Electronic Supplementary Information

ROS activatable prodrug for ALDH overexpressed cancer stem cells

Miae Won,[a†] Ji Hyeon Kim,[a†] Myung Sun Ji,[a†], Jong Seung Kim[a,*]

[a] Department of Chemistry, Korea University, Seoul 02841, Korea.
*Corresponding: jongskim@korea.ac.kr (JSK)
†These authors contributed equally.

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1. Experimental Section

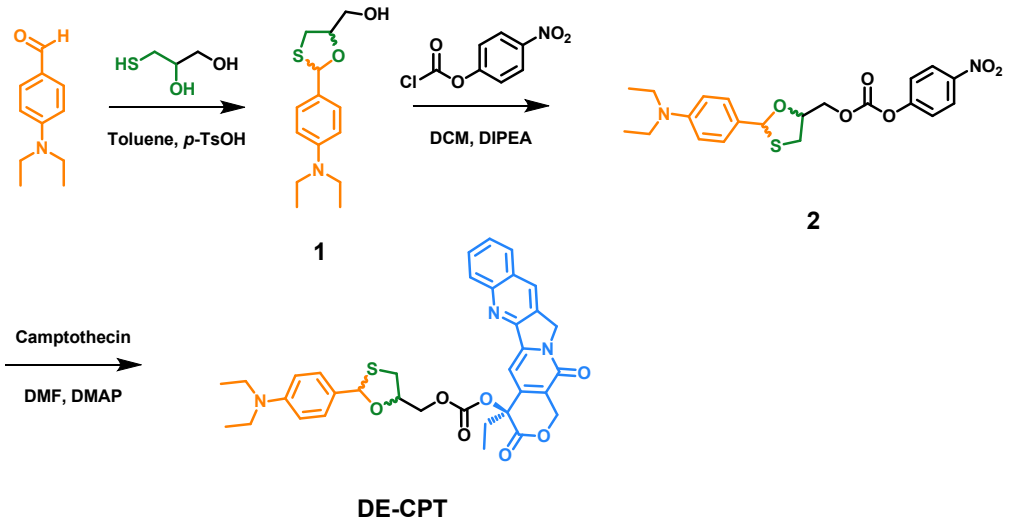
1.1 General information and materials

Unless otherwise noted, all the materials used for the synthesis were purchased from the commercial suppliers. 4-Diethylaminobenzaldehyde (Alfa aesar), α-Thioglycerol (TCI), *p*-Toluenesulfonic acid monohydrate (Alfa aesar), 4-Nitrophenylchloroformate (Carbosynth), Camptothecin (Carbosynth), *N,N*-Diisopropylethylamine (Aldrich), 4-(Dimethylamino)pyridine (Daejung) were purchased and used without further purification. TLC Silica gel 60 F_{254}, 0.25 mm (Merck) was used for analytical thin layer chromatography. PLC Silica gel 60 $F_{254,}$ 1 mm (Merck) was used for preparative thin layer chromatography. Column chromatography was performed with silica gel 60 (Merck, 0.063~0.2 mm) as a stationary phase. ^{1}H and ^{13}C NMR spectra were collected in NMR solvents ($CDCl_3$) on a Bruker 500 MHz spectrometer. All chemical shifts are reported in ppm values using the peak of TMS as an internal reference. The mass spectra were collected on LC/MS-2020 Series (Shimadzu). Analytical or preparative high-performance liquid chromatography (HPLC) were performed using YL9101S (YL-Clarity) and the reverse phase column (C18, 5 mm, waters) was equipped. UV-Vis spectra were recorded on a Scinco S-3100 spectrometer, and fluorescence spectra were obtained using a Shimadzu RF-5301PC instrument. Stock solutions of **DE-CPT** were prepared in DMSO. All excitation and emission slit widths were set at 5 nm. The concentration of each of the samples was fixed at 10 μM in a total volume of 3 mL.

1.2 UV/Vis and Fluorescence spectroscopic method

All UV-vis absorption spectra were obtained using Jasco V-750 spectrophotometer. All fluorescence spectra were collected in Jasco FP-8500 spectrofluorometer. 10 mM stock solutions of **DE-CPT** and other compounds were prepared in ACN or DMSO. 100 mM hydrogen peroxide stock solution was prepared by diluting 27% hydrogen peroxide solution (Alfa aesar). Sodium hypochlorite stock solution was prepared by diluting sodium hypochlorite, 11-15% available chlorine solution (Alfa aesar) and the concentration of sodium hypochlorite was confirmed by measuring absorbance at 292 nm (ε = 350 M^{-1} cm^{-1}).[1] And stock solutions of other analytes such as metal perchlorate and amino acid were prepared in distilled water.

1.3 Synthesis



Scheme S1. Synthetic route for **DE-CPT**.

Compound 1: To a 100 mL round-bottom flask, α-thioglycerol (1.99 g, 18.4 mmol, 1.0 eq) and 4-diethylaminobenzaldehyde (3.58 g, 20.2 mmol, 1.1 eq) were dissolved in toluene. A catalytic amount of *p*-toluenesulfonic acid monohydrate (400 mg) was added to the solution. Then the solution was refluxed and stirred overnight. The progress of the reaction was checked by thin-layer chromatography. After completion of the reaction, the organic solvent was removed. The reaction mixture was dissolved in ether and washed with sat. $NaHCO_3$ solution and brine. The organic phase was dried using anhydrous $NaSO_4$ and evaporated under reduced pressure. The mixture was purified by silica column chromatography (eluent: 1% acetone in DCM). The diastereomeric mixture was obtained as a yellow-red oil (1.19 g, 24%). ^{1}H NMR (500 MHz, $CDCl_3$) δ(1:3.3 mixture of diastereomers): 7.39-7.30 (m, 2H), 6.68-6.64 (m, 2H), 6.12 (s, 0.24H), 6.03 (s, 0.76H), 4.61-4.56 (m, 0.24H), 4.25-4.19 (m, 0.76H), 3.96-3.74 (m, 2H), 3.35 (q, 4H, J = 7.1 Hz), 3.28-3.12 (m, 2H), 2.00-1.88 (m, 1H, -OH), 2.15 (t, 6H, J = 7.1 Hz) ppm.; ^{13}C NMR (125 MHz, $CDCl_3$) δ(diastereomers): 148.5(148.2), 128.7(128.3), 123.5, 111.3, 87.6(86.5), 83.5(82.4), 63.7(63.2), 44.42, 34.8(34.7) ppm.; ESI-MS: *m/z* calcd for $C_{14}H_{21}NO_2S$: 267.13; found: 268.25 $[M+H]^+$

Compound 2: Compound **1** (1.10 g, 4.11 mmol, 1.0 eq) was dissolved in DCM and the solution was cooled in an ice bath. To the solution, DIPEA (2.1 mL, 12 mmol, 3.0 eq) was added and sequentially 4-nitrophenylchloroformate (1.24 g, 6.15 mmol, 1.5 eq) dissolved in DCM was

added dropwise. The progress of the reaction was checked by thin-layer chromatography. After completion of the reaction, the organic solvent was removed. The reaction mixture was dissolved in EtOAc and washed several times with sat. $NaHCO_3$ solution. The organic phase was dried using anhydrous $NaSO_4$ and evaporated under reduced pressure. The mixture was firstly purified by flash column chromatography (eluent: DCM only) for removing unreacted 4-nitrophenylchloroformate. And the mixture was secondly purified by silica column chromatography (eluent: EtOAc:Hexanes = 1:4). The diastereomeric mixture was obtained as a yellow-red oil (950 mg, 54%). ^{1}H NMR (500 MHz, $CDCl_3$) δ(1:2.9 mixture of diastereomers): 8.30-8.24 (m, 2H), 7.40-7.31 (m, 4H), 6.66-6.61 (m, 2H), 6.18 (s, 0.26H), 6.07 (s, 0.74H), 4.84-4.34 (m, 3H), 3.42-3.27 (m, 5H), 3.18-3.06 (m, 1H), 1.18-1.12 (m, 6H) ppm.; ^{13}C NMR (125 MHz, $CDCl_3$) δ(diastereomers): 155.4, 152.4, 148.5, 145.5, 128.6(128.3), 125.3, 123.2, 121.8, 111.3, 87.9(86.8), 79.7(78.9), 69.0(68.5), 44.4, 35.4(35.0), 12.5 ppm.; ESI-MS: *m/z* calcd for $C_{21}H_{24}N_2O_6S$: 432.14; found: 433.25 $[M+H]^+$

DE-CPT: To the solution of compound **2** (248 mg, 0.57 mmol, 1.0 eq) in DMF, camptothecin (200 mg, 0.57 mmol, 1.0 eq) and 4-(dimethylamino)pyridine (281 mg, 2.3 mmol, 4.0 eq) was added at room temperature. Then the solution was stirred overnight. The progress of the reaction was checked by thin-layer chromatography. After completion of the reaction, the organic solvent was removed. The reaction mixture was dissolved in EA and washed several times with sat .$NaHCO_3$ solution. The organic phase was dried using anhydrous $NaSO_4$ and evaporated under reduced pressure. The mixture was purified by silica column chromatography (eluent: acetone:DCM = 1:9). The diastereomeric mixture was obtained as a yellow solid (110 mg, 30%). ^{1}H NMR (500 MHz, $CDCl_3$) δ (1:1.1:2.7:3.1 mixture of diastereomers): 8.39-8.31 (m, 1H), 8.24-8.19 (m, 1H), 7.96-7.90 (m, 1H), 7.86-7.80 (m, 1H), 7.69-7.64 (m, 1H), 7.37-7.09 (m, 3H), 6.60-6.42(m, 2H), 6.10 (s, 0.13H), 6.07 (s, 0.14H), 5.99 (s, 0.33H), 5.97 (s, 0.40H), 5.75-5.67(m, 1H), 5.42-5.35(m, 1H), 5.32-5.14(m, 2H), 4.76-4.17(m, 3H), 3.39-3.21(m, 5H), 3.12-2.95 (m, 1H), 2.34-2.23 (m, 1H), 2.21-2.10 (m, 1H), 1.15-1.09 (m, 6H), 1.01 (t, 3H, J = 7.6 Hz) ppm.; ^{13}C NMR (125 MHz, $CDCl_3$) δ(diastereomers): 167.3(167.2), 157.3, 153.5(153.6, 153.3), 152.2(152.3), 148.9, 148.3(148.1, 148.0), 146.5, 145.6, 131.2(131.0), 130.7(130.6), 129.7(129.8), 128.4(128.5), 128.1 (m), 123.6(125.0, 124.9, 123.4), 120.1(120.4, 120.3, 120.2), 111.1(111.2), 96.0, 87.7(87.8, 86.9, 86.5), 80.1(79.7, 79.2, 78.9), 78.2(78.1), 68.8(68.3, 68.1, 68.0), 67.1, 50.0, 44.4(44.3), 35.5(35.7, 35.1, 35.0), 31.9, 12.5(12.6), 7.7 ppm.; ESI-MS: *m/z* calcd for $C_{35}H_{35}N_3O_7S$: 641.22; found: 642.35 $[M+H]^+$, 664.40 $[M+Na]^+$.

1.4. Cell culture

Human breast cancer cell lines BT474 and the non-cancerous cell lines MCF10A were purchased from Korea Cell Line Bank (Seoul, Korea). BT474 cells were cultured in RPMI 1640 media (HyClone, Chicago, IL, USA). MCF10A cells were cultured Mammary Epithelial Cell Growth basal medium (Lonza, Alpharetta, GA, USA) supplemented with Endothelial Cell Growth Medium Bullet Kit (Lonza). All media were supplemented with 10% FBS (GIBCO, Grand Island, NY, USA) and 1% penicillin /streptomycin (GIBCO) and cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2.

1.5. Cell viability

BT474 and MCF10A cells were seeded in 96 well plates at 1×10^4 cells per well and were allowed to adhere for at least 24 hours. Subsequent to incubation, the cells were treated with **DE-CPT**, CPT, DEAB or 1% DMSO as a control for 72 hours. To determine the cytotoxicity, a Cellomax Cell Viability Kit (Precaregene, Hanam, Gyeonggi-do, Korea) was used according to the manufacturer's instructions in the presence of the three test agents under study. The absorbance of the wells was detected at 450 nm by a Hidex Sense microplate reader (Hidex, Cranbourne, Victoria, AU). Cell viability assays were performed in triplicate and the cytotoxicity was recorded as a percentage calculated for the treated cells relative to the control group.

1.6. Western blot analysis

Protein expression levels of ALDH1, γH2A.X, cleaved PRAP and cleaved caspase 3 were determined by western blotting analyses. Briefly, BT-474 cells were seeded at a density of 3×10^5 cells in 60 mm dish and incubated for 24 h. The attached cells were treated with 30 μM **DE-CPT**, CPT, DEAB or 1% DMSO as a control for 48 hours. After treatment, the cells washed with ice-cold PBS three times and scraped to create cell pellets. After removal of the PBS, a radioimmunoprecipitation assay (RIPA) lysis buffer containing protease inhibitors provided by the manufacturer (Biosesang, Seongnam, Gyeonggi-do, Korea) was added into the cell pellets to obtain protein lysates. A Bradford assay was conducted to measure the protein concentration of each cell lysates. Then protein (30 μg/lane) from each cell lysate was loaded onto a sodium dodecyl sulfate poly-acrylamide gel electrophoresis (SDS-PAGE) set up to

separate the proteins into individual bands. The separated protein bands were transferred to polyvinylidene difluoride (PVDF) membranes (Merck Millipore) and these membranes were incubated with ALDH1 antibodies (Cell Signaling technology; #54135, 1:1000), γH2A.X antibodies (Cell Signaling technology; #9718, 1:1000), cleaved PARP antibodies (Cell Signaling technology; #5625, 1:1000), cleaved caspase 3 antibodies (Cell Signaling technology; #9664, 1:1000) and β-actin (Santa Cruz Biotechnology; SC-47778, 1:1000) overnight at 4 °C. The resulting membranes were washed with Tris-buffered saline containing Tween-20 (TBS-T) and then incubated with the anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibodies (Santa Cruz Biotechnology) and anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibodies (GeneTex, Irvine, CA, USA) overnight at 4 °C. To detect immunoreactive protein bands, enhanced chemiluminescence reagents (Luminate, Merk Millipore) were used according to the manufacturer's instructions.

1.7. PI/Annexin V staining for cell death

Apoptotic cells were evaluated by fluorescence imaging co-stained with calcein-AM and PI (calcein acetoxymethyl ester and propidium iodide, respectively). For this, BT-474 cells (3×10^4 cells) were seeded in confocal 35 mm dishes and were allowed to adhere for at least 24 h. The cells treated with 30 μM **DE-CPT**, CPT, DEAB or Control (1% DMSO) for 48 h followed by double staining with calcein-AM (4 μM) and PI (10 μg/mL). After 30 min of incubation, the medium was removed followed by a gentle wash with PBS. The fluorescent signals for live/dead cells were colorized in green/red, respectively. The excitation wavelength was at 488/530 nm, and the fluorescence emission was collected with filters at 500–600/600–700 nm spectral ranges.

1.8. Spheroid formation

In order to quantify tumor spheroid formation, briefly, BT-474 cells (seeding density at the first day of tumor spheroid formation was 1×10^4 cells/ml) were plated in ultralow-attach 24 well plates (CORNING, Corning, NY, USA) and cultured in RPMI1640 (Gibco), supplemented with B27 (1:50, Invitrogen, Carlsbad, CA, USA), 10 ng/mL basic fibroblast growth factor (bFGF, Invitrogen), 20 ng/mL human epidermal growth factor (EGF, Invitrogen), 1% antibiotic agent, and 15 μg/mL gentamycin for second day. The tumor spheroids were then washed twice with PBS. The tumor spheroid treated with 30 μM **DE-CPT**,

CPT, DEAB or control (1% DMSO) in fresh CSC culture medium, allowed to incubate for 5 days. The number of tumor spheroids were counted based on spheroid sizes larger than 150 μm in diameter under an Olympus X53 inverted microscope.

1.9. Aldefluor assay

BT474 (3 $\times 10^5$ or 1 $\times$ 10^4/ml) cells were seeded in 60 mm dishes for adhesion cells and ultralow-attach 24 well plates for spheroids. After incubation for 24 h, the cells were pre-treated 30 μM **DE-CPT**, CPT, DEAB or control (1% DMSO) for 72 h (adhesion) and 5 days (spheroids), before an ALDEFLUOR^{TM} assay kit (StemCell Technologies, Duham, NC, USA) was used to assess ALDH1 activity according to the manufacturer's protocol. Briefly, cells were incubated for 45 min at 37 ^oC in aldefluor assay buffer containing the ALDH protein substrate BODIPY-aminoacetaldehyde (BAAA, 1 μM per 0.5 $\times$ 10^6 cells). As a specific inhibitor of ALDH1, 50 mM diethylamino-benzaldehyde (DEAB) was used to define the aldefluor-positive population. aldefluor stained cells were analyzed with a flow cytometer.

1.10. RNA extraction and realtime q-PCR analysis

Total RNAs from cell lines were isolated by the PureLinkTM total RNA isolation kit (Invitrogen) and TRIzol reagent (Invitrogen), following the manufacturer's instructions. Reverse transcription to cDNA was performed using the iScriptTM cDNA synthesis kit (BioRad, Hercules, CA, USA). All cDNAs used in real-time PCR were normalized with GAPDH. Quantitative real-time PCRs were performed using iQTMSYBR Green Supermix (BioRad). Gene expression was quantified by the delta-delta-CT method, and real-time PCRs were performed in a CFX-96 thermal cycler (Applied Biosystems, Foster City, CA, USA) and detection system. The following primers for Human target genes were used for realtime PCR: GAPDH sense 5'-AACCATGAGAAGTATGACAACAGC-3' and antisense 5'-GAGTCCTTCCACGATACCAAA-3'; OCT4 sense 5'-TGGGCTCGAGAAGGATGTG-3' and antisense 5'-GCATAGTCGCTGCTTGATCG-3'; Nanog sense 5'-TGAGCTGGTTGCCTCATGTTAT-3' and antisense 5'-GAAGGAAAAGTATCAAGAAATTGGGATA-3' and SOX2 sense 5'-AGGATAAGTACACGCTGCCC-3' and antisense 5'-TAACTGTCCATGCGCTGGTT-3'.

1.11. Statistical analysis

All the data were presented as mean values ± SEM. The statistical analysis was performed using SAS 9.4 program and one-way or two-way ANOVA test for multiple comparisons followed by Bonferroni's test.

2. NMR and ESI-MS spectra

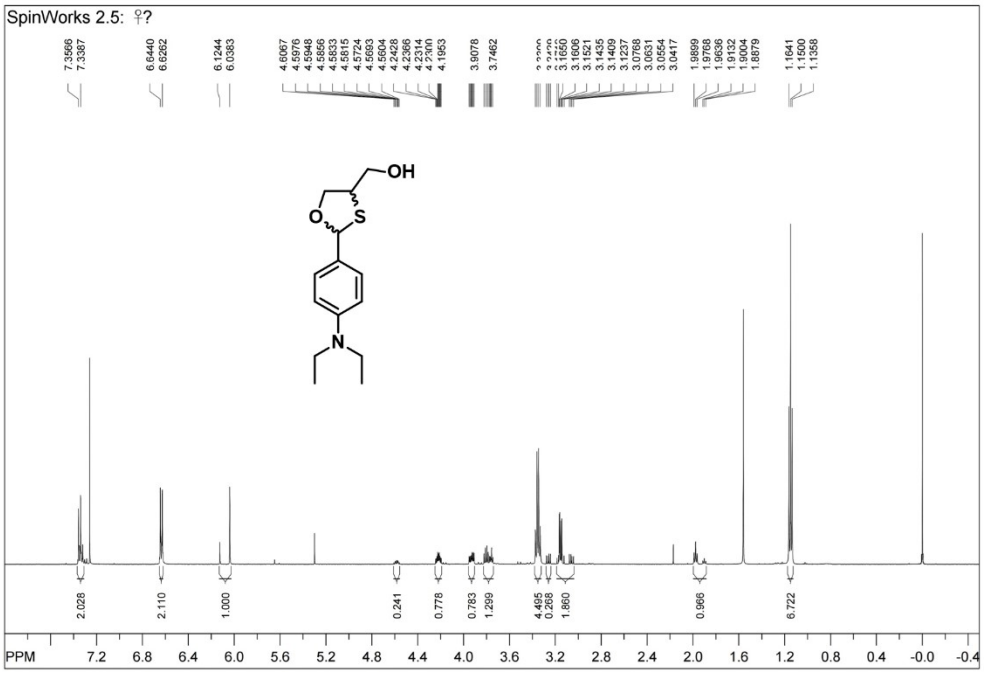


Figure S2. ^{1}H NMR (500 MHz) spectrum of compound **1** in $CDCl_3$.

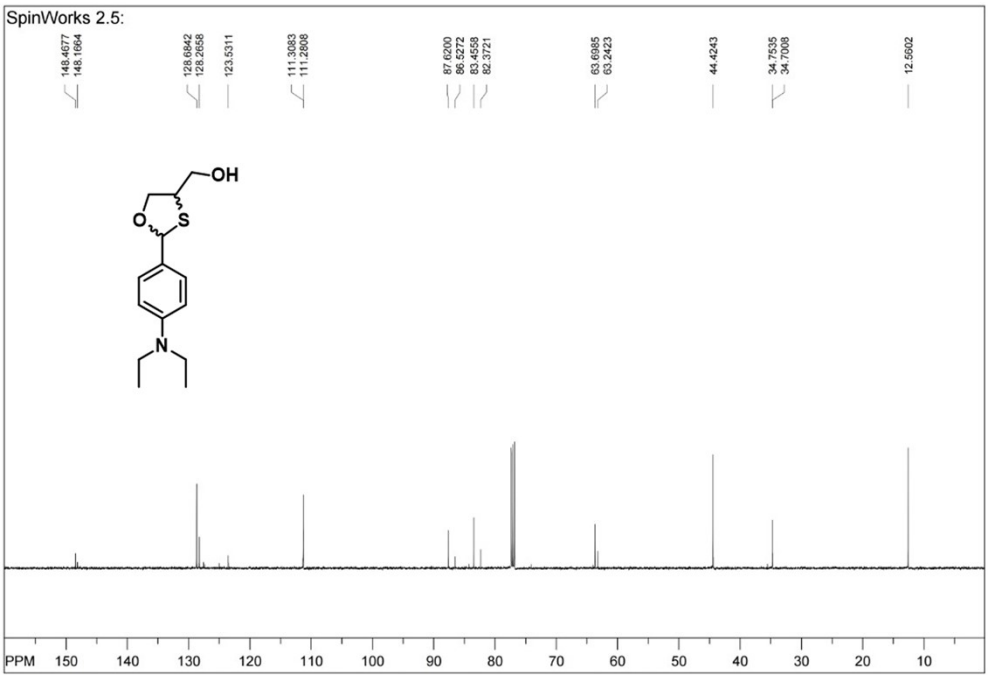


Figure S3. ^{13}C NMR spectrum (125 MHz) of compound **1** in $CDCl_3$.

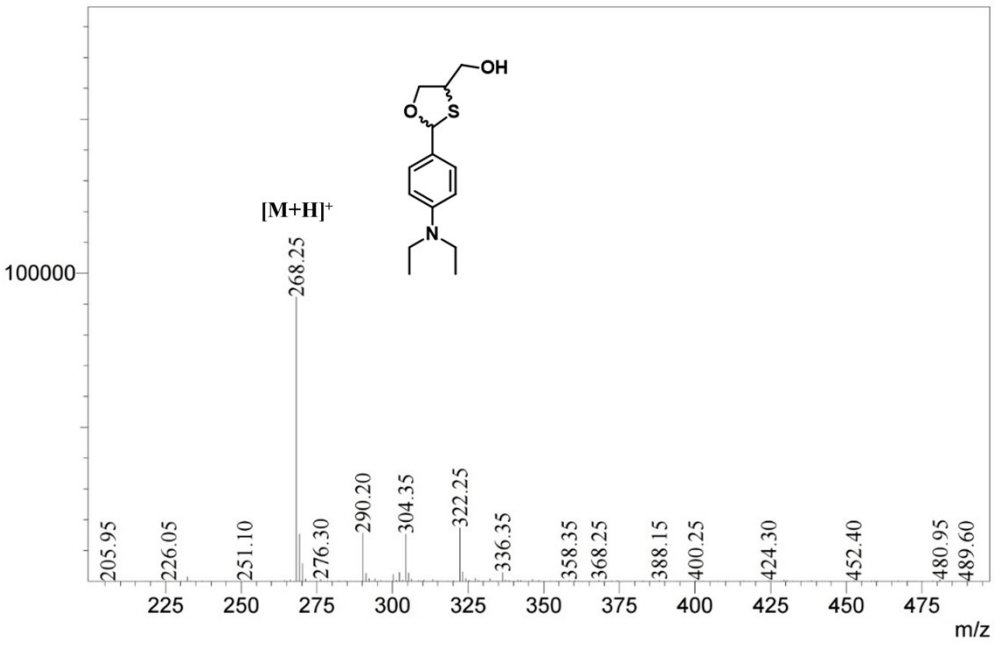


Figure S4. ESI-MS spectrum of compound **1** (positive).

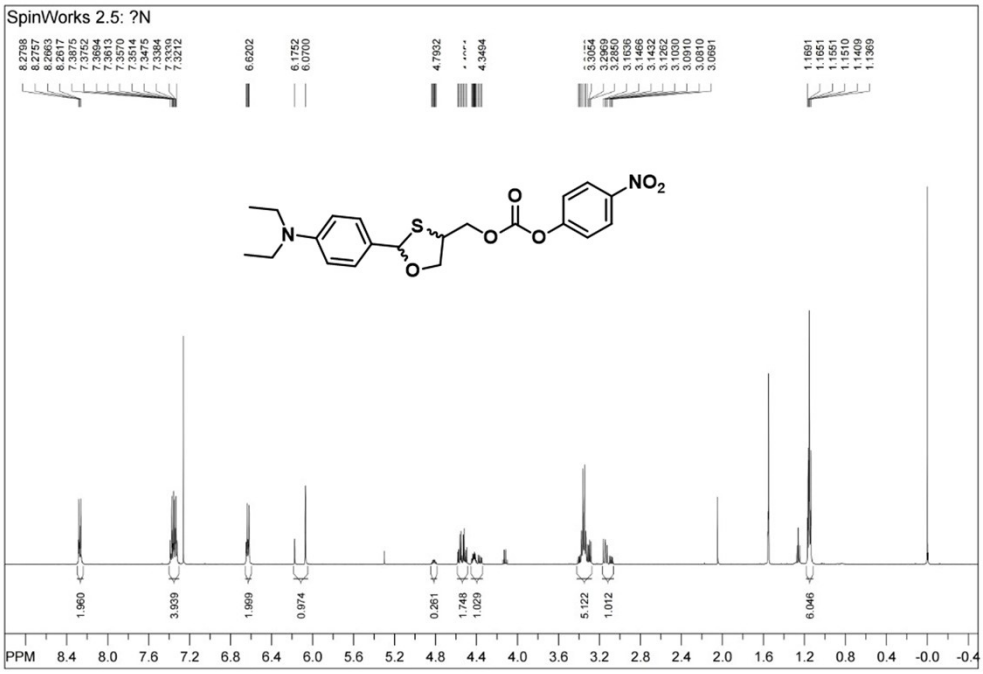


Figure S5. ^{1}H NMR (500 MHz) spectrum of compound **2** in $CDCl_3$.

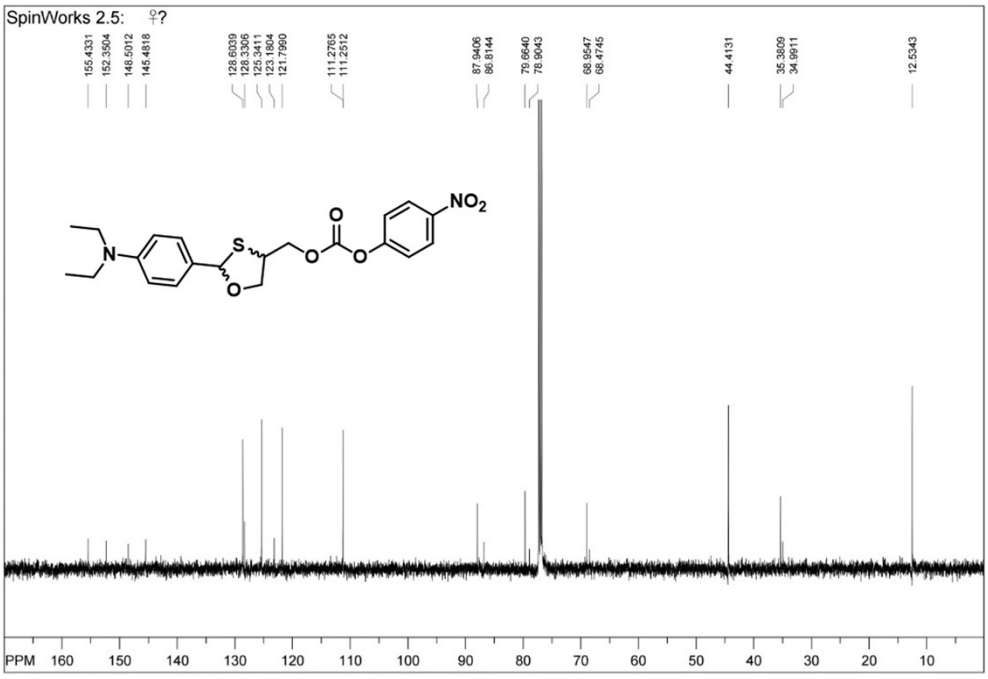


Figure S6. ^{13}C NMR spectrum (125 MHz) of compound **2** in $CDCl_3$.

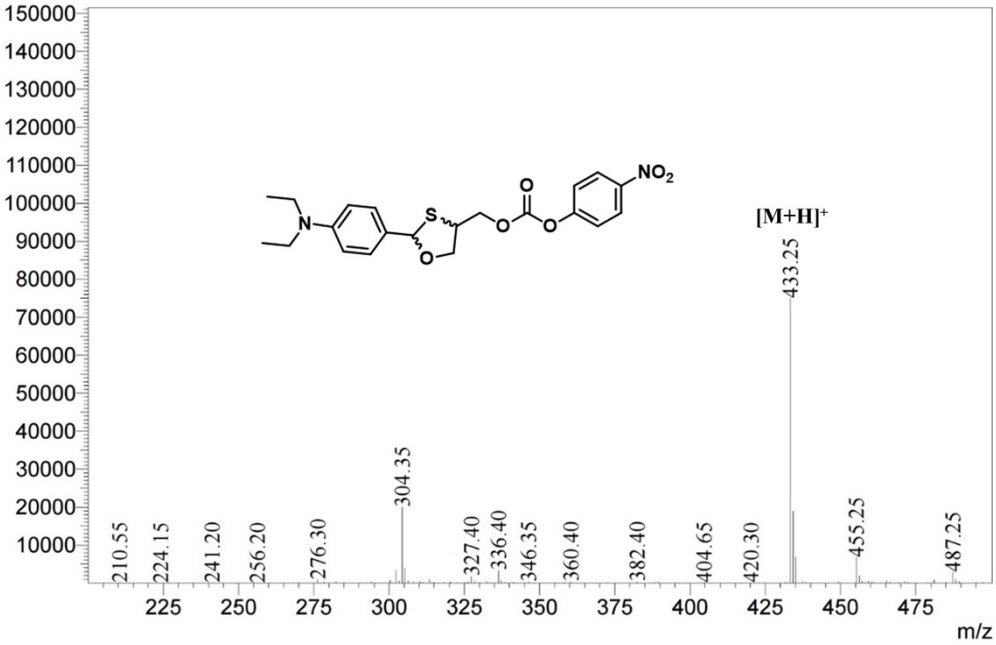


Figure S7. ESI-MS spectrum of compound **2** (positive).

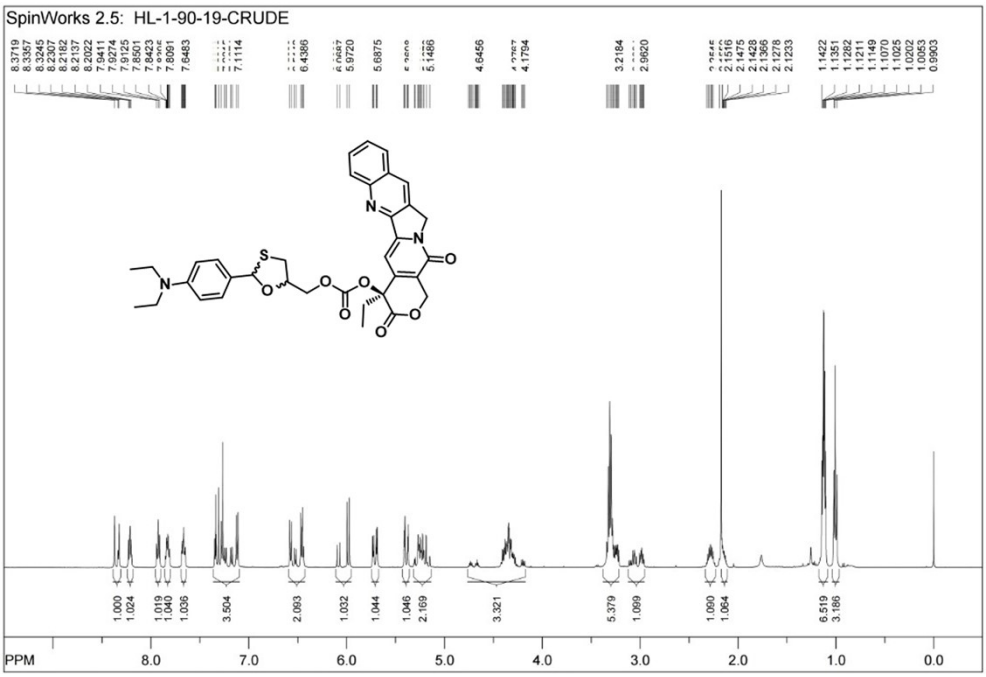


Figure S8. ^{1}H NMR (500 MHz) spectrum of compound **DE-CPT** in $CDCl_3$.

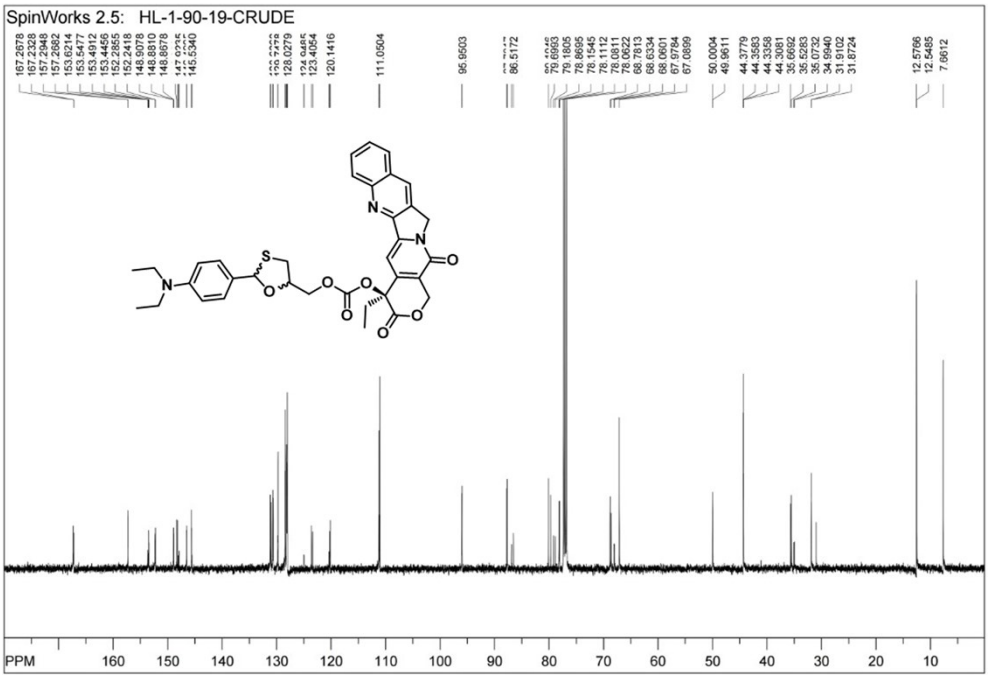


Figure S9. ^{13}C NMR spectrum (125 MHz) of compound **DE-CPT** in $CDCl_3$.

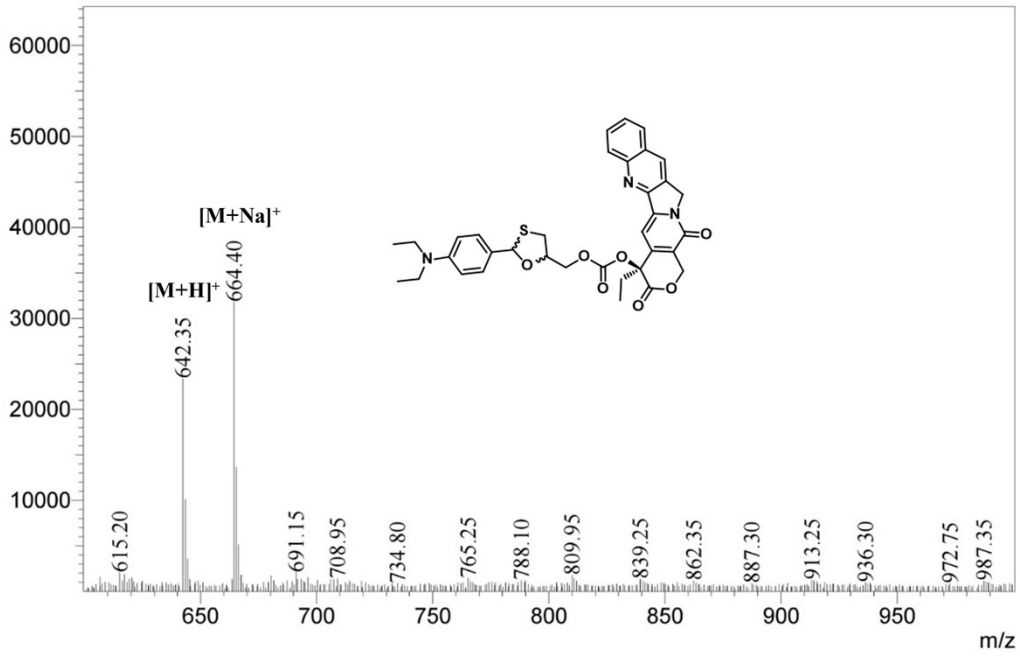


Figure S10. ESI-MS spectrum of compound **DE-CPT** (positive).

3. Solution test experiments

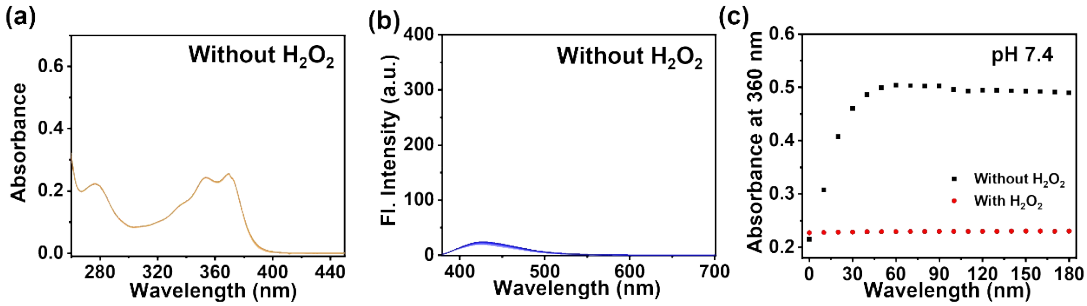


Figure S11. DEAB release of **DE-CPT** under pH 7.4 condition**. (a)** Absorbance spectra of **DE-CPT** (10 μM) treated without 100 eq of H_2O_2. **(b)** Fluorescence spectra of **DE-CPT** (10 μM) treated without 100 eq of H_2O_2. **(c)** The absorbance changes of **DE-CPT** at 360 nm determined from part Figure 2b/ Figure S11a data. The solution was incubated up to 3 h at 37 °C in ACN/PBS (pH 7.4) buffer (1:3) solution.

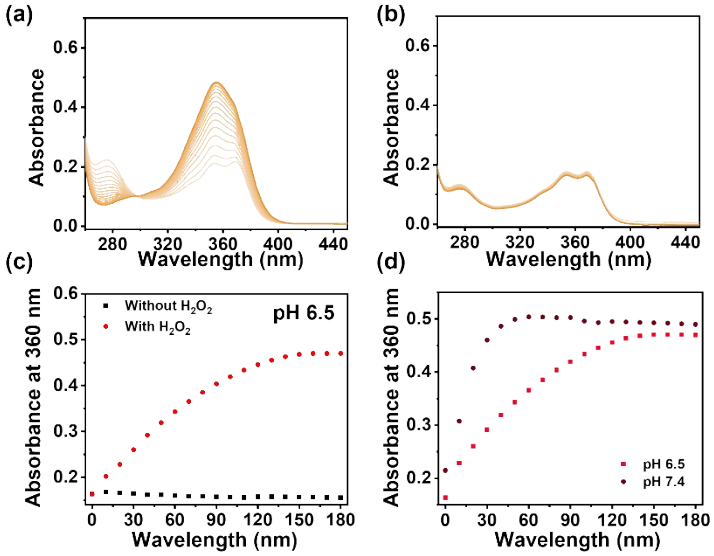


Figure S12. DEAB release of **DE-CPT** under mild acidic condition**.** (a) Absorbance spectra of **DE-CPT** (10 μM) in 37 °C ACN/PBS (pH 6.5) buffer (1:3) solution treated with or (b) without 100 eq of H_2O_2. (c) The fluorescence changes of **DE-CPT** at 435 nm determined from part a /b data. (d) The comparison of **DE-CPT** fluorescence changes at pH 6.5 and pH 7.4 in the presence of 100 eq H_2O_2.

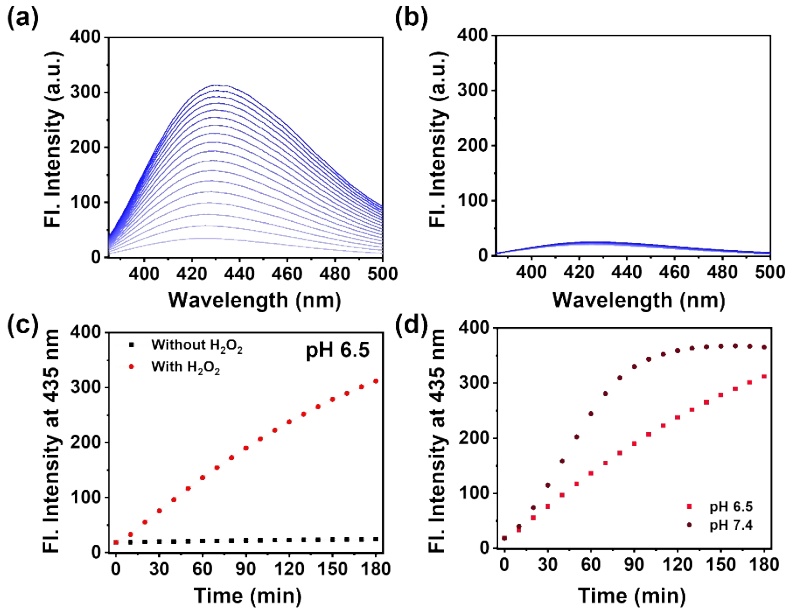


Figure S13. CPT release of **DE-CPT** under mild acidic condition**.** (a) Fluorescence spectra of **DE-CPT** (10 μM) in 37 °C ACN/PBS (pH 6.5) buffer (1:3) solution treated with or (b) without 100 eq of H_2O_2. (c) The fluorescence changes of **DE-CPT** at 435 nm determined from part a /b data. (d) The comparison of **DE-CPT** fluorescence changes at pH 6.5 and pH 7.4 in the presence of 100 eq H_2O_2.

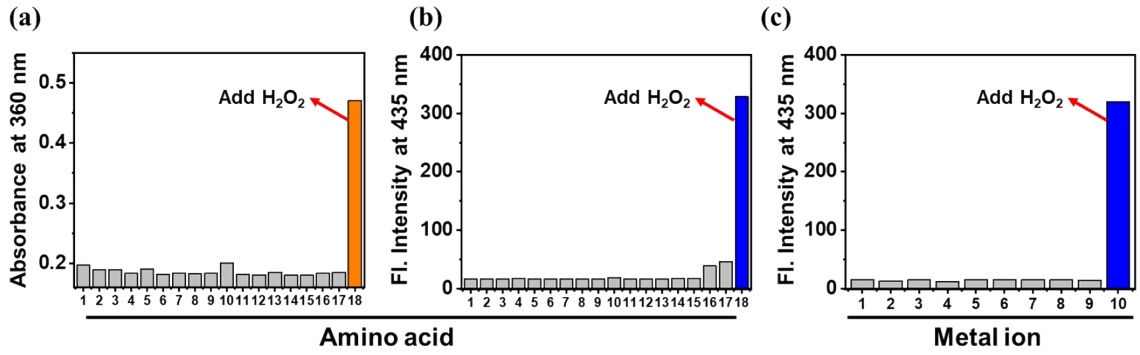


Figure S14. Selectivity study of **DE-CPT** (10 μM) in ACN/PBS buffer (1:4) solution treated with 100 eq various analytes (1: control, 2: glycine, 3: leucine, 4: tyrosine, 5: alanine, 6: methionine, 7: histidine, 8: asparagine, 9: arginine, 10: lysine, 11: serine, 12: glutamine, 13: proline, 14: aspartic acid, 15: glutamic acid, 16: GSH, 17: cysteine, 18: H_2O_2). The solution was incubated up to 2 h at 37 °C. (A) Absorbance intensities of **DE-CPT** at 360 nm. (B) Fluorescence intensities of **DE-CAM** at 435 nm. Selectivity study of **DE-CPT** (10 μM) in ACN/PBS buffer (1:4) solution treated with 100 eq metal ion analytes (1: control, 2: Ni^{2+}, 3: Fe^{3+}, 4: Fe^{2+}, 5: Zn^{2+}, 6: Al^{3+}, 7: Cu^{2+}, 8: Mg^{2+}, 9: Ca^{2+}, 10: H_2O_2). The solution was incubated

up to 2 h at 37 °C. (C) Fluorescence intensities of **DE-CPT** at 435 nm.

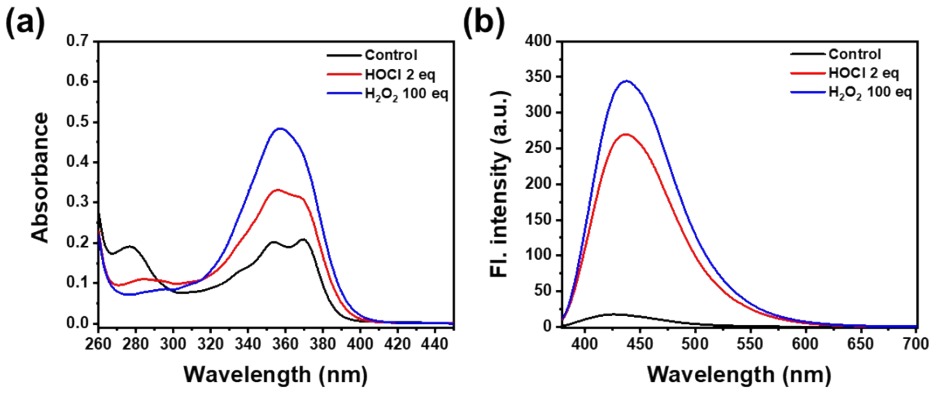


Figure S15. (a) Absorption and (b) fluorescence spectrum of **DE-CPT** (10 μM) in ACN/PBS (pH 7.4) buffer (1:3) solution treated with 2 eq of HOCl or 100 eq of H_2O_2. The solution was incubated up to 2 h at 37 °C.

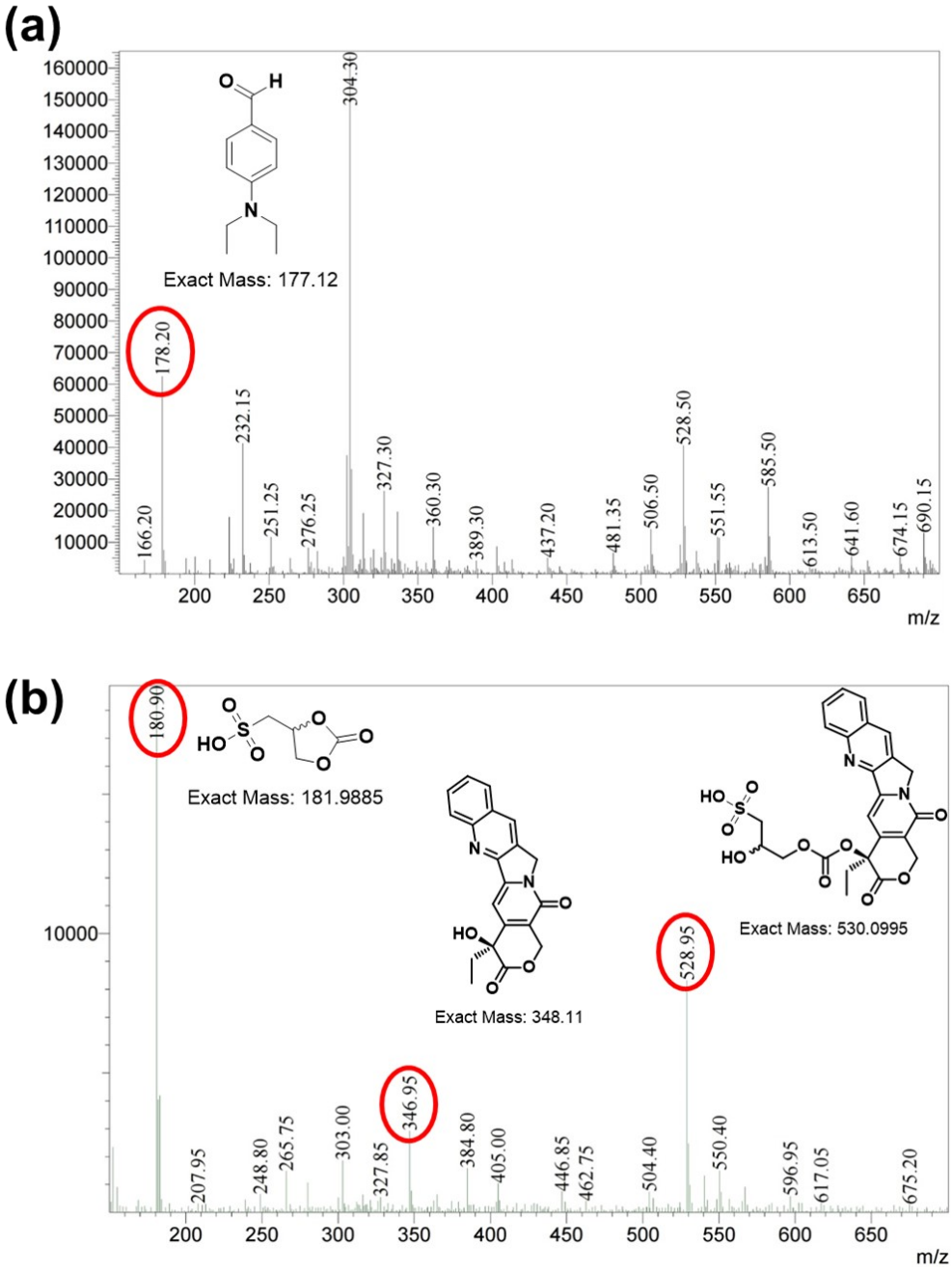


Figure S16. (a) Positive ion mode and (b) negative ion mode mass spectrum of **DE-CPT** (100 μM) in ACN/water (1:4) solution treated with 100 eq of H_2O_2. The solution was incubated to 6 h at 37 °C.

4. In vitro experiments

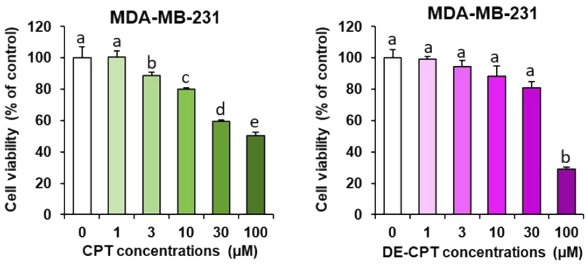


Figure S17. Cell viability of CPT and **DE-CPT** in MDA-MB-231 cells.

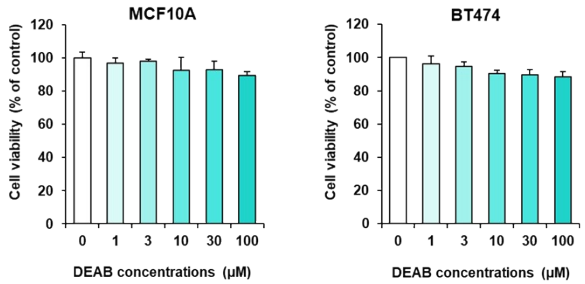


Figure S18. Cell viability of DEAB in MCF10A and BT474 cells.

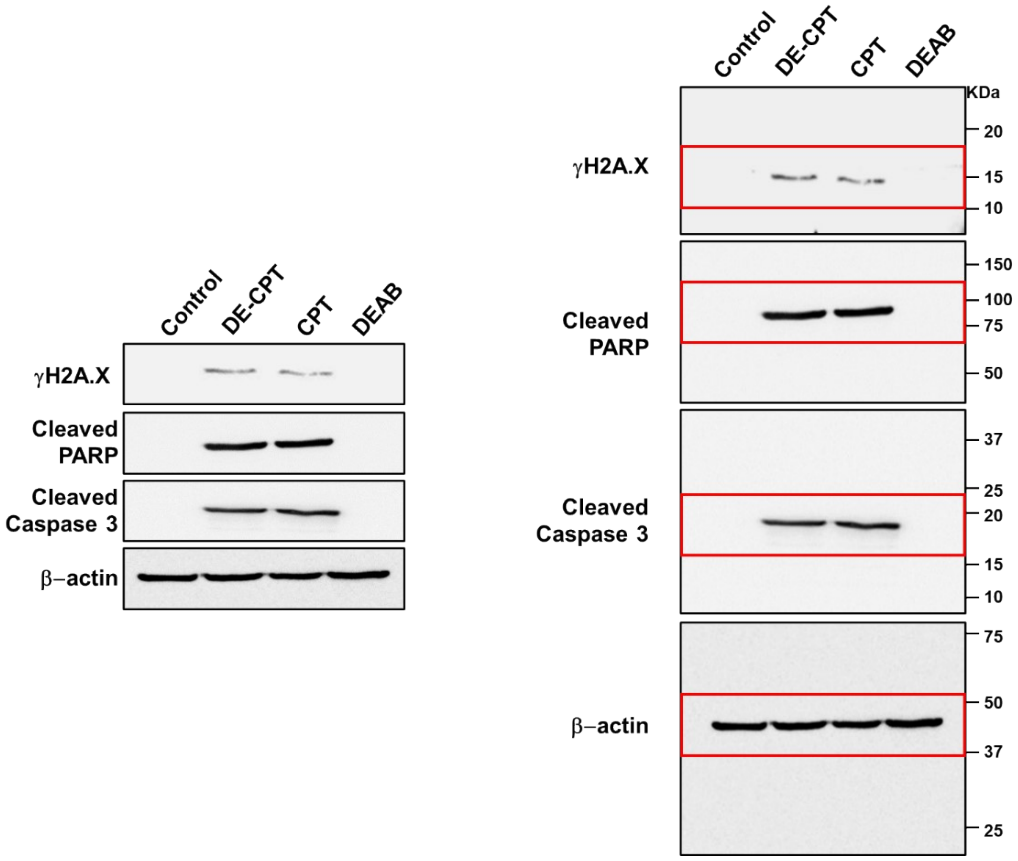


Figure S19. Western blot analysis of Cell death signaling proteins by DE-CPT in BT-474 cells, related to Figure 3d.

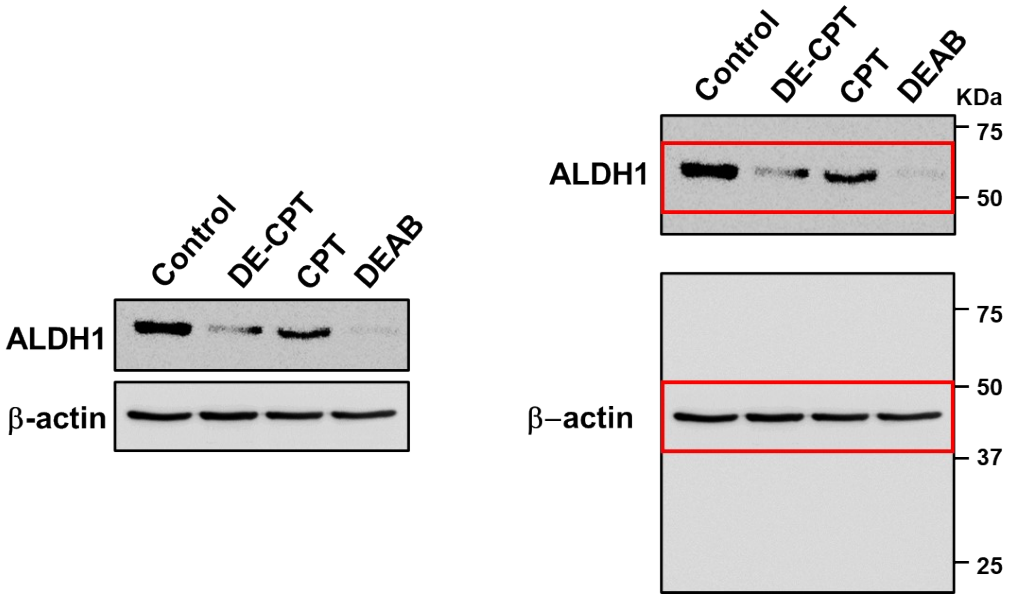


Figure S20. The protein expression of ALDH1 treated with **DE-CPT**, CPT, DEAB, and control in BT474 spheroid cells.

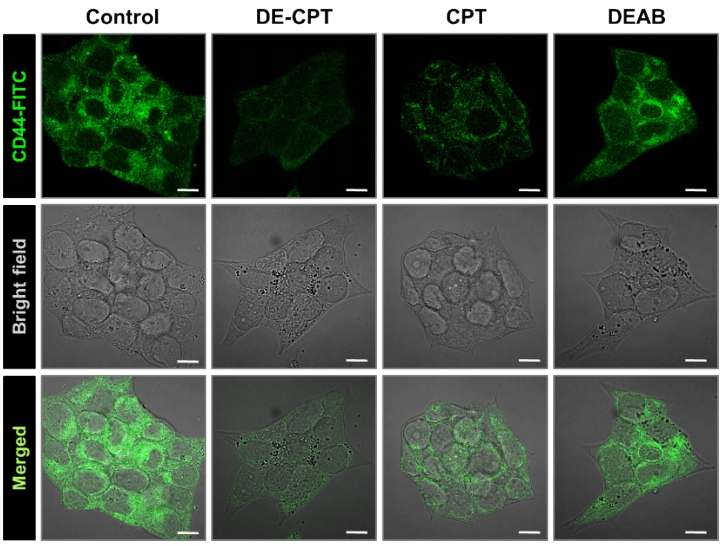


Figure S21. Fluorescence images of CD44-FITC under treated with **DE-CPT**, CPT, DEAB, and control (1% DMSO) in BT474 cells. Scale bar = 100 μm.

5. Reference

1. C. Duan, M. Won, P. Verwilst, J. Xu, H. S. Kim, L. Zeng, and J. S. Kim, *Anal. Chem.*, 2019, **91,** 4172.