Electronic Supplementary Information (ESI)

Regulating tumor glycometabolism and immune microenvironment by inhibiting lactate dehydrogenase with platinum(IV) complexes

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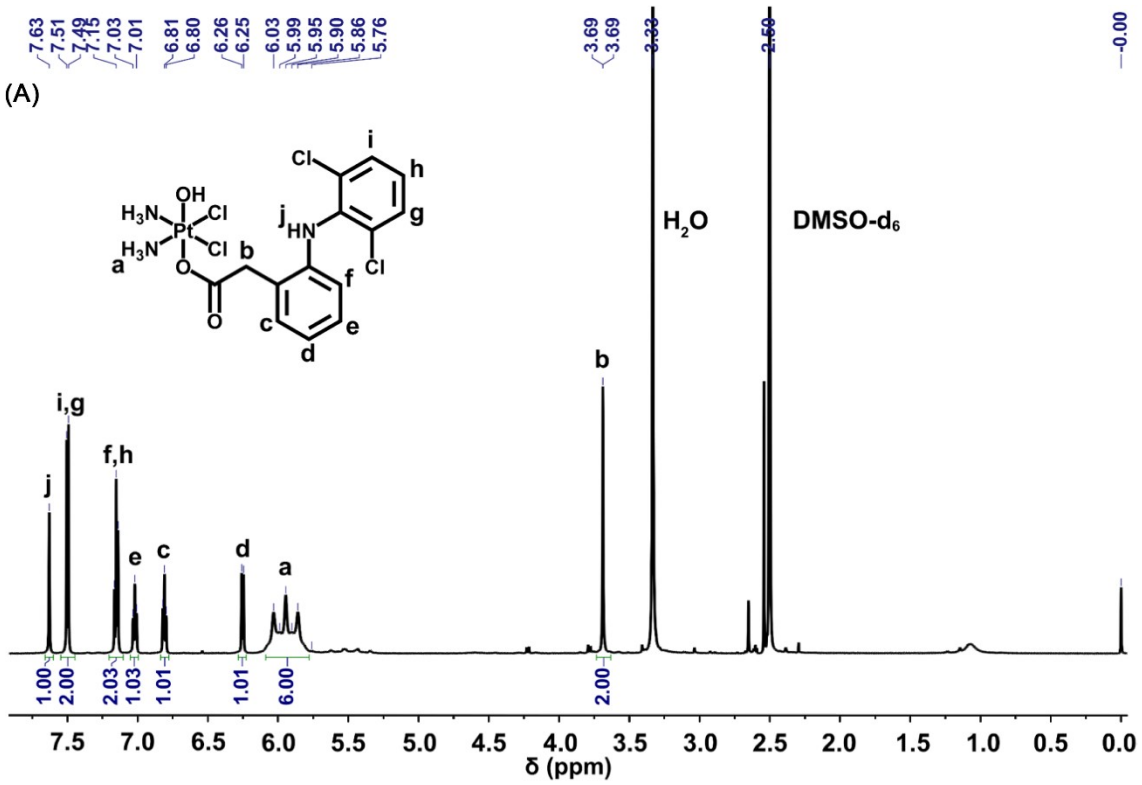
Supplementary Figures and Tables


(A)
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DMSO-d6
i,g
f,h
j
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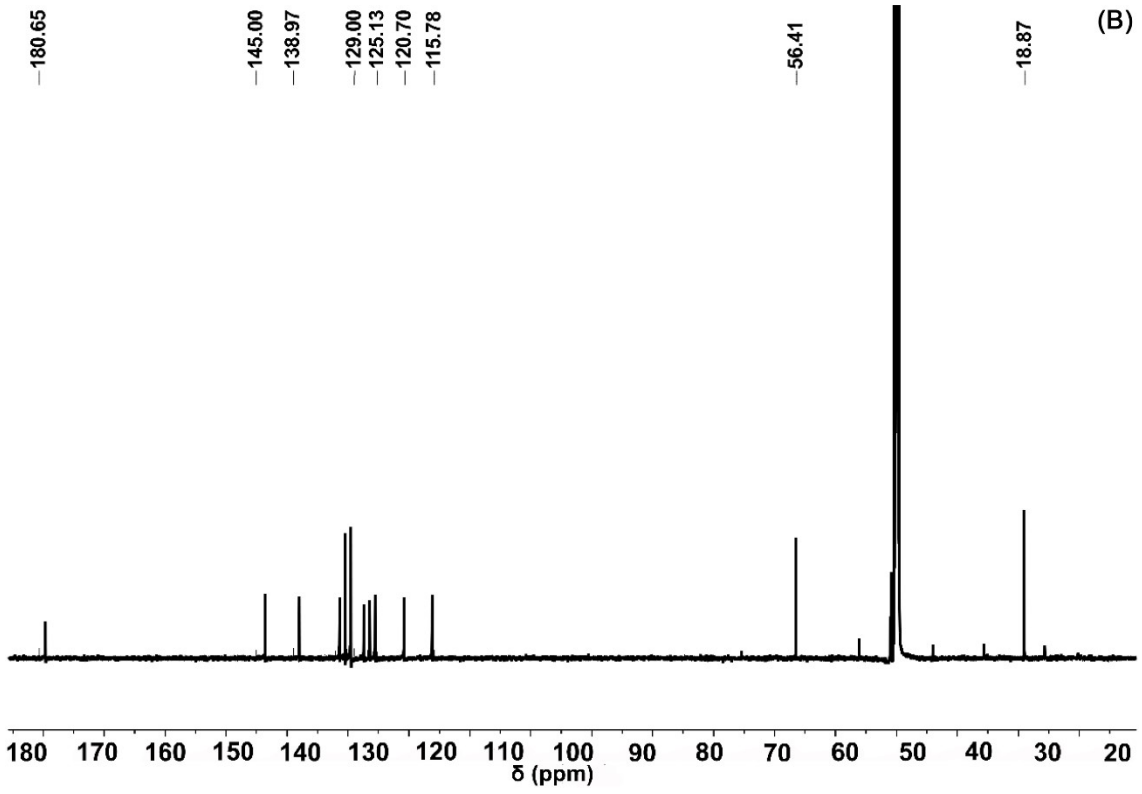
δ (ppm)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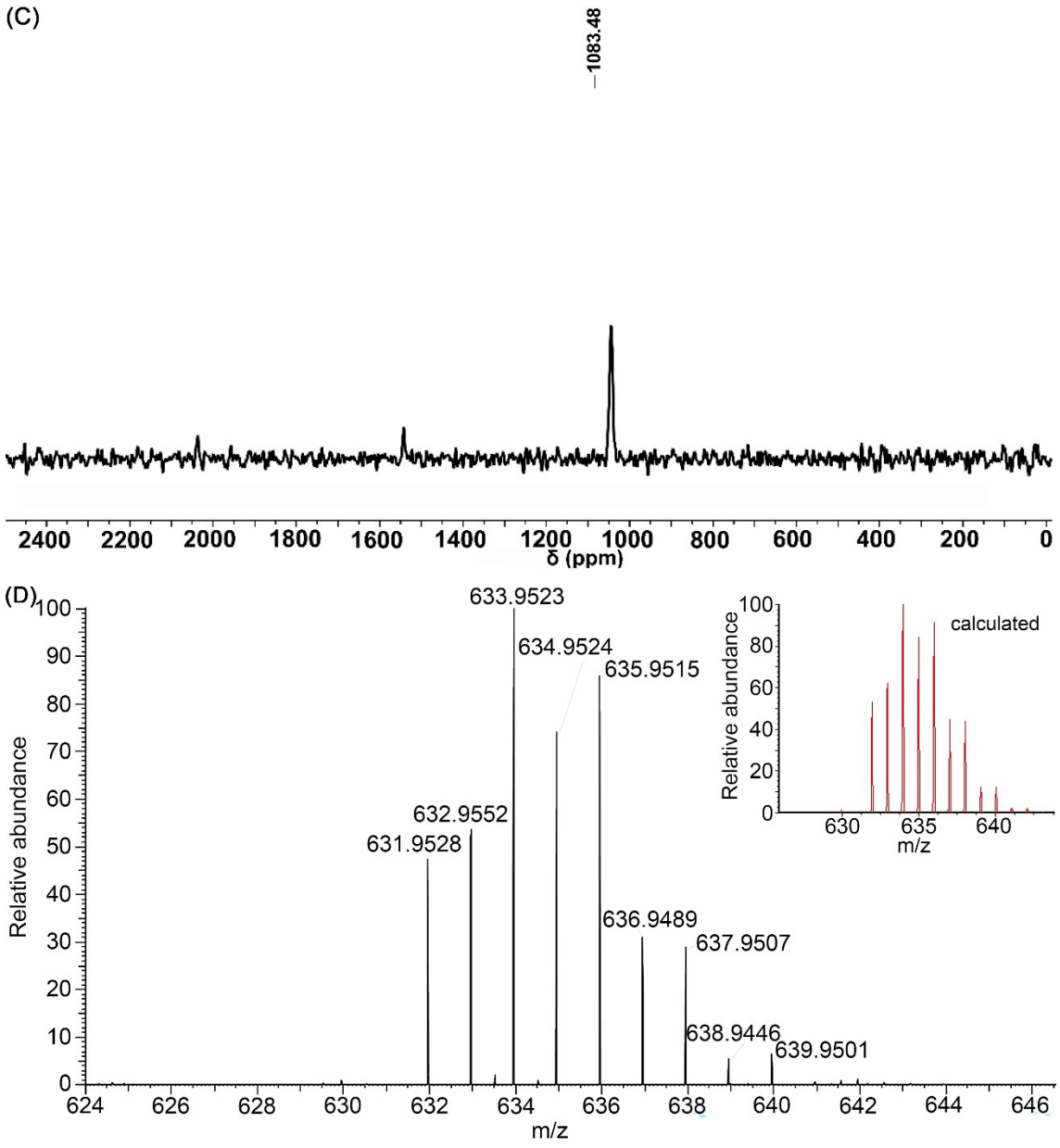
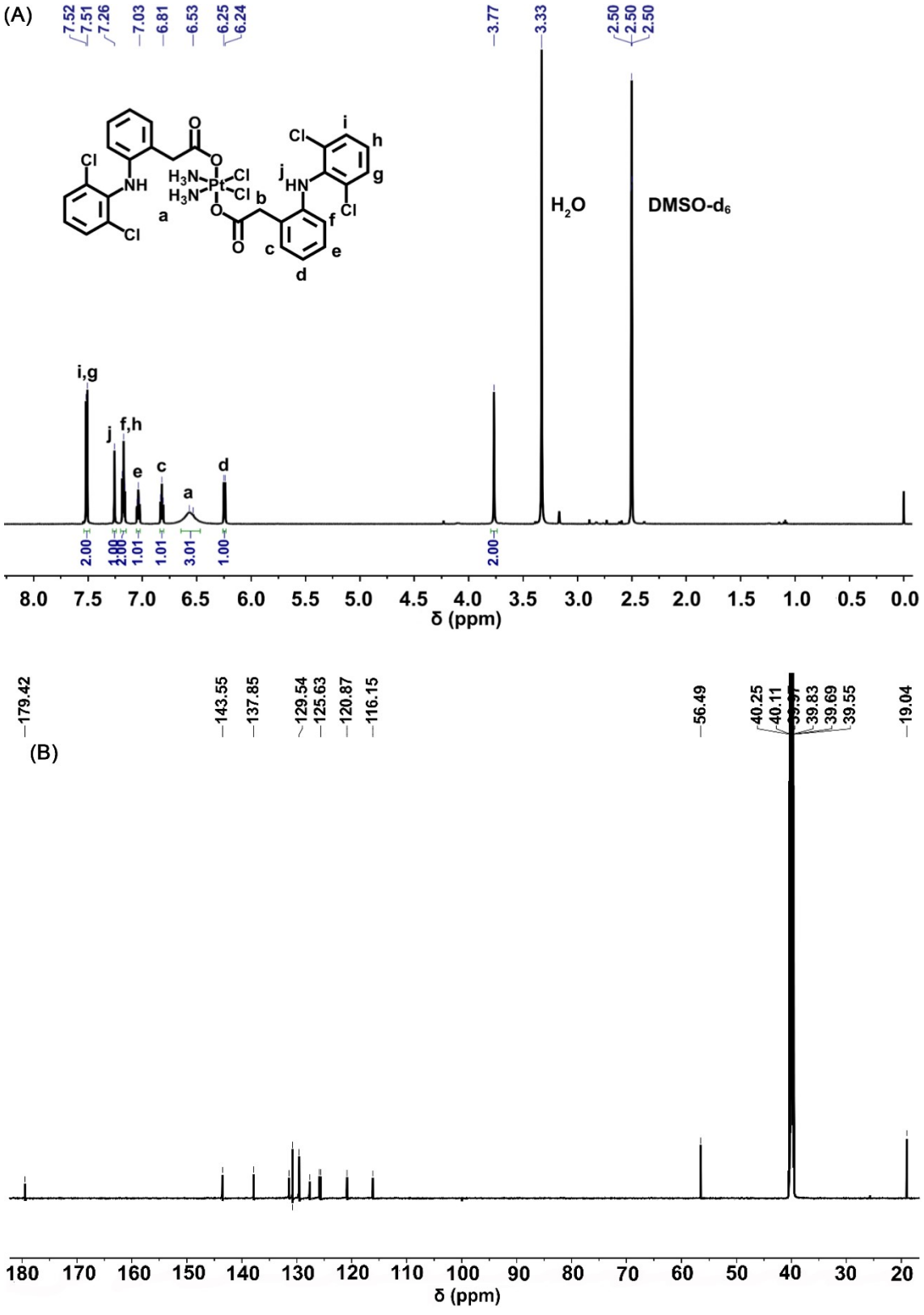


Fig. S1 ^{1}H NMR (400 MHz, DMSO-d_6) (A), ^{13}C NMR (101 MHz, DMSO-d_6) (B), ^{195}Pt NMR (86 MHz, DMSO-d_6) (C) and HR-ESI-MS (positive mode) (D) spectra of MDP.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6.53
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3.77
3.33
2.50
2.50
2.50
H2O
DMSO-d6
i,g
j
f,h
e
c
a
d
2.00
1.01
1.01
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2.00
δ (ppm)
(B)
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39.55
19.04
δ (ppm)

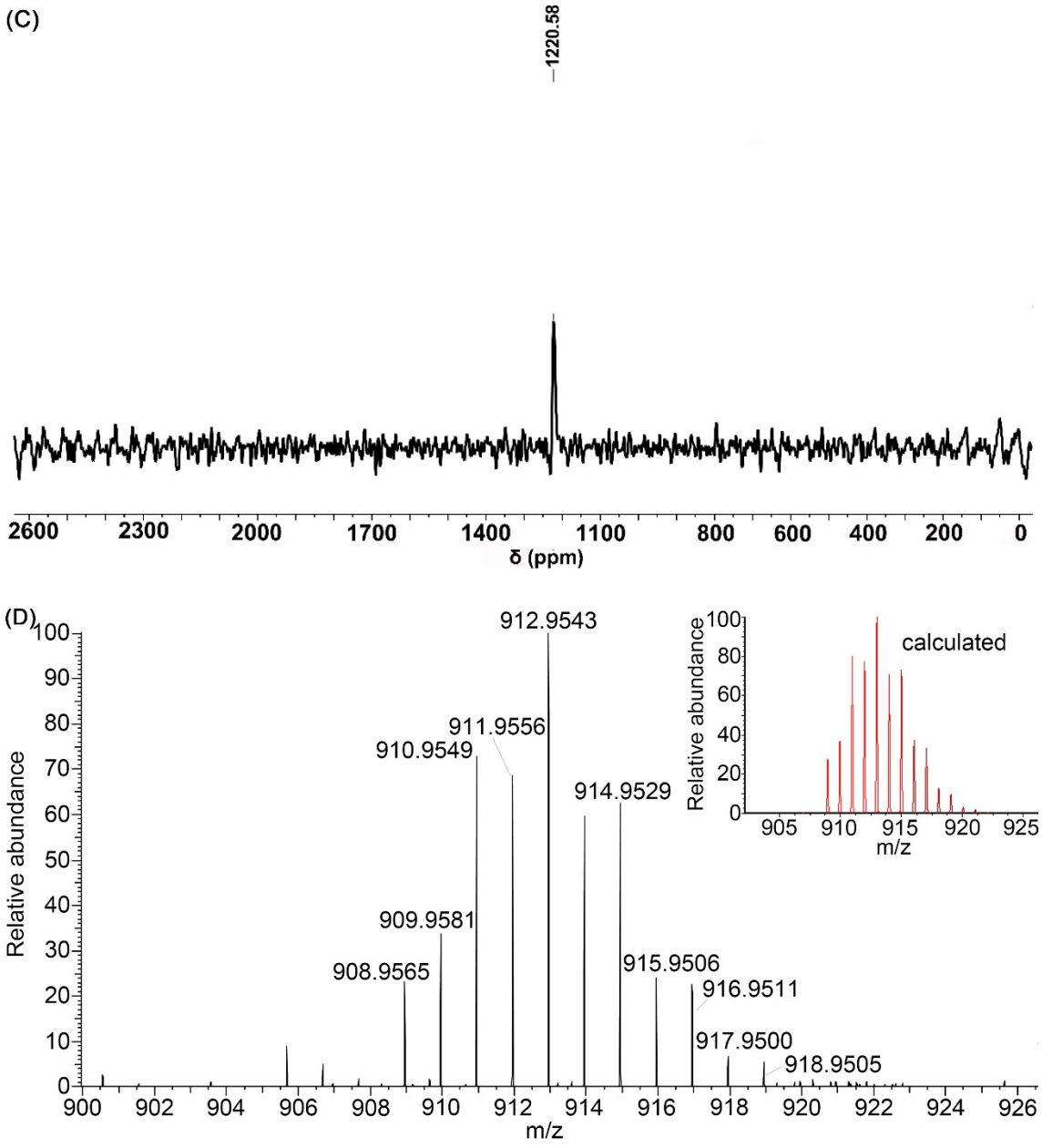

Fig. S2 ^{1}H NMR (400 MHz, DMSO-d_6) (A), ^{13}C NMR (101 MHz, DMSO-d_6) (B) and ^{195}Pt NMR (86 MHz, DMSO-d_6) (C) and HR-ESI-MS (positive mode) (D) spectra of DDP.

Table S1 Partition coefficients of MDP and DDP.

Compounds	MDP	DDP	CDDP[1]
log$P_{O/W}$	–0.13 ± 0.056	–0.046 ± 0.035	–2.35 ± 0.20

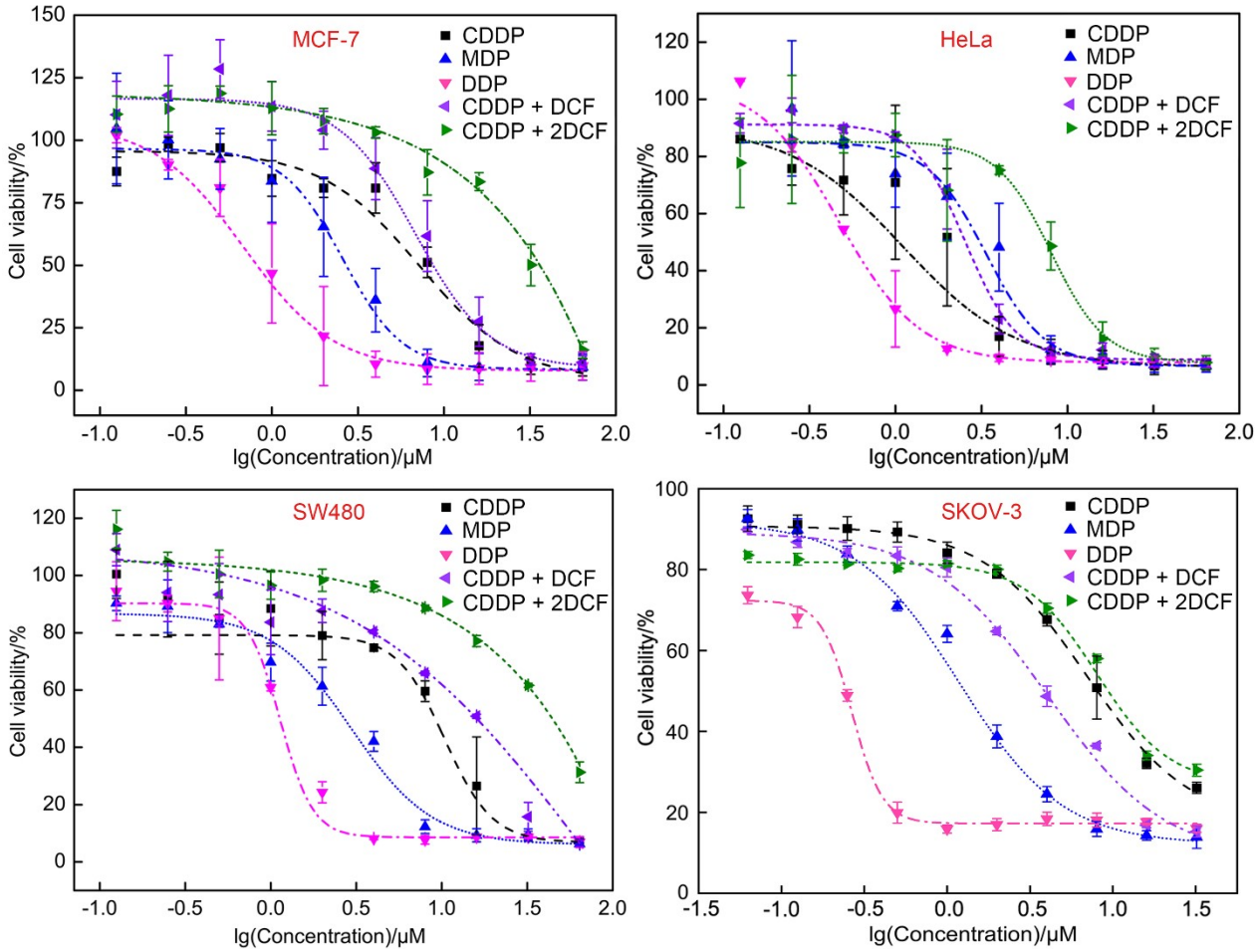

Fig. S3 Cell viability of MCF-7, HeLa, SW480, and SKOV-3 cells after treatment with different concentrations of complexes for 48 h.

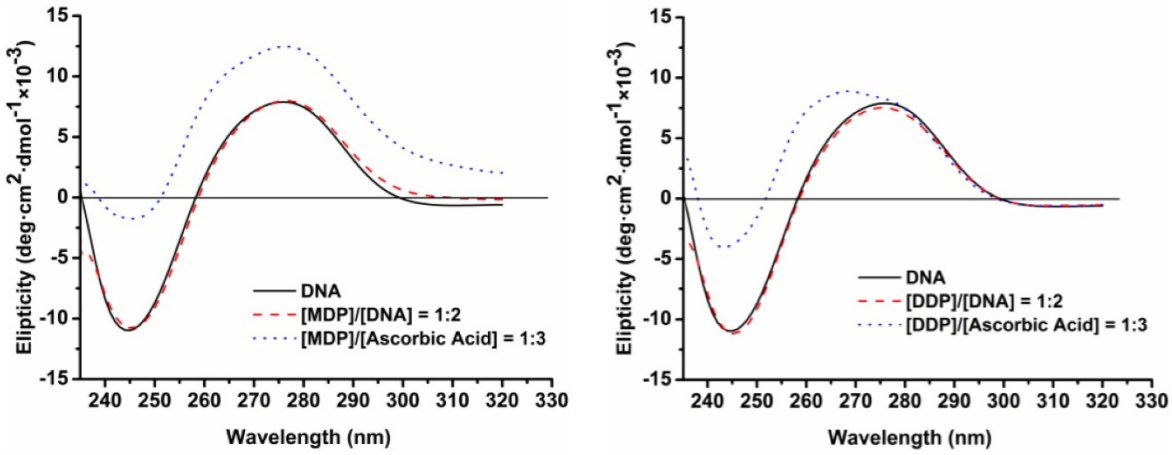

Fig. S4 CD spectra of CT-DNA (100 μM) in the absence and presence of MDP or DDP without or with ascorbic acid after incubation at 37 °C for 24 h.

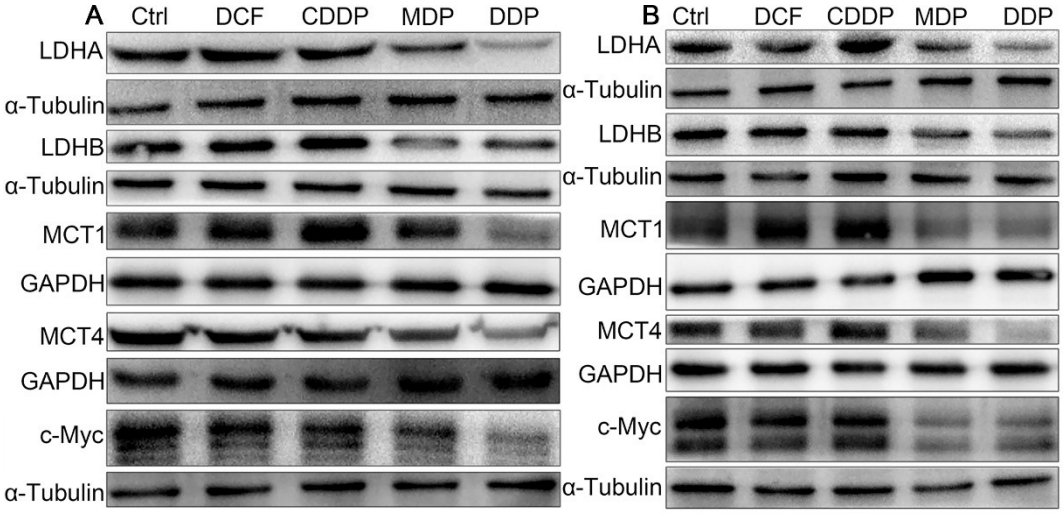

Fig. S5 The expressions of LDHA, LDHB, MCT1, MCT4 and c-Myc in SKOV-3 cells after treatment with CDDP, MDP, DDP (0.6 μM containing 0.6‰ DMSO), and DCF (1.2 μM containing 0.1‰ DMSO), respectively, for 36 h.

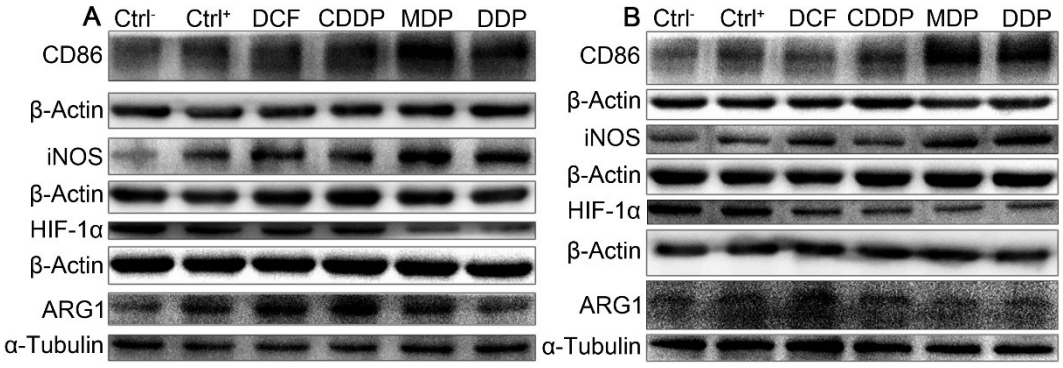

Fig. S6 The expressions of CD86, iNOS, HIF-1α, and ARG1 in THP-1 cells stimulated with PMA (10 ng mL^{-1}) for 48 h and incubated with the compound-treated SKOV-3 cell-conditioned culture supernatant (0.6 μM containing 0.6‰ DMSO) for 36 h. $Ctrl^{-}$: THP-1 cells stimulated with PMA (10 ng mL^{-1}) for 48 h and cultured with RPMI-1640 growth medium; $Ctrl^{+}$: THP-1 cells stimulated with PMA (10 ng mL^{-1}) for 48 h and cultured with SKOV-3 cell-conditioned culture supernatant without compound treatment.

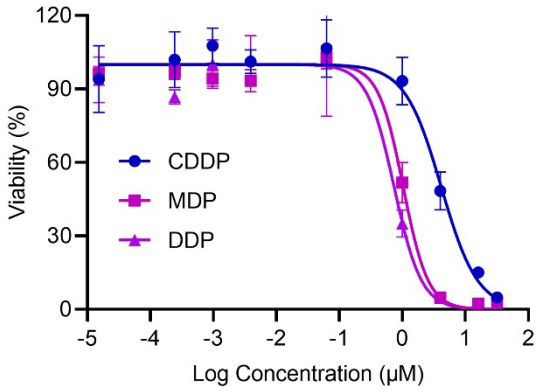

Fig. S7 Cell viability of THP-1 macrophages after treatment with different concentrations of complexes for 48 h.

Table S2 IC_{50} (μM) of MDP, DDP, CDDP, and DCF at 24 h against HUV-EC-C cell line. Data are the average of three measurements.

Compound	CDDP	MDP	DDP	DCF
IC_{50} (μM)	6.55 ± 0.16	3.04 ± 0.07	0.24 ± 0.04	> 64

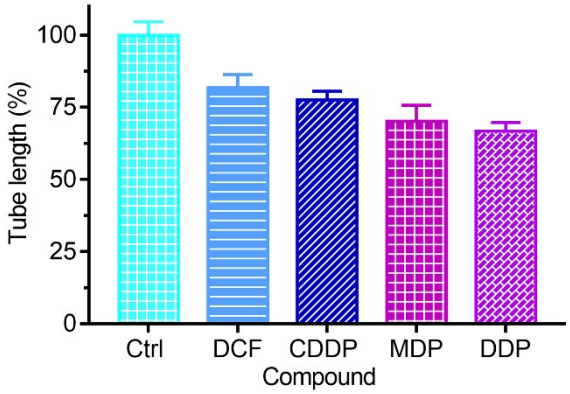

Fig. S8 Tube length of the HUV-EC-C formed tubes in the presence of DCF (0.4 μM containing 0.4‰ DMSO), CDDP, MDP, and DDP (0.2 μM containing 0.2‰ DMSO), respectively, after incubation for 6 h.

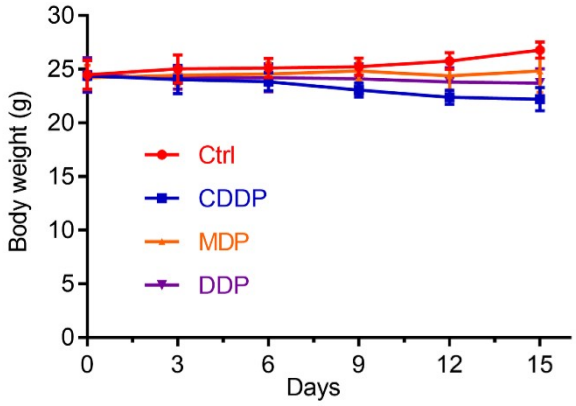

Fig. S9 Body weight of Balb/C nude mice during 15 days of intravenous treatment with each complex every three days (n = 5). PBS was used as a control.

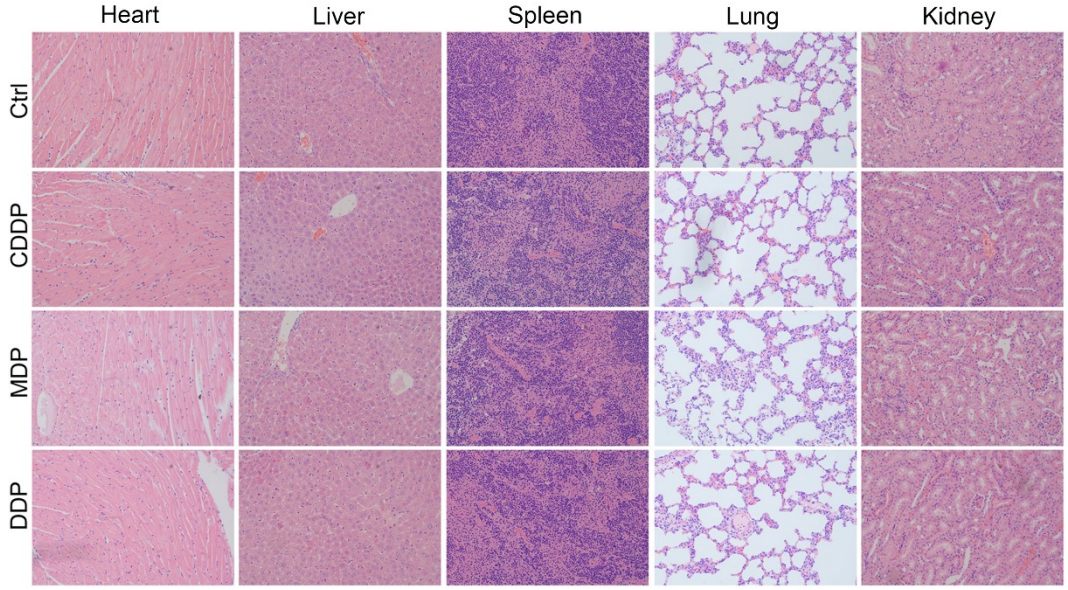

Fig. S10 H&E-stained images of heart, liver, spleen, lung, and kidney sections collected from Balb/C nude mice bearing SKOV-3 xenograft tumor (n = 5) after treatment for 15 days, PBS was used as a control.

1. Experimental

1.1 Materials

All the reagents and solvents were of analytical grade and used as received without further purification. CDDP was purchased from Shandong Boyuan Pharmaceutical Co., Ltd. China. Diclofenac (DCF), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), ascorbic acid (AsA) and calf thymus DNA (CT-DNA) was purchased from Sigma-Aldrich and

used as received without further purification. Dichloromethane (DCM), *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TBTU), triethylamine (TEA), and hydrogen peroxide (H_2O_2, 30%) were purchased from J&K Scientific and used as received. Water was deionized and ultrafiltered by a Milli-Q apparatus (Millipore Corporation, China). Annexin V conjugated with fluorescein isothiocyanate (annexin V-FITC) and propidium iodide (PI), were purchased from KeyGen Biotech. Co. Ltd. (Nanjing, China). Genomic DNA mini preparation kit was purchased from Tiangen Biotech (Beijing) Co., Ltd. LDHB (ab53292), MCT1(ab62331), CD86 (ab239075), β-actin (ab8227), GAPDH (ab181602), *α*-tubulin (ab52866) antibodies and goat anti-rabbit HRP (IgG H&L) (ab97051) secondary antibody were purchased from Abcam. LDHA (3582), c-Myc (5605T),and arginase 1 (93668) antibodies were purchased from Cell Signaling Technology, Inc. HIF-1α (20960-1-AP), MCT4 (22787-1-AP) and iNOS (18985-1-AP) antibodies were purchased from Proteintech Group, Inc. Lactate dehydrogenase (LDH) activity assay kit and L-lactic acid (LA) colorimetric assay kit were purchased from Elabscience Biotechnology Co., Ltd. Human vascular endothelial growth factor (VEGF), human tumor necrosis factor-α (TNF-α), and human interferon-γ (IFN-γ) and human transforming growth factor beta 1 (TGF-β1) ELISA kits were purchased from Genxspan.

2.2 Cell Lines

The human breast cancer MCF-7, human cervical cancer HeLa, human colon cancer SW480, human ovarian cancer SKOV-3, human umbilical vein endothelial cell HUVEC and human monocytic THP-1 cell lines were purchased from American Type Culture Collection (ATCC). Fetal bovine serum (FBS) and all culture media were from Nanjing KeyGen Biotech Co., Ltd.

2.3 General Methods

High resolution mass spectra (HR-ESI-MS) were recorded on an Thermo Fisher Q Exactive mass spectrometer. The isotopic distribution patterns for the complex were simulated using the ISOPRO 3.0 program. ^{1}H-, ^{13}C-, and ^{195}Pt NMR spectra were acquired on a Bruker DRX-400 spectrometer at 298 K. 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.). Flow cytometry was determined by using Cytomics FC500 Flow Cytometry (Beckman Coulter Ltd.). The optical density (OD) of formazan was determined using a Tecan Sunrise ELISA Reader at 570 nm. Elemental analysis was performed using a CHN-O-Rapid elemental analyzer (Heraeus, German). Confocal fluorescence imaging studies were performed on

a ZEISSLaser Scanning Microscope (Zeiss LSM 710). Fluorescence spectra were recorded on a Horiba Fluoromax4 spectrofluorometer.

2.4 Synthesis of Oxoplatin

Oxoplatin was synthesized by oxidizing cisplatin with 30% H_2O_2 as reported in the literature.[2] Yield: 64.7%.

2.5 Synthesis of MDP

MDP was prepared by the addition of DCF (88.9 mg, 0.30 mmol), TBTU (97 mg, 0.30 mmol) and TEA (31 mg, 0.30 mmol) to the solution of oxoplatin (100 mg, 0.30 mmol) in DMSO. After stirring at 60 °C for 72 h, a clear pale yellow solution was obtained. DMSO was removed by excessive addition of DCM and diethyl ether, and the rude product was obtained. The precipitate was dissolved in diethyl ether/methanol (9:1) with diethyl ether several times and dried under vacuum to obtain a white powder. Yield: 53 mg, 29%. ^{1}H NMR (DMSO-d_6): δ (ppm) 3.7 (s, 2H, CH_2), 5.95 (t, 6H, $2NH_3$), 6.26 (d, 1H, Ph), 6.81 (t, 1H, Ph), 7.03 (t, 1H, Ph), 7.14 (t, 2H, Ph), 7.50 (d, 2H, Ph), 7.63 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): δ (ppm) 180.55, 145.00, 138.97, 129.00, 125.13, 120.70, 115.78, 56.41, 18.87. ^{195}Pt NMR (DMSO-d_6): 1083.48 ppm. HR-ESI-MS (positive mode, m/z) found (calcd) for $[M + Na]^+$ 635.9515 (635.1818).

2.6 Synthesis of DDP

Oxoplatin (100 mg, 0.30 mmol) was stirred in DMSO with DCF (195.5 mg, 0.66 mmol), TBTU (222.0 mg, 0.66 mmol) and TEA (66.8 mg, 0.66 mmol) for 72 h at 60 °C in the dark to obtain light yellow solution. Excessive DCM and diethyl ether were added in the solution to get the rude product. The precipitate was dissolved in diethyl ether/methanol (9:1) with diethyl ether several times and dried under vacuum to obtain a white powder. Yield: 101.1 mg, 38%. ^{1}H NMR (DMSO-d_6): δ (ppm) 3.77 (s, 4H, $2CH_2$), 6.25 (d, 2H, Ph), 6.56 (s, 6H, $2NH_3$), 6.81 (t, 2H, Ph), 7.03 (t, 2H, Ph), 7.17 (t, 4H, Ph), 7.25 (s, 2H, Ph), 7.53 (d, 2H, NH). ^{13}C NMR (DMSO-d_6): δ (ppm) 179.42, 143.55, 137.85, 129.54, 125.63, 120.87, 116.15, 56.49, 19.04. ^{195}Pt NMR (DMSO-d_6): 1220.58 ppm. HR-ESI-MS (positive mode, m/z) found (calcd) for $[M + Na]^+$ 912.9543 (913.3138).

2.7 Measurement of Partition Coefficient

Partition coefficient for complexes were measured by the shake-flask method.[3] Briefly, *n*-octanol saturated water was prepared by mixing an equal volume of octanol and water and shaking on a mechanical shaker for 24 h at room temperature. Solutions of complexes (25 μM, 75 μM and

100 μM) were prepared in phosphate buffer (PBS, 10 mM, pH 7.4, presaturated with *n*-octanol, V_{water}). About half of the solutions were mixed with a volume of water-saturated octanol ($V_{octanol}$) and the mixtures were shaken for 24 h at room temperature followed by centrifugation at 2500 rpm for 15 min to separate different phases. The concentration of the solute in the aqueous phase was determined by spectrophotometry (λ_{max} = 267 nm). According to the law of mass conservation, the drug concentration of corresponding 1-octanol phase and the lipo−hydro partition coefficient $P_{o/w}$ ($P_{o/w} = C_o/C_w = A_o/A_w$, where A stands for absorbance) were calculated.

2.8 Reduction Kinetics

The samples were prepared by reacting MDP and DDP with 10 equivalents of AsA in 90% DMSO/10% D_2O solutions, respectively. The time course of the reaction was monitored by ^{195}Pt NMR after the samples were incubated at 37 °C for 0, 6, 12, 24, 36, 48 h, and 72 h, respectively, in the dark.

2.9 *In Vitro* Cytotoxicity Assay

Cytotoxicity was tested on MCF-7, HeLa, SW480, SKOV-3 and HUV-EC-C cell lines. MCF-7 and HeLa cells were cultured in 75 cm^2 cultural flask using DMEM growth medium supplemented with 10% fetal bovine serum, while SW480 and SKOV-3 cells were cultured in RPMI-1640 growth medium supplemented with 10% fetal bovine serum. HUV-EC-C cells were cultured in F-12K growth medium supplemented with endothelial cell growth supplement and 10% fetal bovine serum. Cell cultures were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. The cytotoxicity of different complexes was assessed using the MTT assay. Briefly, cells were seeded in a 96-well plate at a density of 4×10^3 cells per well in 100 μL of growth medium and were preincubated for 24 h before exposure to the complexes. The stock solution of CDDP was prepared in PBS, while MDP, DDP and DCF stock solutions were prepared in DMSO. The stock solutions were diluted to different concentrations and then added in aliquots of 200 μL per well (DMSO concentration < 5 ‰). After exposure for 48 h, the cells were treated with MTT (20 μL, 5 mg mL^{-1} in PBS) for 4 h. The medium was removed and DMSO (150 μL) was added to dissolve the purple formazan crystals. The plates were shaken for 15 min and the absorbance of the solution was measured on a Varioskan flash multimode reader (Tokyo, Japan) at 570 nm.

2.10 Intracellular Accumulation

SKOV-3 cells were seeded in a 6-well plate at a density of 2×10^5 cells per flask. After incubation for 24 h, the cells were treated with 0.5 and 1.0 μM of complexes (0.5‰ v/v DMSO)

at 37 °C for 24 h, respectively. The attached cells were washed twice with PBS (4 °C). Cell pellets were collected by centrifugation and then digested with nitric acid (100 μL) at 95 °C for 2 h, followed by addition of H_2O_2 (50 μL) and HCl (100 μL) to obtain a fully homogenized solution. Water was added to dilute the solution and the final Pt content was determined by ICP-MS.

2.11 Interaction with DNA

SKOV-3 cells were seeded in a 6-well plate at a density of 2×10^5 cells per well. After incubation at 37 °C for 24 h, the cells were treated with the complex (0.5 and 1 μM, 0.5‰ v/v DMSO) for 24 h, respectively. The attached cells were washed twice with PBS (4 °C), harvested by trypsinization (0.5 mL) and washed with PBS (1 mL). Cell pallets were lysed in DNAzol reagent [1 mL, genomic DNA mini preparation kit, Tiangen Biotech (Beijing) Co., LTD] and the genomic DNA was extracted from lysate with pure ethanol (0.5 mL) by incubating the sample at room temperature for 1–3 min. The amount of DNA was determined with Nanodrop 1000 at 260 nm, and the Pt level bound to DNA was quantified by ICP-MS.

The stock solution of CT-DNA was prepared by dissolving DNA in a buffer solution (5 mM Tris-HCl, 50 mM NaCl, pH 7.4), which was stored in a refrigerator overnight at 4 °C to reach homogenous phase and used within 4 days. The concentration of CT-DNA was determined by Nanodrop 1000 at 260 nm taking 6600 M^{-1} cm^{-1} as its absorption coefficient. Samples for CD were prepared by taking fixed concentration of CT-DNA (100 μM) in the absence and presence of MDP or DDP (50 μM) and AsA (150 μM), and incubated at 37 °C for 48 h in the dark. CD spectra were recorded in the range of 235–320 nm at a scan speed of 10 nm min^{-1}.

2.12 Cell Cycle and Apoptosis Assay

SKOV-3 cells were seeded in a 6-well plate at a density of 2×10^5 cells per well and cultured in RPMI-1640 medium for 24 h. The medium was replaced with the fresh one containing CDDP, MDP, or DDP (0.5 μM containing 0.5‰ DMSO). After 24 h of incubation, the cells were collected by trypsinization and washed with PBS, fixed in ice-cold ethanol (70%) for 24 h, pelleted by centrifugation, treated with RNase A and stained with PI in PBS for 30 min and then analyzed by flow cytometry using a FACS.

SKOV-3 cells were seeded in a 6-well plate at a density of 2×10^5 cells per well and incubated in DMEM incubation medium for 24 h. The medium was replaced with the fresh one containing CDDP, MDP, or DDP (0.5 μM containing 0.5‰ DMSO). After incubation for 48 h, the cells were washed twice with cold PBS, trypsinized and centrifuged (2000 g, 5 min). The

supernatant was discarded and the cells were resuspended in binding buffer (500 μL), stained with annexin V and incubated in the dark for 60 min. The cells were treated with PI and analyzed by flow cytometry.

2.13 Detection of LDH and Lactate

SKOV-3 cells were seeded in 6-well micro plates at the density of 2×10^5 cells per well and cultivated for 24 h. The cells were treated with 0.6 μM of complex containing 0.6‰ DMSO for 36 h. The activity of LDH in the cell and amount of lactate in the cell culture supernatant were determined using lactate dehydrogenase (LDH) activity assay kit and L-lactic acid (LA) colorimetric assay kit according to the manufacturer's protocol.

2.14 Mitochondrial Bioenergetics

ECAR and OCR were tested on Seahorse XF^{e}24 cell MitoStress test kit (Seahorse Bioscience, Massachusetts, USA). A total of 5×10^3 cells per well were seeded in 24-well plates, followed by culturing at 37 °C for 18 h. Growth medium was replaced by medium supplemented with MDP and DDP (0.6 μM containing 0.2‰ DMSO), respectively, and incubated at 37 °C for 36 h. XF assay medium (Seahorse Bioscience) containing glucose (25 mM) and pyruvate (2 mM) or glutamine (2 mM) for OCR and ECAR test, respectively, was added to the wells along with each complex to maintain a stimulating environment. The cells were equilibrated at 37 °C in a CO_2-free incubator for 1 h. OCR or ECAR was measured using Seahorse XF^{e}24 extracellular flux analyser, during which oligomycin, FCCP, and rotenone + antimycin A (1 μM each) or glucose (10 mM), oligomycin (1 μM) and 2-DG (130 mM, Sigma–Aldrich) were injected consecutively every 24 min. Data was recorded during the measurement, and the averages of 4 baseline rates and up to 5 test rates were used for data analysis. The OCR data were normalized to per μg protein.

2.15 Western Immunoblot Analysis

Lysates of cells (40 μg protein/lane) were analyzed on 8–12% SDS-PAGE gel. Protein was electrotransferred on PVDF membranes, blocked with a solution of TBS containing 5% milk and 0.1% tween-20 and immunoblotted with primary antibodies anti-LDHA (1:1000), anti-LDHB (1:1000), anti-c-Myc (1:1000), anti-MCT1 (1:8000), anti-MCT4 (1:2000), anti-agrinase 1 (1:1000), anti-CD86 (1:1000), anti-iNOS (1:1000), anti-HIF-1α (1:1000), anti-β-actin (1:5000), anti-GAPDH (1:10000) and anti-α-tubulin (1:8000). Appropriate secondary antibody was used (1:1000 peroxidase-labeled goat anti-rabbit HRP). After incubation, antibodies were washed in TBS and 0.1% tween 20. Bands were detected using chemiluminescent detection reagents.

2.16 Detection of TNF-α, TGF-β1, and VEGF

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 10 ng mL^{-1} PMA over 48 h, and the medium was replaced by the conditioned medium of SKOV-3 cells after treating with each compound (0.6 μM containing 0.6‰ DMSO) for 36 h. The level of TNF-α, TGF-β1, IFN-γ, and VEGF released from the treated cells into the RPMI supernatant was measured using human TNF-α, TGF-β1, IFN-γ, and VEGF ELISA kits, respectively, according to the manufacturer's protocol.

2.17 Tube Formation Assay

Tube formation assay was performed with μ-Slide Angiogenesis (ibidi) according to the manufacturer's instruction. μ-Slide and pipet tips were thawed on ice. After applying 10 μL of Matrigel to each inner well, the μ-Slide was placed in a humid petri dish prepared with water soaked paper towels. The whole assembly was placed into the incubator for polymerization (30 min). In the meantime, HUV-EC-C cell suspension (2×10^5 cells/mL) was prepared. Cell suspension (40 μL) was applied to each well. After 30 min, the medium in the well was replaced with medium containing certain amount of compound. After incubation for 6 h, the supernatant was discarded, and calcein (KeyGEN BioTECH) (10 μM, 20 μL) diluted with PBS was added into each well. The μ-Slide was incubated in the dark for 30 min at room temperature and washed with PBS for three times. The pictures were taken by OLYMPUS IX71 inverted microscope (λ_{ex} = 485 nm, λ_{em} =529 nm).

2.18 *In Vivo* Antitumor Activity

Female Balb/C nude mice (n = 20, 6–8 weeks old) were purchased from Shanghai SLAC Laboratory Animal Co., Ltd., license No.: SCXK (Su) 2016-0010. The animals were housed in animal-holding units in a pathogen-free environment with the temperature and humidity at 22 ± 2 ℃ and 55% ± 5%, respectively. The SKOV-3 single-cell suspension in PBS (3×10^5 per mouse) was injected subcutaneously into the back of the mouse. When the tumor grew to a size of 80–150 mm^3 at 12 days after cell implantation, the mice were randomly divided into four groups. PBS, CDDP (1.5 mg Pt Kg^{-1}), MDP (1.5 mg Pt Kg^{-1}), and DDP (1.5 mg Pt Kg^{-1}) was administered respectively to the mice of each group intravenously via tail vein every three days for 15 days. Tumor growth was monitored by measuring the perpendicular diameter of the tumor using vernier caliper every three days and calculated according to the formula: Tumor volume (mm^3) = 0.5 × $width^2$ × length.

Statistical Analysis

All the results were expressed as mean ± SD where applicable. GraphPad Prism 7 software (GraphPad Software) was used for statistical analysis.

References

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2. S. X. Jin, Y. Guo, D. F. Song, Z. Z. Zhu, Z. Q. Zhang, Y. W. Sun, T. Yang, Z. J. Guo and X. Y. Wang, *Inorg. Chem.*, 2019, **58**, 6507−6516.

3. S. X. Jin, Y. G. Hao, Z. Z. Zhu, N. Muhammad, Z. Q. Zhang, K. Wang, Y. Guo, Z. J. Guo and X. Y. Wang, *Inorg. Chem.*, 2018, **57**, 11135−11145.