Electronic Supplementary Material (ESI) for ChemComm.

Chalcoplatin, a dual-targeting and p53 activator-containing anticancer platinum(IV) prodrug with unique mode of action

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Electronic Supporting Information

Experimental section

1. Materials

Unless otherwise noted, all reactions were carried out under normal atmospheric conditions. Agents and solvents, except acetone, were used as received without additional drying or purification. Acetone was dried by refluxing with K_2CO_3 powder for about 6 h. *c*,*c*,*t*-$[Pt(NH_3)_2Cl_2(OH)_2]$ was synthesized as previously described [1].

2. General measurements

Inductively Coupled Plasma-Optical Emission Spectrometer (ICP-OES, Optima 2100DV, PerkinElmer, USA) was used to determine platinum contents. Elemental analysis was performed using a Vario Micro elemental analyzer. ESI-MS was carried out on an Agilent API 150EX mass spectrometer. ^1H, ^{13}C, and ^{195}Pt NMR spectra were measured by a Bruker Ultrashield 400 MHz NMR spectrometer at room temperature. All NMR chemical shifts (δ) are reported in parts per million (ppm) and referenced as described below. ^1H and $^{13}C\{^1H\}$ NMR spectra were referenced internally to residual solvent peaks using deuterated dimethyl sulfoxide (DMSO-d_6) as the solvent. $^{195}Pt\{^1H\}$ NMR spectrum was referenced externally using standards of K_2PtCl_4 in D_2O (δ=-1628 ppm). Cyclic voltammograms were obtained at room temperature using an electrochemical analyzer system CHI 660C. A three-electrode system was used comprising glassy carbon as the working electrode, a Pt wire as the auxiliary electrode, and Ag/Ag^+ (non-aqueous, 10 mmol/L $AgNO_3$ in CH_3CN) as the reference electrode. Data was obtained using a 0.2 mmol/L solution of chalcoplatin in DMF containing 0.1 mol/L $(n\text{-}Bu_4N)PF_6$ as the supporting electrolyte at room temperature. Trace amount of ferrocene was added as an internal reference. The scan rate was 100 mV/s.

3. Synthesis of chalcone

A 50% KOH solution (2 mL) was added to a vigorously stirred solution of 3',4'-dichloroacetophenone (**1**) (378 mg, 2.0 mmol) dissolved in methanol (14 mL), then a solution of 4-formylphenoxyacetic (**2**) (360 mg, 2.0 mmol) in 5 mL methanol was added dropwise. The reaction was kept at room temperature for about 12 h, and the reaction completeness was monitored by TLC. After adding 50% KOH solution (5 mL), the solid was filtered and washed by diethyl ether. Moderately diluted HCl solution was added to the solid, and the mixture was filtered and washed by cold deionized water and dried in evaporation vacuum. Chalcone (**3**) was obtained as pale solid. Yield: 561.2 mg, 80%. ^{1}H NMR (400 MHz, DMSO-d_6): δ_{ppm} 13.09 (br, 1H, COOH), 8.39 (d, 1H, J=2.0 Hz, H_a), 8.10 (dd, 1H, J=8.4, 2.0 Hz, H_c), 7.90-7.73 (m, 5H, H_b, H_d, H_e, H_f, H_i), 7.00 (d, 2H, J=8.8 Hz, H_g, H_h), 4.77 (s, 2H, CH_2, H_j).

4. Synthesis of chalcoplatin

Oxalyl chloride (2.5 mL) was added to **3** (517 mg, 1.45 mmol), and the color of the mixture changed from pale to light yellow. Catalytic amount of DMF (20 μL) was added to the mixture, which was further stirred at 50 °C for 2 h. Unreacted oxalyl chloride was removed under vacuum to yield a pale yellow residue. The crude product, obtained in a quantitative yield, was used directly in the next step without further manipulation. A solution of **3** in dried acetone (10 mL) was added to *c,c,t*-[$Pt(NH_3)_2Cl_2(OH)_2$] (87 mg, 0.26 mmol) in dried acetone (10 mL). The yellow mixture was heated to reflux for about 20 mins to get a bright yellow solution. The desired product precipitated as white solid upon addition of deionized water (20 mL). To maximize the yield, the mixture was left at 4 °C for about 16 h. Acetone was removed by reduced pressure. The crude product was filtered, lyophilized, and then purified by column chromatography

(EtOAc/MeOH= 1:1) to yield a yellow solid chalcoplatin (**4**). Yield: 65.03 mg, 25%. ^{1}H NMR (400 MHz, DMSO-d_6): δ_{ppm} 8.40 (s, 4H, J=2.0 Hz, H_a), 8.10 (d, 2H, J=8.4, H_c), 7.87-7.74 (m, 10H, H_b, H_d, H_e, H_f, H_i), 7.01 (d, 4H, J=7.6 Hz, H_g, H_h), 6.61 (br, 6H, NH_3), 4.74 (s, 4H, H_j). ^{13}C{^{1}H}NMR (100 MHz, DMSO-d_6): δ_{ppm} 187.30, 175.70, 160.92, 145.68, 138.44, 136.23, 132.35, 131.55, 131.47, 130.77, 128.92, 127.83, 119.29, 115.64, 64.90. ^{195}Pt{^{1}H}NMR (86 MHz, DMSO-d_6): δ_{ppm} 1230.13. MS (ESI$^+$) m/z: $[M+Na]^+$ calculated for $C_{34}H_{28}Cl_6N_2O_8PtNa$: 1023.4, found: 1023.3. Anal. Calcd for $C_{34}H_{34}Cl_6N_2O_{11}Pt$: C, 38.73; H, 3.25; N, 2.66 Found: C, 38.46, H, 3.40, N, 2.78.

5. Cyclic votametry measurement of chalcoplatin

Due to the limited solubility of chalcoplatin in aqueous solution, the cyclic voltammogram was recorded in DMF containing 0.1 mol/L (n-Bu_4N)PF_6 as the supporting electrolyte at room temperature. Trace amount of ferrocene was added as an internal reference. The scan rate was 100 mV/s. The result indicated that chalcoplatin exhibited a single irreversible reduction peak, Ep= -0.73 V (vs. Fc^+/Fc= +0.05 V).

6. Reduction of chalcoplatin

The reaction was carried out by mixing calf-thymus DNA with cisplatin or chalcoplatin in 10 mM PBS (pH 7.4) containing 5% DMF. The final concentrations of DNA and Pt were 0.5 mg/mL and 2 mM, respectively. Ascorbic acid was added to the mixture at a final concentration of 2 mM. The mixture was incubated at 37 °C for 48 h. After reaction, DNA was ethanol precipitated and washed twice with 70% ethanol to remove excess amount of Pt. DNA was quantitated by Nanodrop Spectrophotometer (Thermo Scientific ND-1000) and Pt levels were

measured by ICP-OES. The results were expressed as μg Pt per mg DNA.

7. In vitro cytotoxicity

7.1 Cell lines and cell culture conditions

HeLa, A549, MCF-7, HCT116, and MRC-5 cells were cultured in DMEM containing 10% FBS and 100 units penicillin/streptomycin. HL-60 cells were cultured in RPMI 1640 with 10% FBS and 100 units penicillin/streptomycin. All cells were incubated at 37 ºC under 5% CO_2. MRC-5 cells were cultured in MEM containing 10% FBS supplemented with 1% L-glutamine, 1% non-essential amino acids, 1% sodium pyruvate, and 100 units penicillin/streptomycin. Cisplatin-resistant cells, A549cDDP, were generated from their parental A549 cells following a previous report [2]. Briefly, A549 cells were cultured in complete medium containing 0.5 μg/mL cisplatin at the beginning for the first screening, and the remaining cells were cultured in complete medium containing 1.0 μg/mL cisplatin for at least 4 weeks until the resistance was obtained.

7.2 Cytotoxicity test

MTT assay was used to evaluate the cytotoxicity of the compounds against HeLa, A549, A549cDDP, MCF-7, HCT116, and MRC-5 cells. Cells were seeded in 96-well plates at a density of 1,500 cells per well and incubated till their confluency reached about 30%. Then, medium containing different concentrations of compounds was added to each well. The final concentration of DMF was 1%. After 72 h incubation, the original medium was replaced by fresh medium containing 1 mg/mL MTT. After staining for 4 h, the medium was removed and DMSO was added to each well. The absorbance was measured at 570 and 630 nm. Medium containing 1% DMF without compound was used as a control.

MTS assay was used to assess the cytotoxicity of compounds against HL-60 cells. Cells were seeded in 96-well plates at a density of 40,000 cells per well in phenol-red free medium, and different levels of compounds were added. The final concentration of DMF was 1%. After 72 h incubation, a mixed solution of MTS (50 mg/L) and phenazine methosulfate (PMS, 50 mg/L) was added, and the mixture was incubated at 37 ºC under 5% CO_2 for 4 h. The absorbance was subsequently measured at 490 and 630 nm.

8. Cellular uptake tests

HeLa and HL-60 cells were seeded in 100 mm dishes. After the cells reached about 80% confluency, 10 μM of cDDP or chalcoplatin was added. The final concentration of DMF was 1%. After 12 h incubation, cells were collected and washed 4 times with ice-cold PBS. Cells after washes were centrifuged at 700×g for 10 min and resuspended in 1 mL PBS. A volume of 100 μL was taken out to determine the cell density. The rest of the cells was spun down and digested at 65 ºC in 0.3 mL 65% HNO_3 overnight. The Pt level in cells was determined by ICP-OES. DNA was extracted from HeLa cells treated with 10 μM cisplatin or chalcoplatin for 12 h. DNA level was determined by Nanodrop Spectrophotometer (Thermo Scientific ND-1000) and Pt level was measured by ICP-MS.

9. Cell cycle analysis and apoptosis determination

9.1 Cell cycle analysis

HeLa cells were seeded in 6-well plates at a density of 4×10^5 cells per well for 24 h. After incubation, medium was replaced by fresh medium containing cDDP, chalcoplatin, or chalcone at concentrations of 0.4, 2, 10 μM. The final concentration of DMF was 1%. Cells treated with 1%

DMF only were set as the negative control. After incubation for another 24 h, cells were collected and washed twice by ice-cold PBS. Then the cells were centrifuged and resuspended in 0.5 mL PBS, followed by fixing with 4.5 mL 70% ethanol overnight at -20ºC. After spun down and washed with PBS again, 1 mL propidium iodide (PI) solution (20 μg/mL, containing 0.1% Triton X-100 and 200 μg/mL RNase A, pH 7.4) was added and cells were stained for 15 min at 37 ºC. Cell cycle distribution was analyzed by a flow cytometer.

9.2 Apoptosis determination

HeLa cells were seeded in 6-well plates at a density of 2×10^5 cells per well and incubated for 24 h. Medium containing cDDP, chalcone, or chalcoplatin was added individually and incubated. Cells treated only with 1% DMF were set as the negative control. After 72 h incubation, all the cells were collect and washed twice with ice-cold PBS, followed by washing once with annexin-V binding buffer (10 mM HEPES, 140 mM NaCl, and 2.5 mM $CaCl_2$, pH 7.4). Cell density was adjusted to 1×10^6 cells/mL. Then, annexin-V (5 μL of stock solution) and PI solution (1 μL of 100 μg/mL solution) were added to 100 μL of the cell suspension for staining according to manufacturer's instructions. After 15 min incubation at room temperature, the samples were diluted with 400 μL annexin-V binding buffer for flow cytometer analysis.

9.3 JC-1 and DAPI double staining assay

HeLa cells were seeded on 8-well glass chamber slides and incubated overnight for attaching. Cells were treated with 0, 10, 20 μM cDDP and chalcoplatin (final concentration of DMF: 1%) for 24 h. After treatment, cells were stained with JC-1 (10 μg/mL in medium containing 5% DMSO) at 37 °C for 15 min, followed by washing once with PBS. Then cells were stained with

DAPI (5 μg/mL in medium) at 37 °C for 5 min, followed by washing three times with PBS. After staining, cells were fixed with 70% ethanol for 5 min and kept in PBS for imaging. Fluorescent samples were recorded on a laser confocal scanning microscope (Leica SPE).

10. Protein extraction, quantification, SDS PAGE and Western Blotting

Proteins were extracted from the treated cells or the control dish with 500 μl of RIPA buffer containing protease inhibitor cocktail and PMSF, and centrifuged at 12,000 rpm at 4°C for 10 min. The supernatants were collected as whole cell extracts and stored at -80^{o}C. Protein concentration was determined by the Bradford protein assay (Bio-Rad, Hercules, CA, USA). 40 μg of total proteins and prestained molecular weight markers were separated on 10% sodium dodecyl sulfate-containing polyacrylamide gel and followed by transferring onto nitrocellulose membranes (Pall Life Sciences, Pensacola, Fl, USA). The membranes were blocked in PBST (phosphate-buffered saline with 0.1% Tween-20) containing 5% nonfat milk (Carnation), and probed with p53 (PAb 240, Abcam, Cambridge, MA, USA) and actin (Cell Signaling Technology, Danvers, MA, USA) antibodies at 4°C overnight. After the incubation the membranes were washed with PBST, further incubated with secondary antibody conjugated with horseradish peroxidase, and extensively washed, before the immuno reactivity was visualized by enhanced chemiluminescence (WesternBright Quantum, Menlo Park, CA) according to the manufacturer's protocol. Finally the results were recorded by Fujifilm LAS-4000 Image Analyzer.

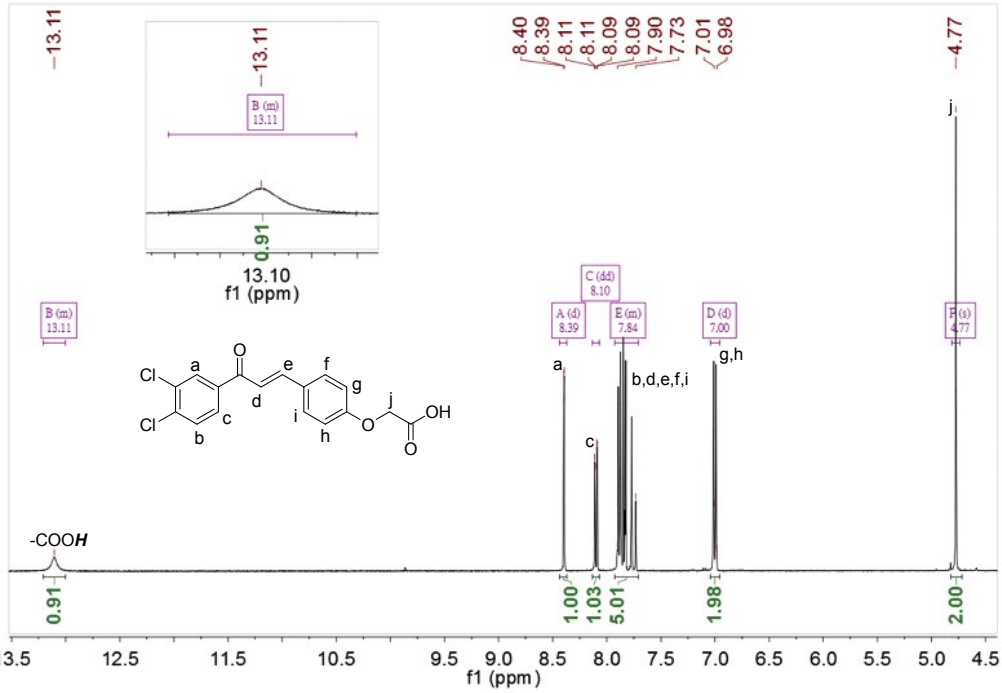

Figure S1 ^{1}H NMR spectrum of chalcone (**3**) in DMSO-d_6.

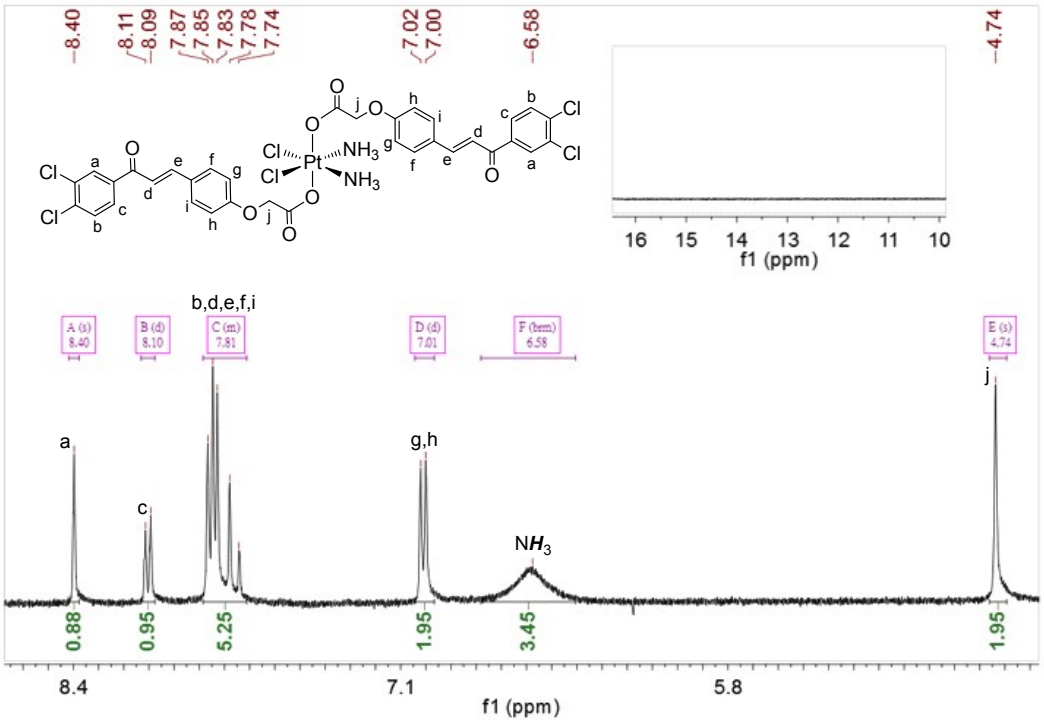

Figure S2 ^{1}H NMR spectrum of chalcoplatin (**4**) in DMSO-d_6.

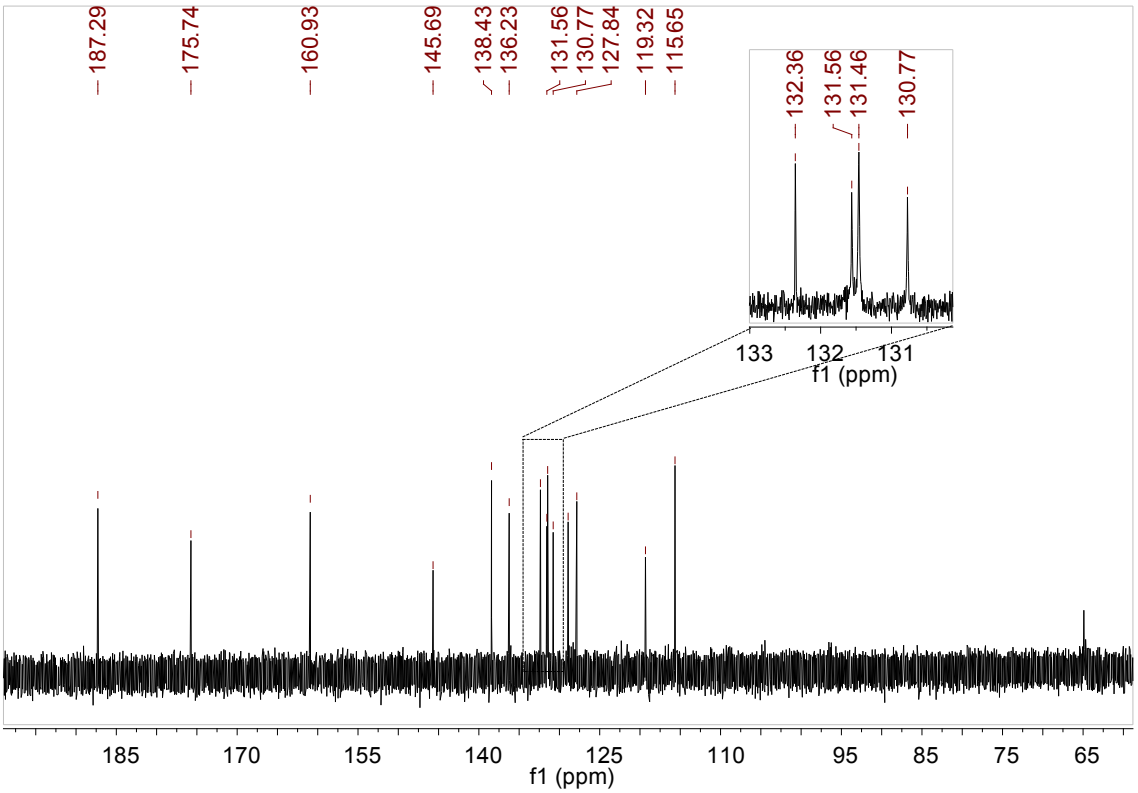

Figure S3 ^{13}C NMR spectrum of chalcoplatin (**4**) in DMSO-d_6.

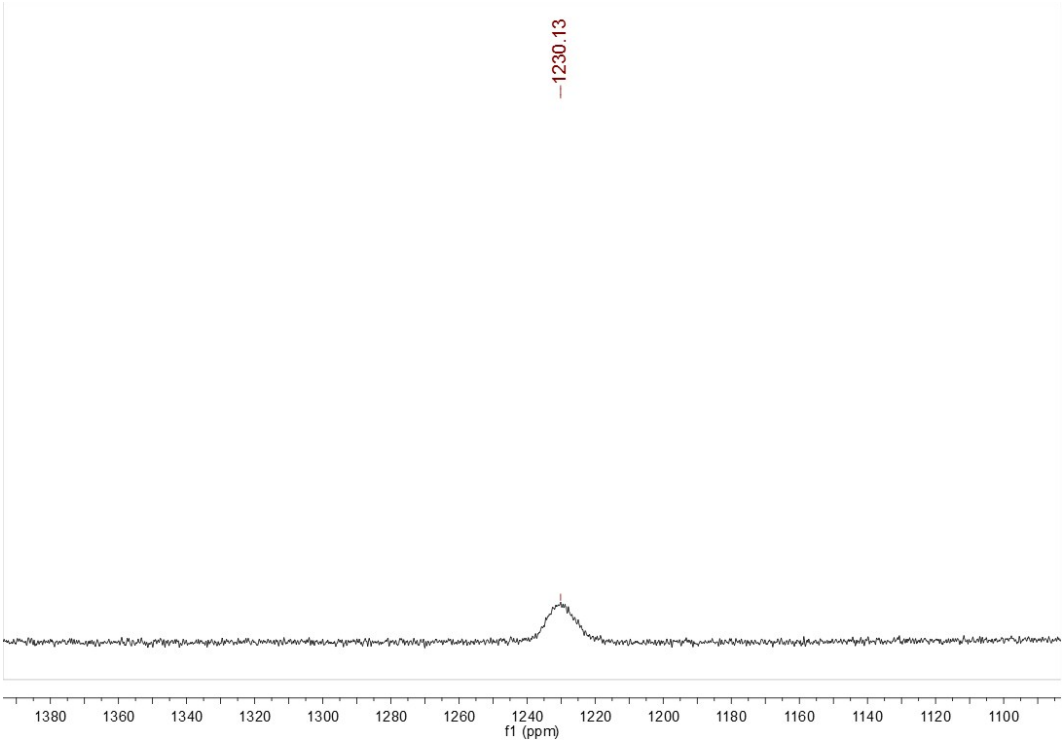

Figure S4 ^{195}Pt NMR spectrum of chalcoplatin (**4**) in DMSO-d_6.

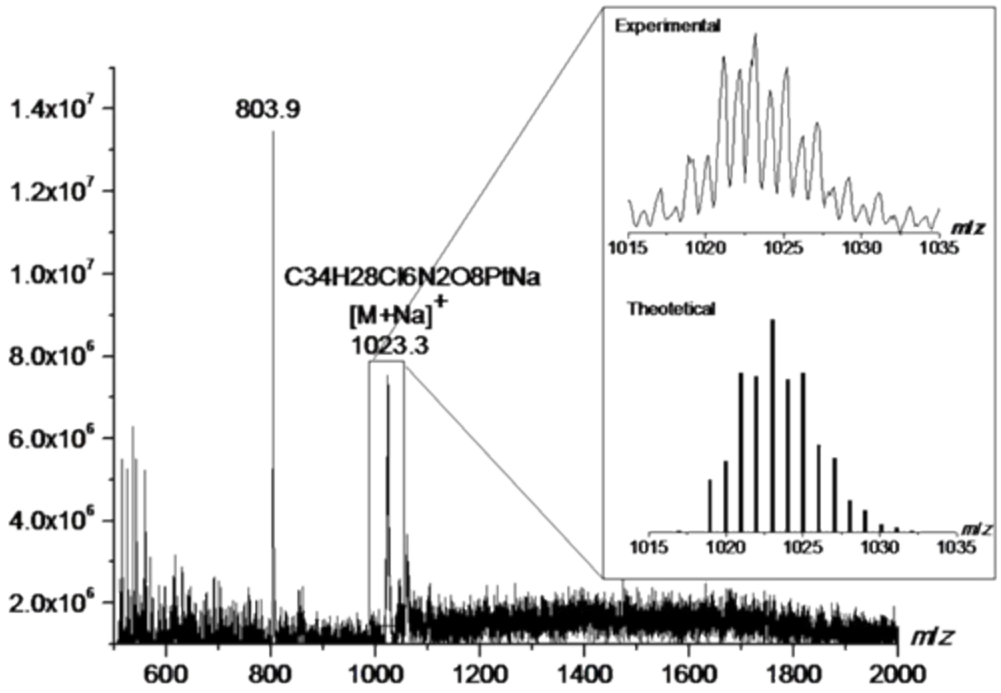

Figure S5 ESI-MS of chalcoplatin (**4**) in methanol.

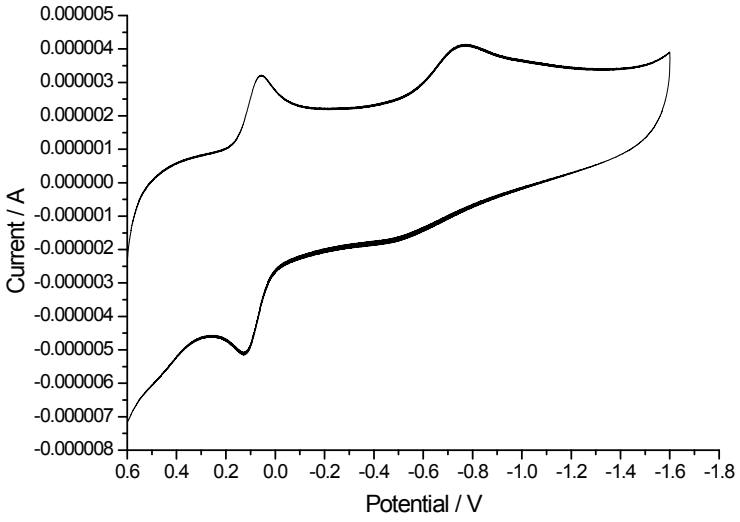

Figure S6 Cyclic voltammetry of chalcoplatin (**4**) with ferrocene as an internal reference.

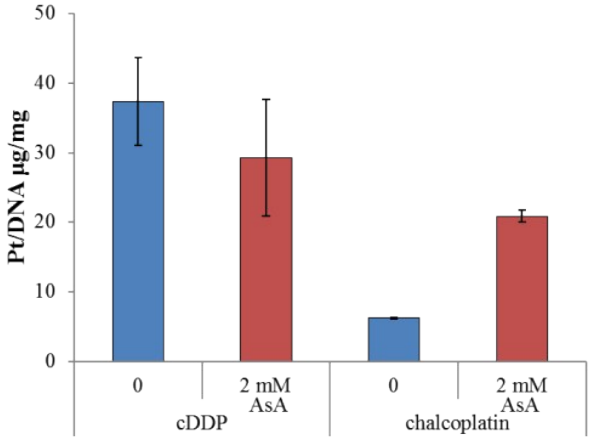

Figure S7 Levels of Pt on ct-DNA without and with the presence of AsA after 48 h incubation at 37 °C.

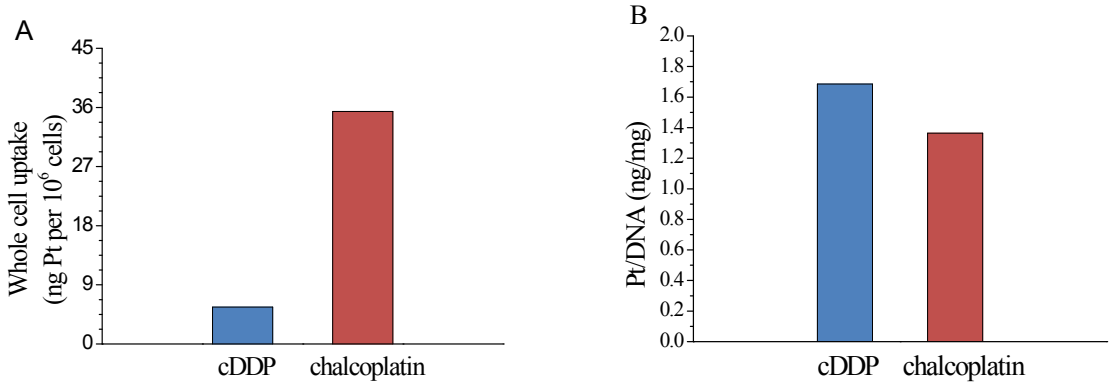

Figure S8 (A) Whole cell uptake of platinum and (B) levels of Pt on genomic DNA of HeLa cells upon treatment with 10 µM cisplatin or chalcoplatin for 12 h.

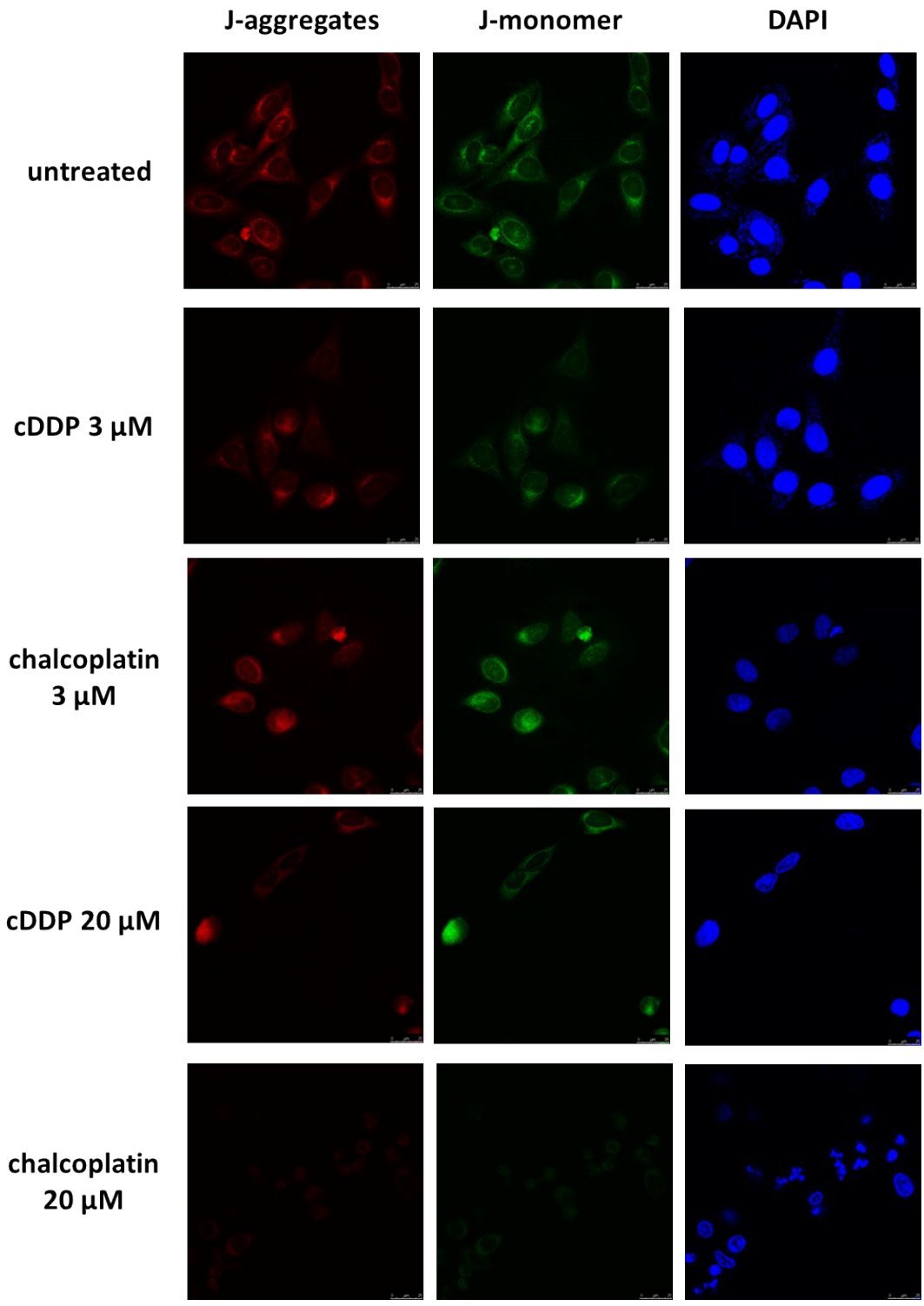

Figure S9 Confocal images of JC-1 monomer and aggregates in HeLa cells upon treatment of 0, 3, and 20 μM cDDP or chalcoplatin.

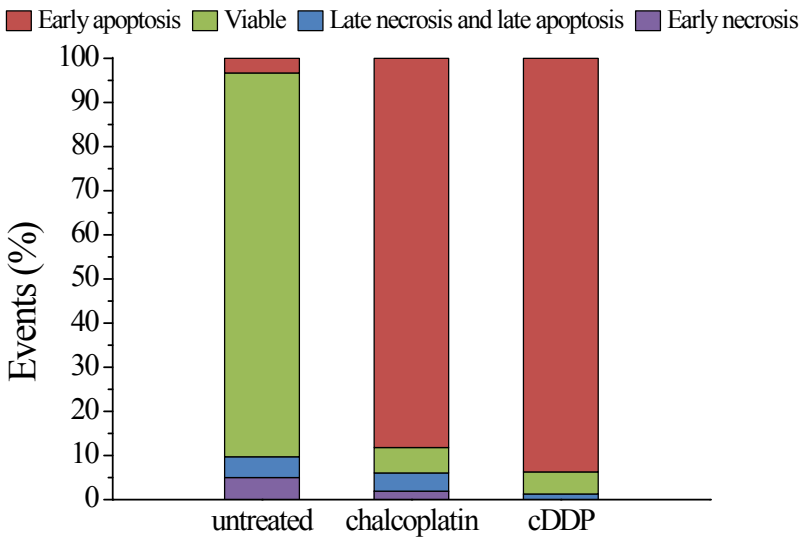

Figure S10 HeLa cell apoptosis upon treatment of 15 μM cisplatin or chalcoplatin of 24 h, followed by 48 h incubation.

References:

[1] M. D. Hall, C. T. Dillon, M. Zhang, P. Beale, Z. H. Cai, B. Lai, A. P. J. Stampfl, T. W. Hambley, *J. Inorg. Biochem.*, 2003, **8**, 726-732.

[2] X.-J. Liang, H. Meng, Y. Wang, H. He, J. Meng, J. Lu, P. C. Wang, Y. Zhao, X. Gao, B. Sun, C. Chen, G. Xing, D. Shen, M. M. Gottesman, Y. Wu, J. Yin, and L. Jia, *Proc. Natl. Acad. Sci. U.S.A.*, 2010, **107**, 7449-7454.