Electronic Supplementary Information (ESI)

Immunogenicity and cytotoxicity of a platinum(IV) complex derived from capsaicin

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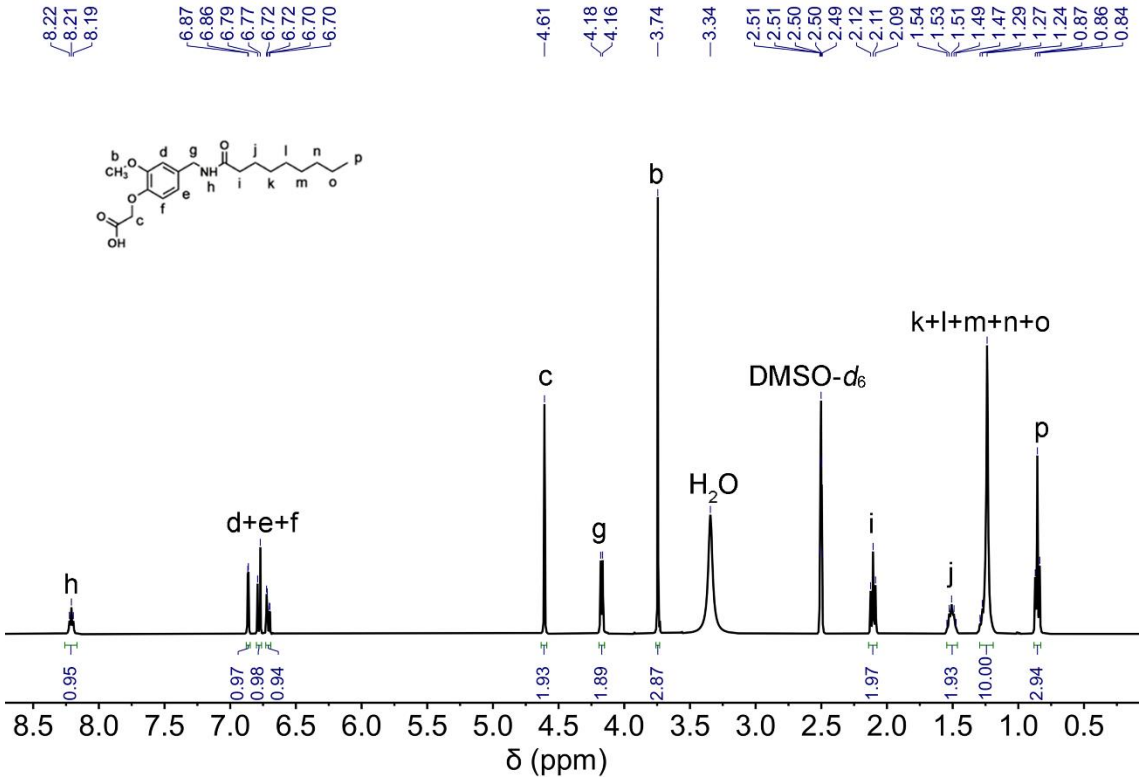
1. Supplementary Figures and Tables



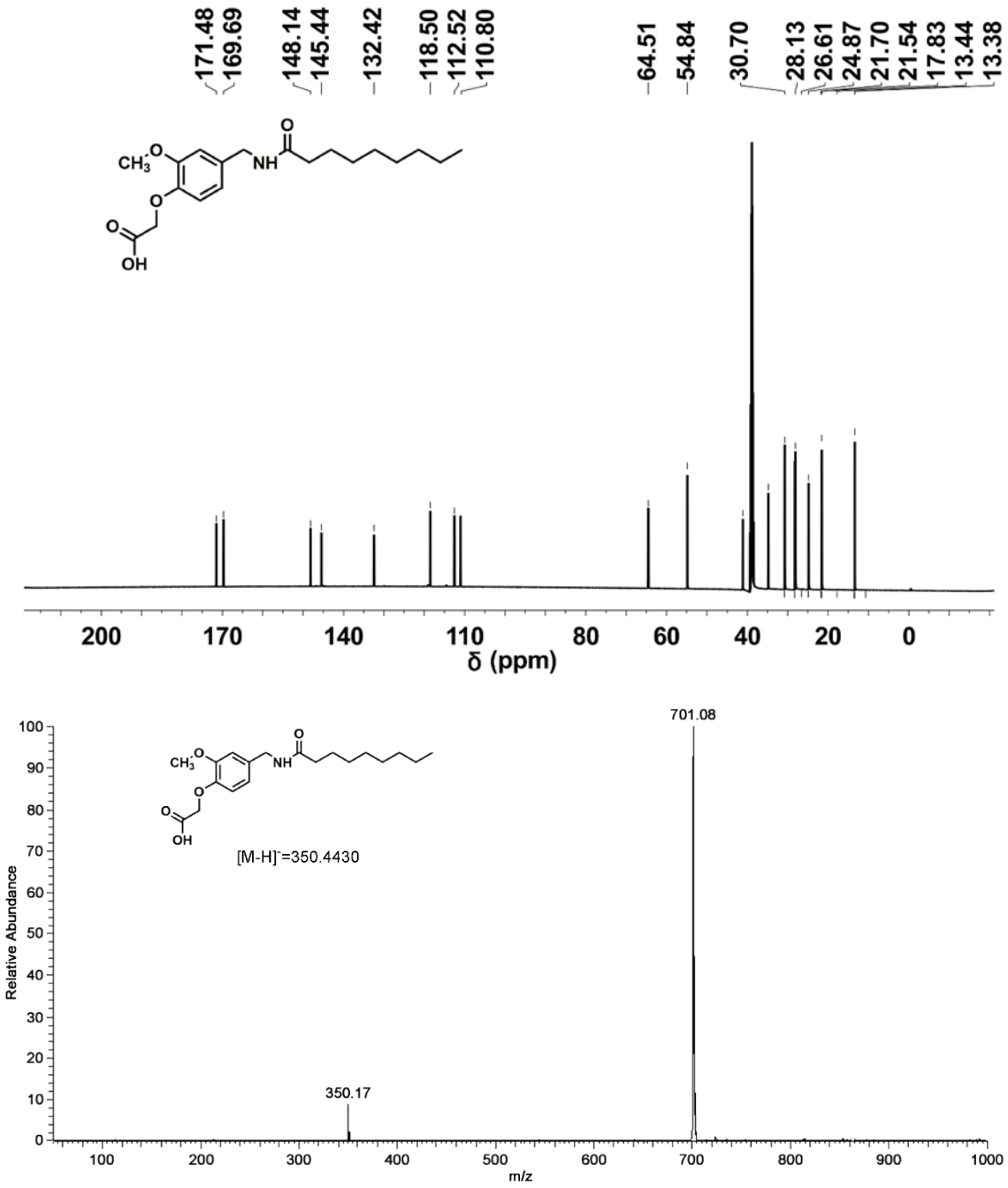
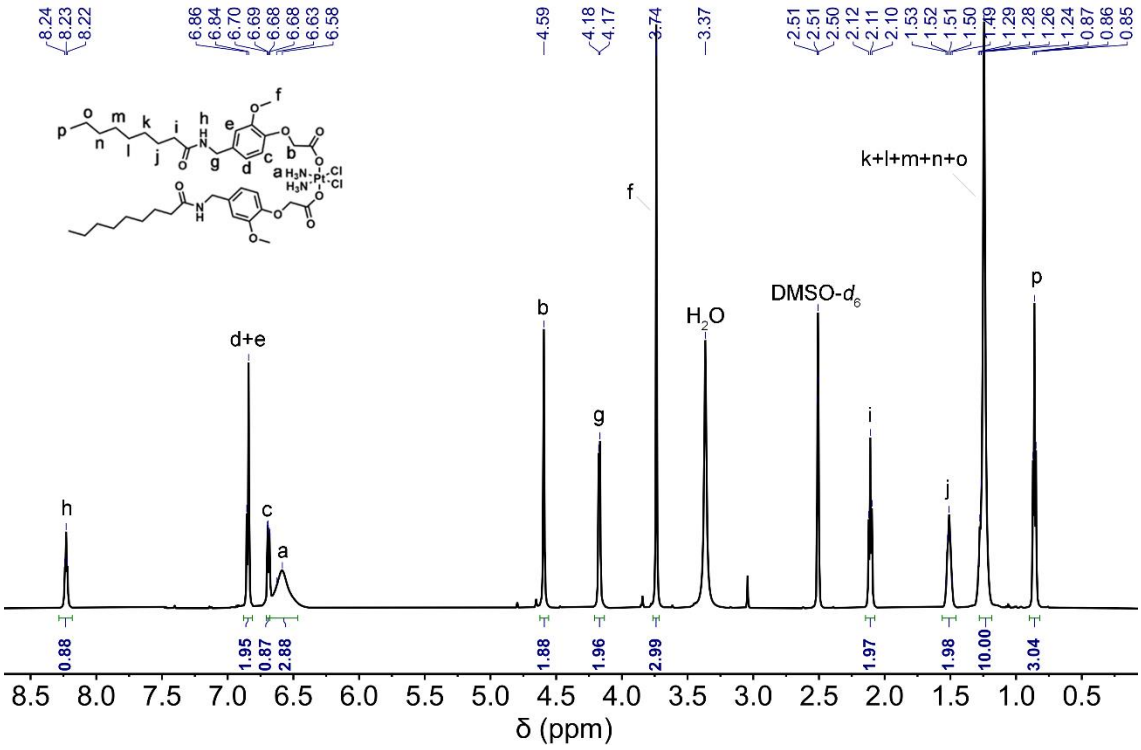


Fig. S1 ^{1}H NMR (600 MHz, DMSO-d_6), ^{13}C NMR (101 MHz, DMSO-d_6) and ESI-MS (negative mode) spectra of SCAA.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8.22
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4.17
3.74
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2.51
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1.53
1.52
1.51
1.50
1.49
1.29
1.28
1.26
1.24
0.87
0.86
0.85
k+l+m+n+o
f
DMSO-d_6
H_2O
b
d+e
g
i
p
j
h
c
a
0.88
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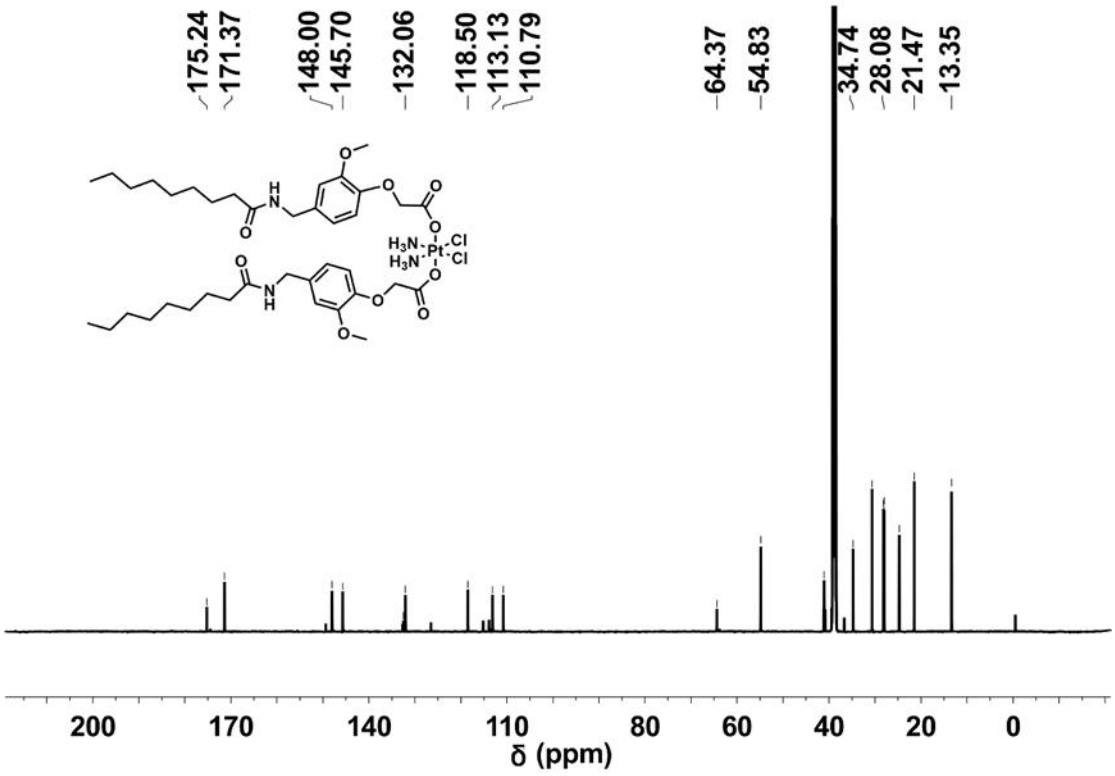
δ (ppm)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δ (ppm)

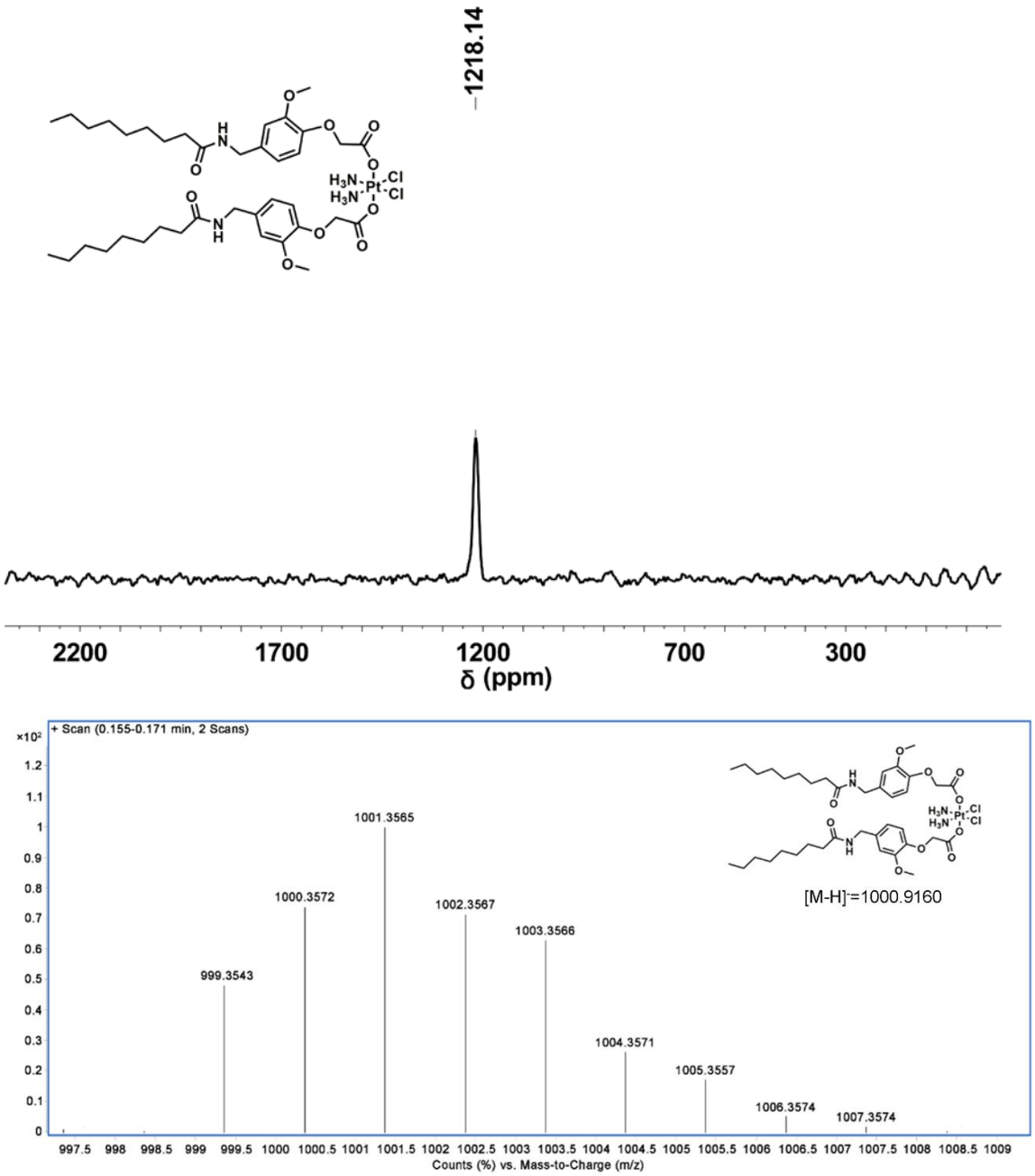

Fig. S2 ^{1}H NMR (600 MHz, DMSO-d_6), ^{13}C NMR (101 MHz, DMSO-d_6), ^{195}Pt NMR (86 MHz, DMSO-d_6) and HR-ESI-MS (negative mode) spectra of DCP

2. Experimental

2.1 Materials

All chemicals were used as received without further purification unless otherwise noted. Cisplatin was purchased from Shandong Boyuan Pharmaceutical Co. Ltd.

Triethylamine (TEA), *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TBTU), *N*-dimethylformamide (DMF), dimethyl sulfoxide (DMSO), hydrogen peroxide (30% H_2O_2), sodium sulfate anhydrous, methanol and ether were purchased from J&K Scientific. AsA, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), phorbol 12-myristate 13-acetate (PMA) and synthetic capsaicin (SC) were purchased from Sigma-Aldrich. Annexin V conjugated with fluorescein isothiocyanate (Annexin V-FITC) and propidium iodide (PI) were purchased from KeyGen Biotech. Co. Ltd. (Nanjing, China). ROS, enhanced ATP and CCK-8 assay kits and cell-lysis RIPA buffer were purchased from Beyotime Institute of Biotechnology. HMGB1, IFN-γ and TNF-α ELISA kits were purchased from Genxspan, USA. CRT antibody (ab2907) and Alexa Fluor 488-labeled goat anti-rabbit IgG (ab150077) were purchased from Abcam. Calf thymus (CT) DNA, CellTrace™ Far Red and CellTrace™ CFSE were purchased from ThermoFisher Scientific. Ultrapure water was prepared using a Millipore Simplicity System (Millipore).

The human PANC-1, MCF-7, MG-63, HepG2, PBMC and human monocytic THP-1 cells were purchased from American Type Culture Collection (ATCC). Fetal bovine serum (FBS) and all culture media were from Nanjing KeyGen Biotech Co., Ltd. The percentage of DMSO used in all cell tests was less than 1‰. All the control groups contain equal amount DMSO with the sample groups.

2.2 Measurements

^{1}H-, ^{13}C- and ^{195}Pt-NMR spectra were measured on Bruker DRX-400/-500 and Bruker Avance III 600 spectrometers at 298 K. ESI-MS spectrum was measured on an LCQ spectrometer (Finnigan). HR-MS spectrum was recorded on an Agilent 6540Q-TOF LC/MS spectrometer. The content of Pt was determined on an inductively coupled plasma mass spectrometer (ICP-MS) using a standard Plasma-Quad II instrument (VG Elemental, Thermo OptekCorp.). Fluorescence confocal imaging was carried out on a laser scanning confocal imaging system (Olympus TH4-200) consisting of ZEISS Laser Scanning

Microscope (LSM 710) and a 20 mW-output 488 nm argon ion laser. Flow cytometric analysis was performed on Cytomics FC500 flow cytometer. The optical density was determined by a Tecan Sunrise ELISA Reader. CD spectra were measured by a Jasco J-810 spectropolarimeter (Japan Spectroscopic, Japan).

2.3 Synthesis of oxoplatin

Oxoplatin was synthesized according to the literature.[1] Briefly, cisplatin (0.4g, 1.33 mmol) was stirred in water (12 mL) at 60 ℃, and 30% H_2O_2 (20 mL) was added dropwise to the suspension. The reaction solution was cooled 4 h later at room temperature overnight. After setting at 4 °C for 2 d, light yellow crystals were obtained, which were filtered, washed with cold water and dried under vacuum to obtain pure product. Yield: 67.8% (0.301 g, 0.901 mmol).

2.4 Synthesis of SCAA

SC (1.5 g, 5 mmol) was dissolved in DMF (30 mL), then K_2CO_3 (1.4 g, 10 mmol) and ethyl bromoacetate (1.7 g, 10 mmol) were added and reacted for 30 min and 12 h at 60 ℃, respectively. DMF was removed by rotary evaporation to obtain a light yellow oily solid. After cooling, ethanol (100 mL) and 10% NaOH (3 mL) were added to react for 5 h at room temperature. Water (400 mL) was added and pH was adjusted to 2 to obtain a white precipitate, which was filtered and washed with water to obtain a white solid. After recrystallizing in ethanol and drying, a white powder was obtained. Yield: 85.4% (1.537 g, 4.4 mmol). ESI-MS (negative, m/z): $[M–H]^–$, 350.17, calcd, 350.45; $[2M–H]^–$, 701.08, calcd, 701.88. ^{1}H NMR (600 MHz, DMSO-d_6, ppm): δ 8.22–8.19 (t, 1H, CO-NH-), 6.87–6.86 (m, 1H, PhH-), 6.79–6.77 (m, 1H, PhH-), 6.72–6.70 (m, 1H, PhH-), 4.61 (s, 2H, O-CH_2-), 4.18–4.16 (d, 2H, NH-CH_2-), 3.74 (s, 3H, CH_3-O), 2.12–2.09 (t, 2H, CH_2), 1.54–1.49 (m, 2H, CO-CH_2-), 1.29–1.24 (m, 10H, -C_5H_{10}-), 0.87–0.84 (m, 3H , -CH_3). ^{13}C NMR (101MHz, DMSO-d_6, ppm): δ 171.48, 169.69, 148.14, 145.44, 132.41, 118.47, 112.52, 111.00, 64.51, 54.83, 41.11, 34.79, 30.70, 28.21, 28.07, 24.79, 21.54, 13.38.

2.5 Synthesis of DCP

Oxoplatin (100 mg, 0.3 mmol) was dissolved in DMSO (2.5 mL) ultrasonically (100 Hz, 40 min). SCAA (422 mg, 1.2 mmol), TBTU (386 mg, 1.2 mmol), TEA (122 mg, 1.2 mmol) and DMSO (1 mL) were added and stirred for 24 h at 60 ℃ in the dark to obtain a clear brown yellow reaction solution. Ether was added to the reaction solution (100 μL) up to 7 mL, followed by addition of methanol (900 μL). The solution was centrifuged to collect the yellow precipitate. The steps were repeated several times until the reaction solution was used up. The precipitate was collected, washed with ether/methanol (9:2) three times and dried under vacuum to obtain a light yellow product. Yield: 36.7% (110.2 mg, 0.110 mmol). HR-ESI-MS (negative mode, m/z): 999.3543, 1000.3572, 1001.3565, 1002.3567, 1003.3566, 1004.3571, 1005.3557, 1006.3574. ^{1}H NMR (600 MHz, DMSO-d_6, ppm): δ 8.24–8.22 (t, 2H, 2 CO-NH-), 6.86–6.84 (m, 4H, 2PhH-), 6.70–6.68 (m, 2H, 2PhH-), 6.68–6.58 (m, 6H, 2NH_3-), 4.59 (s, 4H, 2O-CH_2-), 4.18–4.17 (d, 4H, 2NH-CH_2-), 3.74 (s, 6H, 2CH_3-O), 2.12–2.10 (t, 4H, 2CO-CH_2), 1.53–1.40 (m, 4H, 2-CH_2-), 1.40–1.24 (m, 20H, 2-C_5H_{10}-), 0.87–0.85 (t, 6H, 2CH_3-). ^{13}C NMR (101 MHz, DMSO-d_6, ppm): δ 175.24, 171.37, 149.35, 148.00, 145.70, 132.48, 132.06, 118.50, 113.13, 110.79, 64.37, 54.83, 40.59, 34.74, 26.79, 21.47, 13.55, 11.78. ^{195}Pt NMR (86 MHz, DMSO-d_6, ppm): δ 1218.14.

2.6 Reduction

DCP (20 mg, 0.02 mmol) and AsA (35.2 mg, 0.2 mmol) were dissolved in 90% DMSO-d_6/10% D_2O (500 μL) and incubated at 37 ℃ in the dark. The reduction of DCP was monitored by ^{195}Pt NMR at 0, 6, 12, 24, 48, and 72 h, respectively.

2.7 Interaction with DNA

CT-DNA stock solution was prepared by dissolving DNA in Tris-HCl buffer solution (5 mM Tris, 50 mM NaCl, pH 7.4), which was shaken at 4 ℃ overnight. The purity and concentration of CT-DNA were determined by UV absorption spectrometry before use. The stock solution of CT-DNA was diluted to 100 μM with Tris-HCl buffer solution. DCP (50 μM) was mixed with CT-DNA (100 μM) in the presence or absence of AsA, and

incubated at 37 ℃ for 24 h in the dark. CD spectra were recorded in the range of 235–320 nm at a scan speed of 10 nm min^{-1}.

2.8 Cytotoxicity

MCF-7, HepG2, and PANC-1 cells were cultured in DMEM containing 10% FBS, 100 U mL^{-1} penicillin and 100 U mL^{-1} streptomycin, while MG-63 cells were cultured in MEM. All cultures were maintained in an incubator under 5% CO_2 at 37 °C. Cells were seeded into 96-well plates with 100 μL medium per well and incubated with each complex dissolved in fresh media at different concentrations. After incubation for 72 h, MTT (20 mL, 5 mg mL^{-1}) was added to each well and removed 4 h later, and DMSO (150 μL) was added. The absorbance (OD) at 570 nm was recorded on an ELISA plate reader. The cell viability (%) of cells was calculated by the following formula: $(OD_{sample} - OD_{blank})/(OD_{control} - OD_{blank}) \times 100\%$.

2.9 Apoptosis and cell cycle arrest

PANC-1 cells were seeded into 6-well plates and incubated for 18 h. The cell culture medium was replaced with fresh one containing each compound. After incubation for 72 h, the cells were washed twice with cold PBS, trypsinized and centrifuged. The cells were collected and resuspended in 1× binding buffer (500 μL), stained with Annexin V-FITC and incubated in the dark for 60 min. PI (5 μL) was added and incubated in the dark for 15 min. Apoptotic status was analyzed by flow cytometry.

PANC-1 cells were seeded in a 6-well plate and cultured in DMEM overnight. The medium was replaced with fresh one containing compound (1 μM). After incubation for 72 h, the cells were collected by trypsinization and washed with PBS, fixed in ice cold ethanol (70%) at –20 °C for 24 h, centrifuged to get rid of ethanol, and incubated with RNase A solution (100 μL) at 37 °C for 30 min. After incubation, PI (400 μL) was added and incubated at 4 °C in the dark for 30 min. The samples were washed with PBS and resuspended in PBS (500 μL). Flow cytometry was used to detect the cells in different cell cycle phases. Cell cycle profiles were modeled using Modfit LT software (Verity Software

House, Topsham, ME, USA).

2.10 Cellular uptake

PANC-1 cells were treated as described above. After centrifugation, the cell pellets were digested with HNO_3 (100 μL, 2 h), 30% H_2O_2 (50 μL, 1.5 h) and HCl (100 μL, 2 h) at 95 °C, respectively. The solution was diluted with water, and the Pt content was determined by ICP-MS.

2.11 Visualization of cell surface CRT

PANC-1 cells were seeded in a 20 mm glass bottom cell culture dish (Nest) and incubated at 37 °C for 24 h. The cells were treated with compound for 24 h, washed with cold PBS twice, fixed with 1% formaldehyde and incubated with anti-CRT for 1 h at 4 °C. The cells were then washed with cold PBS twice, and incubated with a FITC-labeled secondary antibody for 1 h at room temperature in the dark. After washing with PBS twice, the cells were fixed with 4% paraformaldehyde for 15 min, followed by counterstaining with Hoechst 33342 for 5 min and visualized with confocal microscopy immediately. The wells were emptied and washed with PBS twice, and fluorescent images were taken by a confocal laser scanning microscope (Zeiss LSM 710).

2.12 Detection of HMGB1 and ATP

PANC-1 cells were seeded into 6-well plates and incubated for 18 h. The cells were treated with each compound for 36 h and the supernatant was carefully extracted. The amount of secreted HMGB1 and ATP in the cell culture supernatant was measured by human HMGB1 ELISA kit and enhanced ATP assay kit, respectively, according to the manufacturer's protocol.

2.13 Extracellular IFN-γ and TNF-α

Human PBMCs were cultured in RPMI 1640 medium containing 10% FBS. The cells were seeded into 6-well plates at a density of 2×10^6 cells per well, and the medium was replaced by the conditioned medium of PANC-1 cells after treating with the compound for 24 h. The level of IFN-γ and TNF-α secreted from the treated cells into the RPMI

supernatant was measured using human IFN-γ and TNF-α ELISA kits according to the manufacturer's protocol.

2.14 Cytotoxicity of activated PBMCs

Human PBMCs were seeded in a 6-well plate and incubated with the conditioned medium of PANC-1 cells that were treated with each compound for 24 h. The activated PBMCs were washed twice with complete DMEM medium to remove the residual medium, and then resuspended in the complete DMEM. PANC-1 cells were seeded into 96-well plates at a density of 4.0×10^3 cells per well and incubated for 24 h. The above PBMCs cells (4.0×10^4) were added to each well and co-cultured with PANC-1 cells for 24 h. Enhanced CCK-8 solution (20 μL) was added to each well and incubated for 2 h. The absorbance of the solution at 450 nm was recorded on an ELISA plate reader, and cell viability was calculated on the absorbance by the formula: $[A - C - (B - C)]/(D - C) \times 100\%$, where A is the absorbance of tumor cells cultured with activated or non-activated PBMCs, B is the absorbance of PBMCs, C is the absorbance of DMSO, and D is the absorbance of tumor cells.

2.15 In vitro phagocytosis

THP-1 monocytic cells (2×10^6 cells) were differentiated into macrophages in a 6-well plate containing 2 mL of the RPMI 1640 medium with 5 ng mL^{-1} PMA over 48 h. PANC-1 cells were seeded in 6-well plate at the density of 1×10^5 cells per well and incubated overnight; and the cells were then incubated with CDDP (25 μM), DCP (150 μM) and SC (300 μM), respectively, in complete DMEM for 24 h. The differentiated THP-1 cells were stained with CellTrace™ Far Red (1 μM) for 20 min, and the PANC-1 cells were stained with CellTrace™ CFSE (5 μM) for the same time. The two kinds of cells were washed twice with sterile PBS and co-incubated for 4 h, harvested, and fixed for 10 min with 4% formaldehyde solution in PBS. Phagocytosis was evaluated by flow cytometry on two channels ($\lambda_{ex,\ em}$: 488, 530 nm; and $\lambda_{ex,\ em}$: 640, 670 nm). The proportion of phagocytosis was calculated on the number of double-stained macrophages with

phagocytosed PANC-1 cells to the total number of macrophages. The results were presented as change folds relative to the untreated cells.