, 0.1 mM, 0.5 mM and 1 mM) for 24 h in fresh medium.