Electronic Supplementary Information (ESI)

Towards rational design of RAD51-targeting prodrug: platinum^{IV}-artesunate conjugates with enhanced cytotoxicity against BRCA-proficient ovarian and breast cancer cells

Shuren Zhang, Hao Yuan, Yan Guo, Kun Wang, Xiaoyong Wang* and Zijian Guo*

1. Supplementary figures

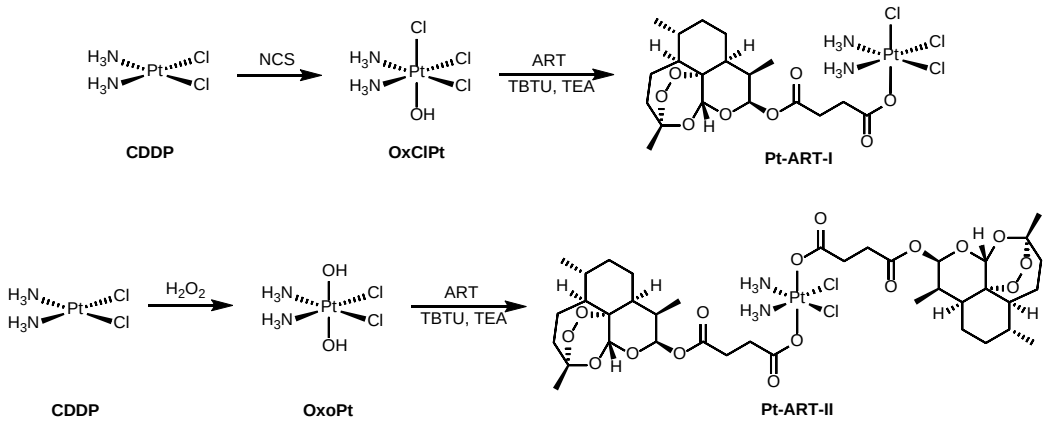
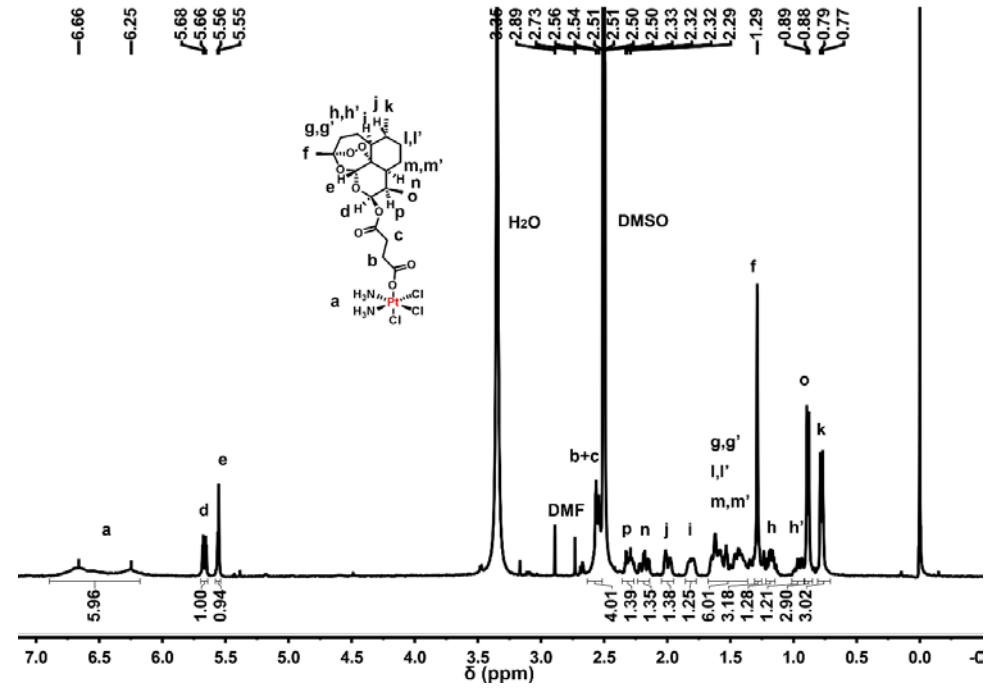
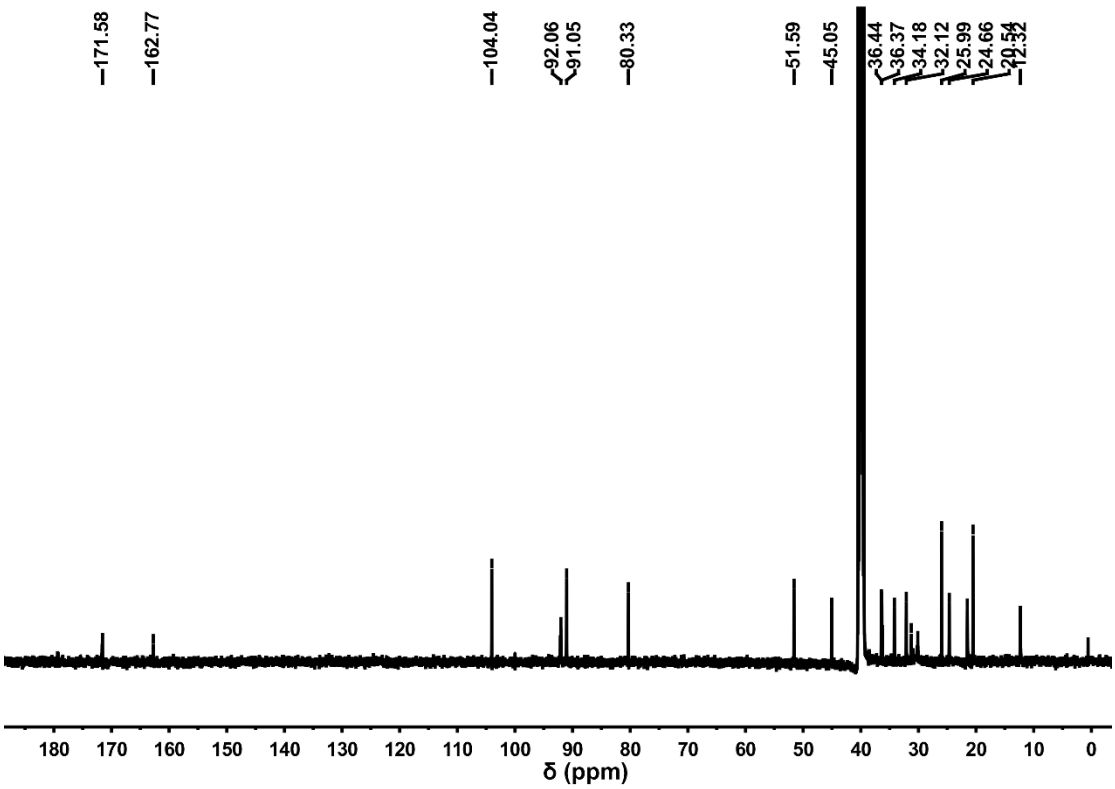


Fig. S1 Synthetic routes to Pt-ART-I and Pt-ART-II.

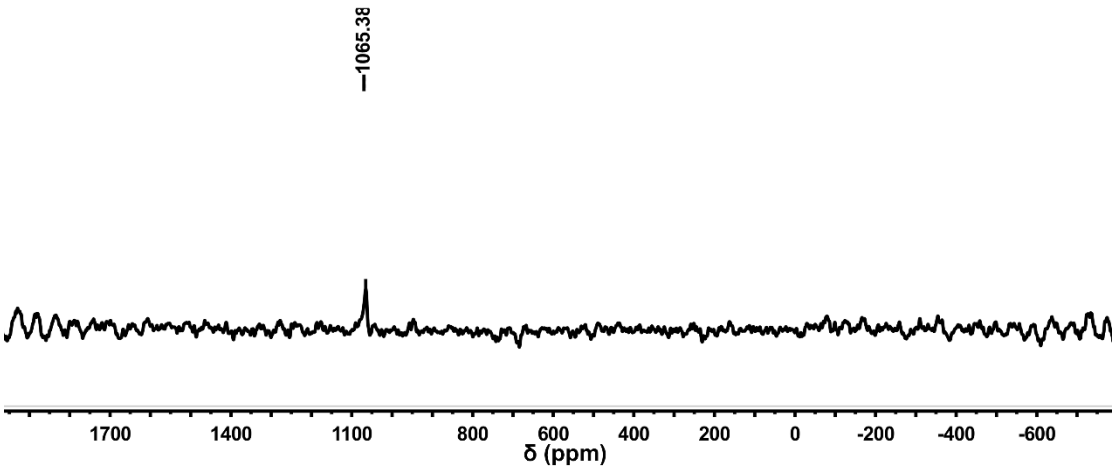
(A)



(B)


171.58
162.77
104.04
92.06
91.05
80.33
51.59
45.05
36.44
36.37
34.18
32.12
25.99
24.66
20.54
12.32
180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
δ (ppm)

(C)


1065.38
1700 1400 1100 800 600 400 200 0 -200 -400 -600
δ (ppm)

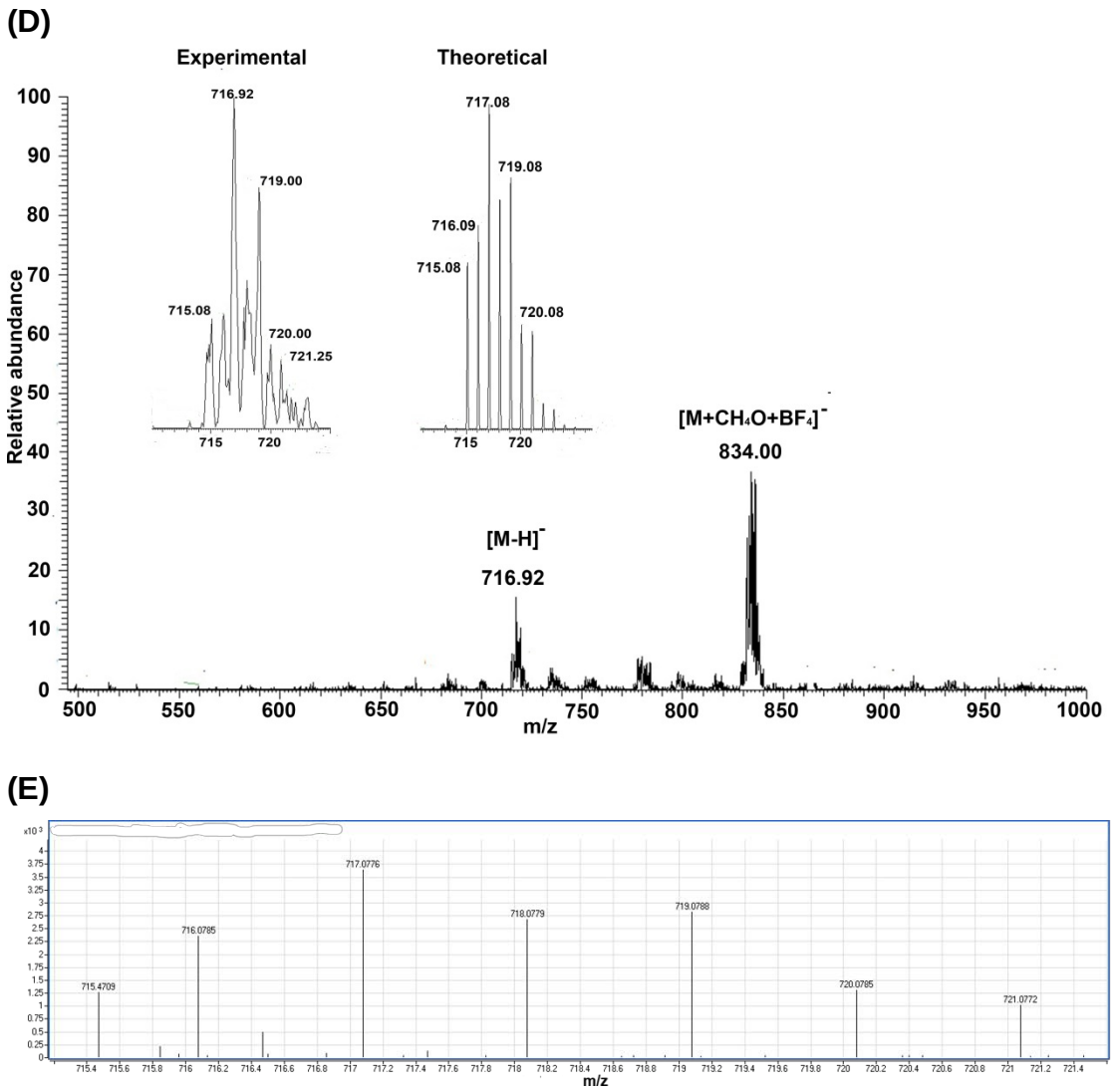
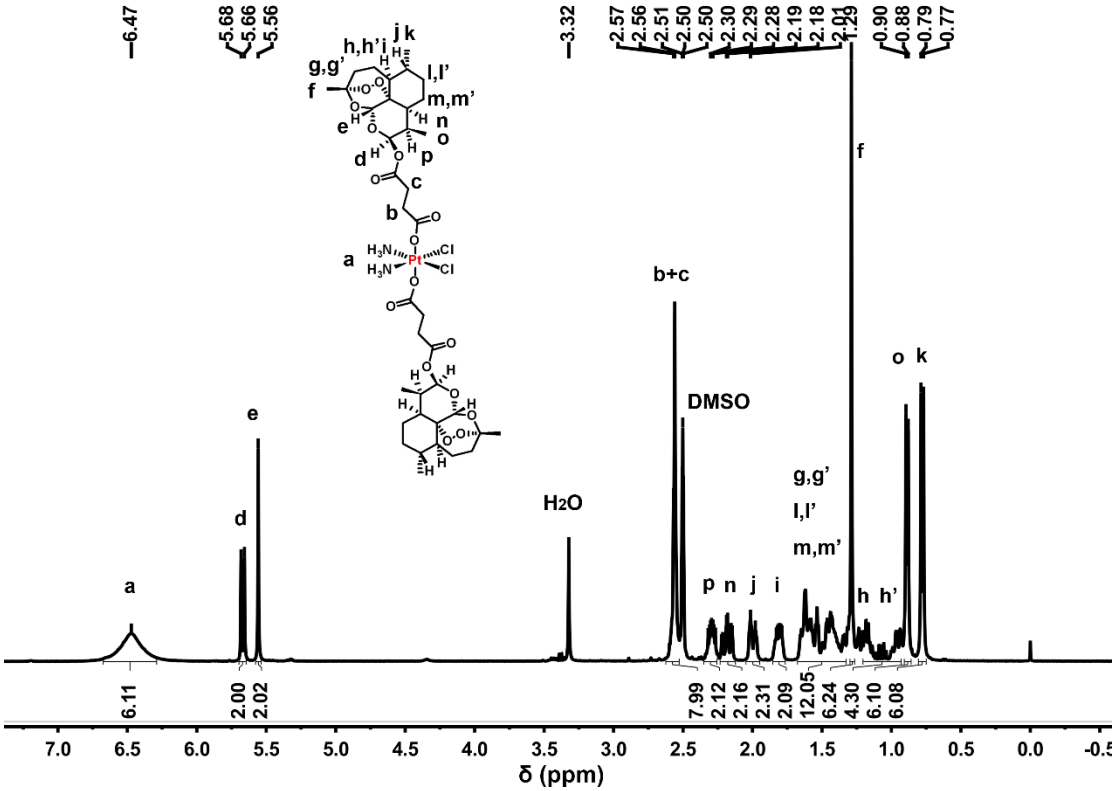
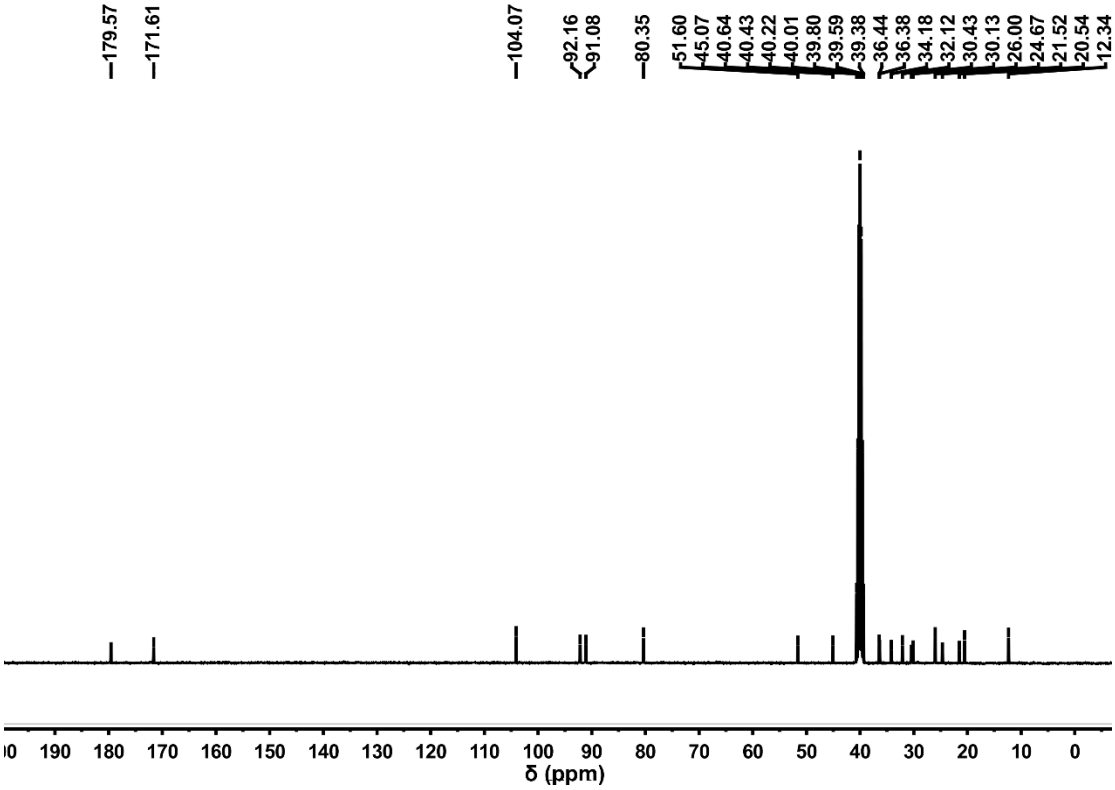


Fig. S2 ^{1}H NMR (400 MHz, DMSO-d_6) (A), ^{13}C NMR (100 MHz, DMSO-d_6) (B), ^{195}Pt NMR (69 MHz, DMSO-d_6) (C), ESI-MS (D) and HR-MS (E) spectra of Pt-ART-I.

(A)


6.47
5.68
5.66
5.56
3.32
2.57
2.56
2.51
2.50
2.50
2.30
2.29
2.28
2.19
2.18
2.01
1.29
0.90
0.88
0.79
0.77
h,h'
j k
g,g'
l,l'
f
m,m'
e
n
o
d
p
c
b
a
b+c
DMSO
f
o k
e
d
H2O
g,g'
l,l'
m,m'
a
p n j i
h h'
6.11
2.00
2.02
7.99
2.12
2.16
2.31
2.09
12.05
6.24
4.30
6.10
6.08
7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0
δ (ppm)

(B)


179.57
171.61
104.07
92.16
91.08
80.35
51.60
45.07
40.64
40.43
40.22
40.01
39.80
39.59
39.38
36.44
36.38
34.18
32.12
30.43
30.13
26.00
24.67
21.52
20.54
12.34
190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
δ (ppm)

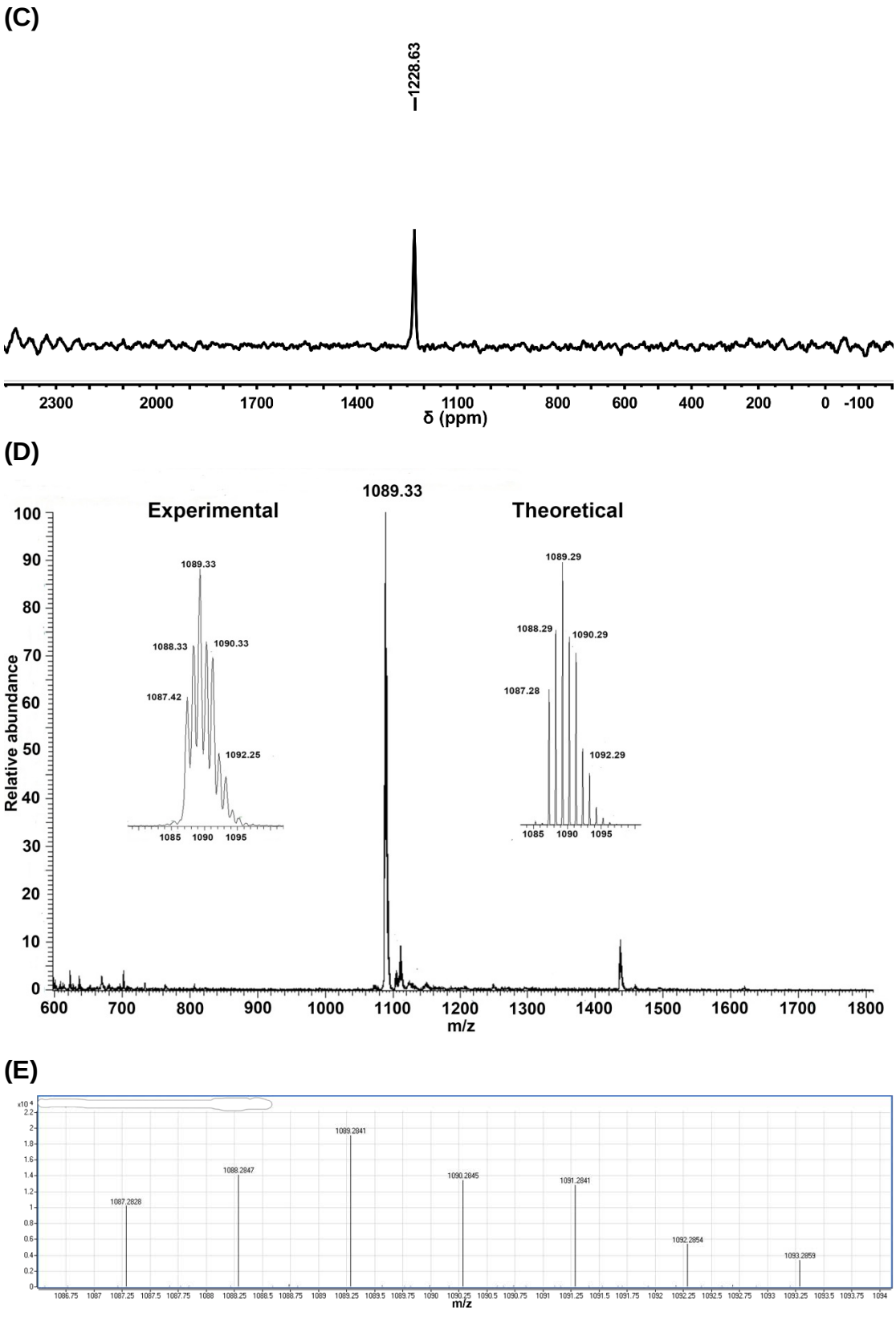


Fig. S3 ^{1}H NMR (400 MHz, DMSO-d_6) (A), ^{13}C NMR (100 MHz, DMSO-d_6) (B), ^{195}Pt NMR (69 MHz, DMSO-d_6) (C), ESI-MS (D) and HR-MS ($[M+Na]^+$) (E) spectra of Pt-ART-II.

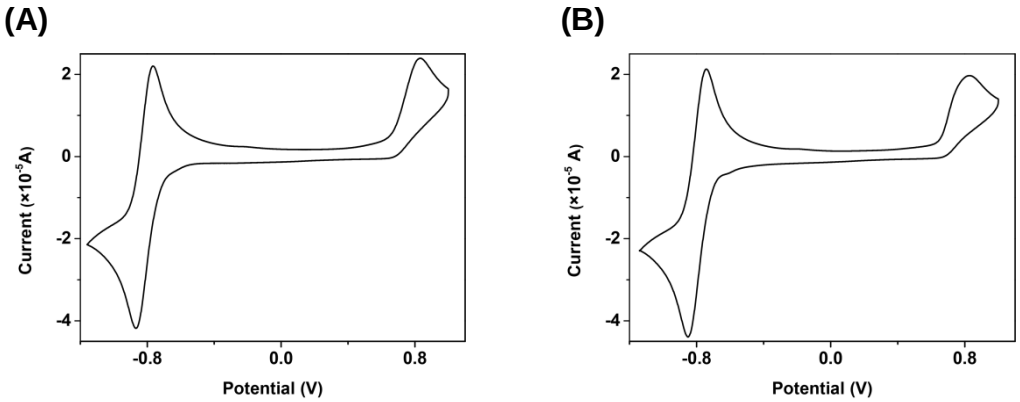


Fig. S4 Cyclic voltammogram of Pt-ART-II (A) and Pt-ART-I (B) in DMF containing 0.1 M (n-Bu_4N)PF_6 at a scan rate of 100 mV S^{-1} using a glassy carbon as a working electrode.

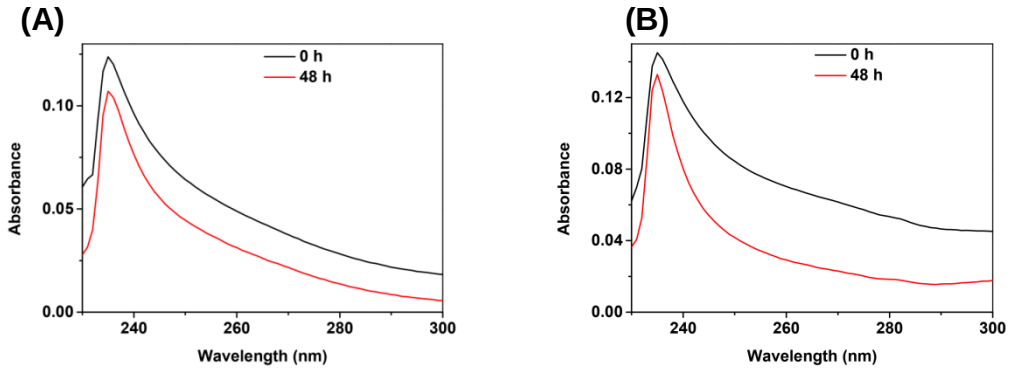


Fig. S5 UV-Vis spectra of Pt-ART-II (A) and Pt-ART-I (B) in PBS with 5% DMSO under physiological conditions (pH 7.4, 37 °C) at 0 and 48 h.

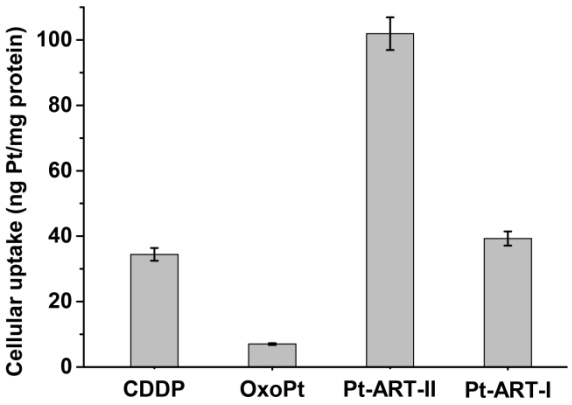


Fig. S6 Cellular uptake of Pt in Caov3 cells after exposure to CDDP, OxoPt, Pt-ART-II and Pt-ART-I (10 μM) for 12 h.

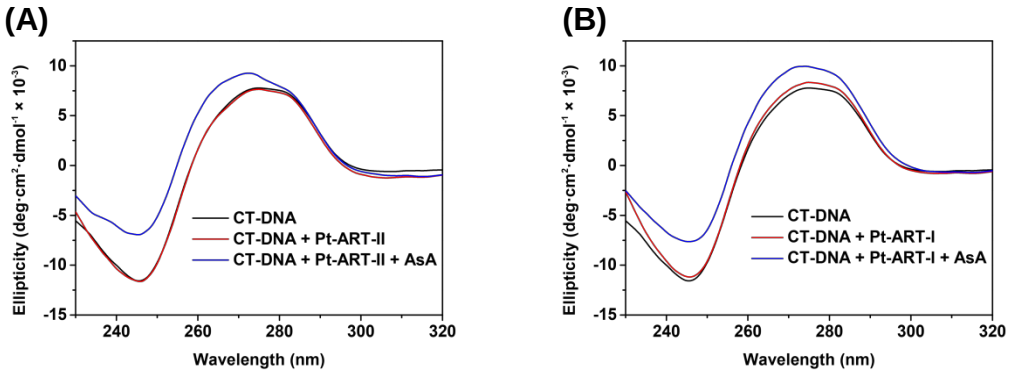


Fig. S7 CD spectra of CT-DNA (100 μM) in the absence and presence of Pt-ART-II (20 μM, A) and Pt-ART-I (20 μM, B) without or with ascorbic acid (80 μM) after incubation at 37 ℃ for 72 h.

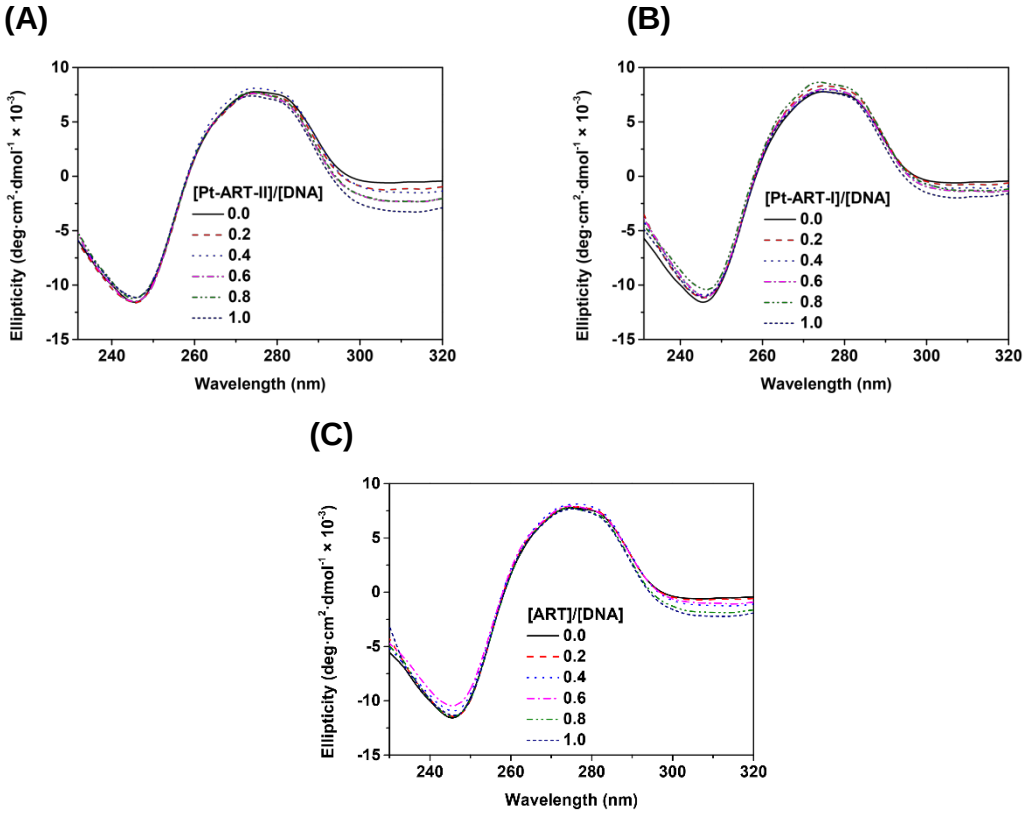


Fig. S8 CD spectra of CT-DNA (100 μM) in the presence of Pt-ART-II (A), Pt-ART-I (B) and ART (C) at different [complex]/[DNA] molar ratios after incubation at 37 ℃ for 72 h.

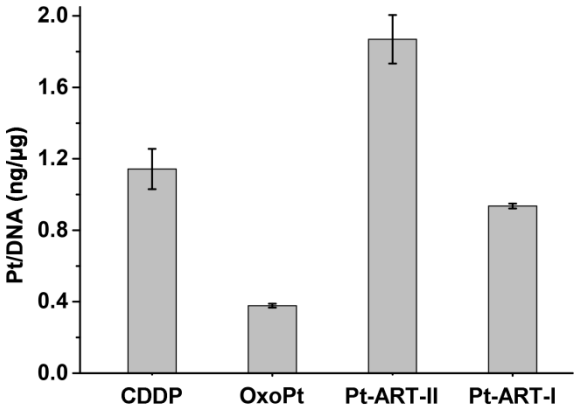


Fig. S9 Platination of cellular DNA (ng Pt/μg DNA) in Caov3 cells induced by the complexes (10 μM) after incubation for 48 h.

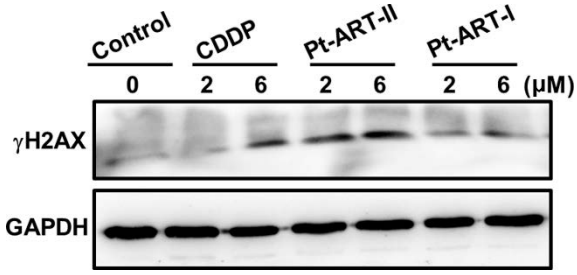


Fig. S10 Expression of γH2AX in Caov3 cells after treatment with the complexes for 60 h.

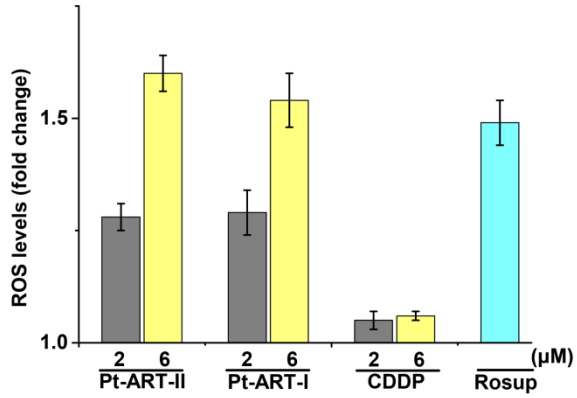


Fig. S11 Production of ROS induced by the complexes after incubation for 12 h.

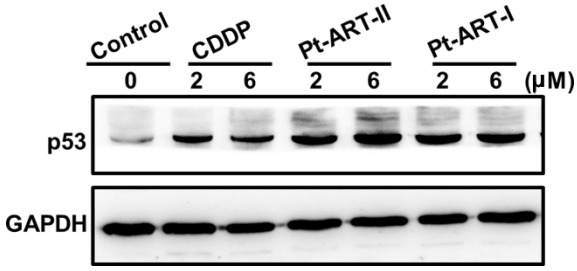


Fig. S12 Expression of p53 in Caov3 cells after treatment with the complexes for 60 h.

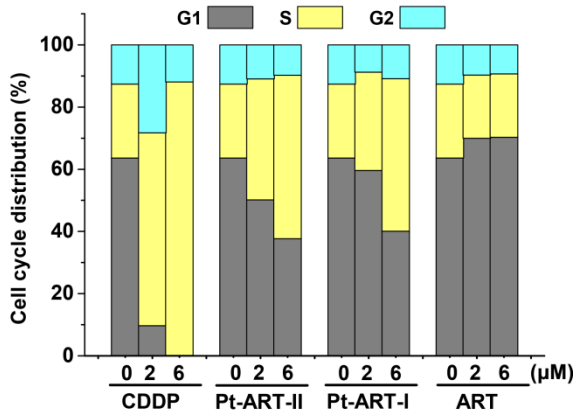


Fig. S13 Cell cycle arrest of Caov3 cells treated with CDDP, Pt-ART-II, Pt-ART-I, and ART, respectively, for 48 h.

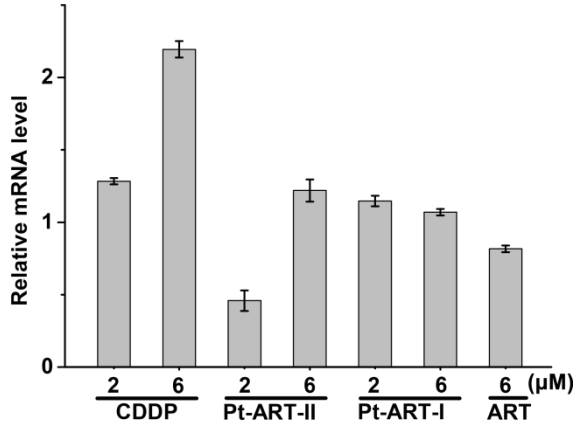


Fig. S14 Level of RAD51 mRNA in Caov3 cells after treatment with the complexes for 60 h.

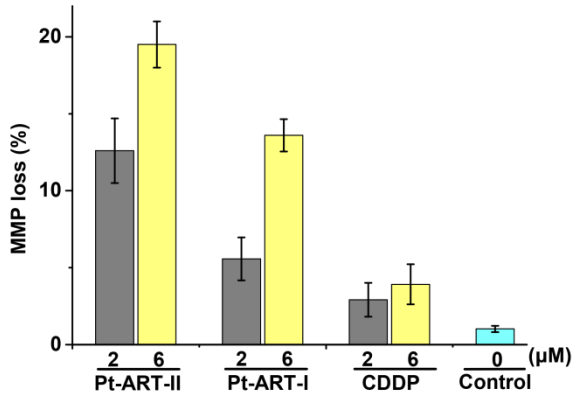


Fig. S15 Mitochondrial membrane potential (MMP) changes in Caov3 cells after treatment with the complexes for 60 h.

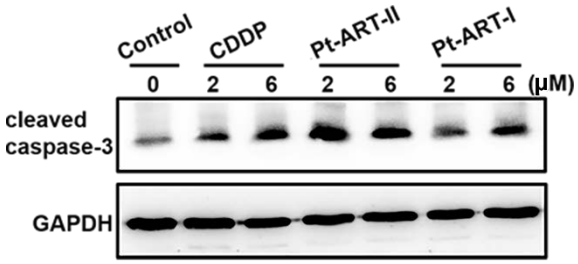


Fig. S16 Expression of cleaved caspase-3 in Caov3 cells after treatment with the complexes for 60 h.

2. Experimental

2.1. Chemical reagents

All the reagents were of analytical grade and used as received without further purification. Cisplatin (CDDP) was purchased from Shandong Boyuan Pharmaceutical Co., Ltd. China. Artesunate (ART), 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. 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). Ascorbic acid, 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), rhodamine123, Hoechst 3342, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma-Aldrich. Calf thymus (CT) DNA and DNAzol reagent were purchased from ThermoFisher Scientific. The antibodies used for western blotting were purchased from Abcam. *cis*-Diamminedichloro-*trans*-dihydroxyplatinum(IV) (OxoPt) and *cis*-diamminetrichlorohydroxyplatinum(IV) (OxClPt) were prepared according to the literature procedure.[1, 2]

2.2. Cell lines

The human ovarian carcinoma cell line Caov3, the human breast cancer cell line MDA-MB-231, and the human kidney epithelial HK-2 cell line were purchased from American Type Culture Collection (ATCC).

2.3. Instruments

Electrospray ionization mass spectra (ESI-MS) were obtained using an LCQ spectrometer (Finnigan). The isotopic distribution patterns for the complex were simulated using the ISOPRO 3.0 program. High resolution mass spectra (HR-MS) were measured on a liquid chromatography mass spectrometry instrument-G6500 (Agilent). ^{1}H-, ^{13}C-, ^{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.). CD spectra were recorded on a JASCO J-810 spectropolarimeter at room temperature using a cylindrical quartz cell (1.0 cm), with a continuous flow of nitrogen purging the polarimeter.

2.4. Synthesis of Pt-ART-I

OxClPt (100 mg, 0.28 mmol) was stirred in dry DMF (5 mL) with ART (119 mg, 0.31 mmol), triethylamine (58 μL, 0.42 mmol), and TBTU (135 mg, 0.42 mmol) at ambient temperature for 24 h. Addition of 40 mL diethyl ether to the mixture resulted in precipitation of the yellow crude product, which was cooled to –20 ℃ and the solution was allowed to stand for 2 h. The precipitate was filtered and resolved in acetone (5 mL). N-hexane (5 mL) and diethyl ether (20 mL) were added to the solution and the resulting precipitate was filtered, washed with diethyl ether and ethanol/water (1:1), and dried under vacuum. Pt-ART-I was obtained as solid with a yield of 40%. ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 6.65–6.25 (m, 6H, H_a), 5.67 (d, J = 9.1, 1H, H_d), 5.55 (s, 1H, H_e), 2.56–2.54 (m, 4H, H_b, H_c), 2.32–2.28 (m, 1H, H_p), 2.22–2.15 (m, 1H, H_n), 2.01–1.99 (m, 1H, H_j), 1.88–1.75 (m, 1H, H_i), 1.71–1.32 (m, 6H, H_g, $H_{g'}$, H_l, $H_{l'}$, H_m, $H_{m'}$), 1.29 (s, 3H, H_f), 1.21–1.14 (m, 1H, H_h), 1.02–0.91 (m, 1H, $H_{h'}$), 0.89 (d, J = 5.9 Hz, 3H, H_o), 0.78 (d, J = 6.7 Hz, 3H, H_k). ^{13}C NMR (100 MHz, DMSO-d_6): δ (ppm) 171.58, 162.77, 104.04, 92.06, 91.05, 80.33, 51.59, 45.05, 36.44, 36.37, 34.18, 32.12, 31.24, 30.12, 25.99, 24.66, 21.51, 20.54, 12.32. ^{195}Pt NMR (69 MHz): 1065.38 ppm. HR-MS (negative mode, m/z): 717.0776, calcd 717.0956.

2.5. Synthesis of Pt-ART-II

OxoPt (100 mg, 0.30 mmol) was stirred in dry DMF (5 mL) with ART (346 mg, 0.90 mmol), triethylamine (125 µL, 0.90 mmol), and TBTU (290 mg, 0.90 mmol) at ambient temperature for 48 h. Addition of 40 mL diethyl ether to the mixture resulted in precipitation of the yellow crude product, which was cooled to –20 ℃ and the solution was allowed to stand for 2 h. The precipitate was filtered and resolved in acetone (5 mL). N-hexane (5 mL) and diethyl ether (20 mL) were added to the solution and the resulting precipitate was filtered, washed with diethyl ether and ethanol/water (1:1), and dried under vacuum. Pt-ART-II was obtained as solid with a yield of 60%. ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 6.47 (s, 6H, H_a), 5.67 (d, J = 9.7 Hz, 2H, H_d), 5.56 (s, 2H, H_e), 2.58–2.55 (m, 8H, H_b, H_c), 2.37–2.24 (m, 2H, H_p), 2.22–2.14 (m, 2H, H_n), 2.03–1.95 (m, 2H, H_j), 1.85–1.76 (m, 2H, H_i), 1.67–1.39 (m, 12H, H_g, $H_{g'}$, H_l, $H_{l'}$, H_m, $H_{m'}$), 1.29 (s, 6H, H_f), 1.25–1.14 (m, 2H, H_h), 1.00–0.92 (m, 2H, $H_{h'}$), 0.89 (d, J = 6.3 Hz, 6H, H_o), 0.78 (d, J = 7.1 Hz, 6H, H_k). ^{13}C NMR (100 MHz, DMSO-d_6): δ (ppm) 179.57, 171.61, 104.07, 92.16, 91.08, 80.35, 51.60, 45.07, 36.44, 36.38, 34.18, 32.12, 30.43, 30.13, 26.00, 24.67, 21.52, 20.54, 12.34. ^{195}Pt NMR (69 MHz): 1228.63 ppm. HR-ESI (positive mode, m/z): $[M+Na]^+$ = 1089.2841, calcd 1089.2860.

2.6. Measurement of partition coefficient

Shake-flask method was used to measure the partition coefficient for complexes Pt-ART-I and Pt-ART-II. Briefly, a mixture of an equal volume of octanol and phosphate buffer (10 mM, pH 7.4) were shaken on a mechanical shaker at room temperature for 24 h. Saturated solutions of both complexes in phosphate buffer (presaturated with n-octanol) were prepared in an ultrasonic bath followed by a filtration through a 0.2 µm nylon filter. Equal volumes of the solution and 1-octanol presaturated with phosphate buffer were mixed and the mixtures were shaken for 24 h at room temperature. Centrifugation was carried out at 2500 rpm for 30 min to separate the two phases. The aqueous layer was carefully separated and the Pt content was analyzed in initial and final aqueous phases by ICP-MS. The log $P_{O/W}$ was measured using the following equation.

$$\log P_{O/W} = \log [(Pt_{initial} – Pt_{final})/ Pt_{final}]$$

Table S1 The partition coefficient of Pt-ART-II and Pt-ART-I. The log *P* of CDDP came from the literature data.[3]

Drugs	Pt-ART-II	Pt-ART-I	CDDP [3]
log $P_{O/W}$	1.10 ± 0.15	0.98 ± 0.20	−2.30 ± 0.15

2.7. Cytotoxicity assay

Tumor cells were cultured over night after inoculation in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (v/v), 2 mM glutamine, 100 U mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin. All cultures were maintained in an incubator in a highly humidified atmosphere of 95% air with 5% CO_2 at 37 °C. The growth inhibition was measured by the MTT assay. Briefly, 2000 cells/well in culture medium (100 μL) were planted in 96-well plates (Falcon, CA). The cells were treated in triplicate with different concentrations of complex at 37 ℃ for 72 h. Aliquot MTT solution (20 μL, 5 mg mL^{-1}) in PBS buffer (8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4 and 0.24 g KH_2PO_4 per liter, pH 7.40) was added to each well and incubated for 4 h. The supernatant was removed and DMSO (150 μL) was added to solubilize the MTT formazan. The amount of MTT formazan was determined using a Tecan Sunrise ELISA Reader at 570 nm after the plates were shaking for 30 min. The optical density (OD) was used to calculate the percentage of cell viability relative to the untreated control values, that is, $(OD_{control} - OD_{test})/(OD_{control} - OD_{blank}) \times 100\%$. The background readings of MTT incubated in a cell-free medium were subtracted from each value before calculation. The half-maximal inhibitory concentration (IC_{50}) of the complexes were obtained from the fitted inhibition curves at 72 h. The mean IC_{50} was calculated using the data from three replicates.

2.8. Flow cytometric analysis

Cell death was analyzed by fluorescence-activated cell sorting (FACS) using Annexin V and propidium iodide (PI) staining assay. Caov3 cells were seeded in a 6-

well plate at a density of 2×10^5 cells per well and incubated in DMEM (2 mL) and allowed to settle for 24 h. The medium was replaced with the fresh one containing Pt-ART-II, Pt-ART-I, ART and cisplatin respectively. After incubation for 72 h, the cells were washed twice with cold PBS, trypsinized and centrifuged (4000 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 45 min. The cells were treated with PI and analyzed by flow cytometry.

2.9. Cell cycle

Caov3 cells were seeded in a 6-well plate at a density of 2×10^5 cells per well and cultured in DMEM and allowed to settle for 24 h. The medium was replaced with the fresh one containing cisplatin, ART, Pt-ART-II, and Pt-ART-I, respectively. After incubation for 48 h, the cells were collected by trypsinization and washed with PBS, fixed in ice-cold ethanol (70%) for 12 h, pelleted by centrifugation, treated with RNaseA, stained with PI in PBS for 30 min and then analyzed by flow cytometry using a FACS.

2.10. ROS assay

Caov3 cells were seeded in a 6-well plate at a density of 2×10^5 cells per well and cultured in DMEM and allowed to settle for 24 h. The medium was replaced with the fresh one containing cisplatin, ART, Pt-ART-II, and Pt-ART-I, respectively. After incubation for 12 h, the cells were washed twice with cold PBS, trypsinized and centrifuged (4000 g, 5 min). The supernatant was discarded and the cells were resuspended in DMEM without FBS (1500 μL), stained with DCFH-DA and incubated in the dark for 30 min and then analyzed by flow cytometry.

2.11. Cellular uptake

Caov3 cells were seeded in a 6-well plate at a density of 2×10^5 cells/well. After incubation for 24 h, the cells were treated with each compound (10 μM) for 12 h. The attached cells were washed twice with PBS (4 °C). Cell pellets were collected by centrifugation and they were then digested with nitric acid (100 μL) for 2 h at 95 °C,

followed by the addition of H_2O_2 (50 µL) and HCl (100 µL) to give a fully homogenized solution. Water was then added to dilute the solutions and the final Pt content was determined by ICP-MS.

2.12. DNA platination

Caov3 cells were seeded in a 150 cm^2 cultural flask at a density of 2×10^7 cells/flask. After incubation at 37 °C for 24 h, the cells were treated with each complex (10 µM) for 48 h. The attached cells were washed twice with PBS (4 °C), harvested by trypsinization (0.5 mL) and washed with 1 mL of PBS. Cell pallets were lysed in DNAzol (1 mL genomic DNA isolation reagent, ThermoFisher Scientific) and the genomic DNA was extracted from lysate with pure ethanol (0.5 mL) by incubating the sample for 1–3 min at room temperature. The isolated DNA was washed with 75% ethanol and re-dissolved in NaOH (1 mL, 8 mM). The DNA concentration was determined using nanodrop spectrophotometer and the Pt content was quantified by ICP-MS.

2.13. CD spectroscopy

The conformational variations of CT-DNA upon binding to Pt-ART-II and Pt-ART-I in the absence and presence of ascorbic acid was analyzed by CD spectra. CT-DNA stock solution 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 and used within 4 days. The concentration of CT-DNA was determined by nanodrop spectrophotometer by taking 6600 $M^{-1}cm^{-1}$ as its absorption coefficient at wavelength of about 260 nm. CD spectra were recorded in the range of 230-320 nm at a scan speed of 10 nm/min. Samples were prepared by taking fixed concentration of CT-DNA (100 µM) in the absence and presence of Pt-ART-II and Pt-ART-I (20 µM) and incubated at 37 °C for 72 h in the dark. To determine the impact of ascorbic acid on the reduction of Pt-ART-II and Pt-ART-I and their subsequent binding to CT-DNA, samples were prepared by using a fixed concentration (100 µM) of CT-DNA, Pt-ART-II/Pt-ART-I (20 µM), and ascorbic acid (80 µM) and incubated for 72 h at 37 °C. In

addition, different concentrations Pt-ART-II, Pt-ART-I, ART were incubated with CT-DNA at 37 °C for 72 h to confirm that the two drugs and ligand cannot bind to DNA and only their reduced Pt^{II} equivalents are able to bind to DNA.

2.14. Western blotting

Total protein was extracted from Caov3 cells pellet using homogenizer with RIPA lysis buffer. Approximately 3.6 mg of total protein was precleared with 20 μL of 50% protein A/G slurry at 4 °C for 15 min. Proteins that were resolved by 2-DE and SDS-PAGE were transferred to PVDF (Millipore, 0.22 μm) in Towbin buffer containing 0.033% SDS. PVDF membranes were blocked at ambient temperature for 1 h in blocking buffer (5% skim milk/0.1% Tween-20/PBS). The primary antibodies in appropriate dilutions were incubated with the membranes at 4 °C overnight. The blots were washed with PBST (0.1% Tween-20/PBS) and incubated with peroxidase-conjugated secondary antibody in washing buffer for 1 h. After washing with PBST, the blots were visualized by enhanced chemiluminescence kit from Millipore.

2.15. Real-time PCR

Total cellular RNA was prepared using TRIzol reagent (Invitrogen). First-strand cDNA was synthesized from 1 μg of total RNA using reverse transcriptase (Thermo Scientific, Beijing, China). The expression levels of RAD51 mRNAs were determined by RT-PCR using SYBR GREEN mix (TOYOBO, Osaka, Japan) and a Light Cycler 480 system (Roche). Human *β*-actin gene was used as an internal control. Primers were:

RAD51: 5′-GGTGAAGGAAAGGCCATGTA-3′ (forward)

5′-GGGTCTGGTGGTCTGTCTT-3′ (reverse)

β-actin: 5′-AGCGAGCATCCCCCAAAGTT-3′ (forward)

5′-GGGCACGAAGGCTCATCATT-3′ (reverse)

The samples were loaded in quadruple, and the result of each sample was normalized to β-actin.

2.16. Immunofluorescence staining of RAD51

Cells grown on coverslips in 6-well plates were treated with the complexes for 24 h before they were processed for determination of the RAD51 level. For the immunofluorescence staining of RAD51, cells were pre-treated with the drugs (2 μM) for 60 h. Cells were fixed with 4% formaldehyde, followed by treatment with 0.2% Triton X-100 in PBS for 5 min, then blocked with 5% bovine serum albumin in PBS containing 0.3% Triton X-100 for 30 min. Rabbit anti-Rad51 antibody was from Abcam at a dilution of 1:500. The specimens were incubated overnight at 4 ℃. Cells were then washed thrice in PBS before incubating in the dark with a FITC-labeled secondary antibody for 60 min. After washing with PBS containing 0.3% Triton X-100 for 3 times, cells were then counterstained with Hoechst 3342 for 5 min. The coverslips were mounted to slides with an antifade solution. Slides were then examined under a confocal microscope.

2.17. Flow cytometric analysis of the mitochondrial membrane potential (MMP)

Caov3 cells were seeded in a 6-well plate at a density of 2×10^5 cells per well and cultured in DMEM and allowed to settle for 24 h. The medium was replaced with the fresh one containing cisplatin, ART, Pt-ART-I, and Pt-ART-II, respectively. After incubation for 60 h, the cells were washed twice with cold PBS, trypsinized and centrifuged (4000 g, 5 min). The supernatant was discarded and the cells were resuspended in DMEM without FBS (1500 μL), stained with rhodamine123 and incubated in the dark for 30 min and then analyzed by flow cytometry.

1. M. Ravera, E. Gabano, G. Pelosi, F. Fregonese, S. Tinello and D. Osella, *Inorg. Chem.*, 2014, **53**, 9326–9335.
2. R. K. Pathak and S. Dhar, *Chem. Eur. J.*, 2016, **22**, 3029–3036.
3. S. P. Oldfield, M. D. Hall and J. A. Platts, *J. Med. Chem.*, 2007, **50**, 5227–5237.