Electronic Supplementary Material (ESI) for ChemComm.

Efficient co-delivery of Pt(IV) prodrug and p53 activator as a convenient strategy to enhance the anticancer activity of cisplatin

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Electronic Supporting Information

Experimental section

1. Materials

All reagents and materials were obtained from commercial resources. Cell culture media, trypsin, PBS, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), phenol/chloroform/isoamyl alcohol, propidium iodide (PI), and annexin V-FITC conjugate were purchased from Life Technologies. Cisplatin was ordered from Boyuan Technology (China). BCA assay kit and Western lysis buffer were obtained from Beyotime Institute of Biotechnology (China). NE-PER Nuclear and Cytoplasmic Extraction Kit was from Thermo Scientific. Proteinase K and RNase A were obtained from Roche Diagnostics. Complete Mini EDTA-free Protease Inhibitor Cocktail Tablets were purchased from Roche Applied Science. Glycine and calf-thymus DNA were obtained from Sigma. TEMED, ammonium persulfate, tween-20, and SDS were purchased from Bio-Rad. Bromophenol blue was brought from Acros. All antibodies were obtained from Cell Signaling Technology.

2. Methods

2.1. Synthesis of LDH, chalcone, and DSCP

LDH was synthesized following a reported method.[1] Briefly, a 40 mL solution of 0.15 M NaOH was added dropwise to a 10 mL mixture of 2 mM $MgCl_2$ and 1 mM $AlCl_3$ with stir. The mixture was stirred for 10 min in a sealed container and spun down. After resuspending the precipitate in 40 mL MilliQ water in an airtight container, the suspension was hydrothermally treated at 80 °C for 4 h. The as-prepared colloid was 0.4% (w/w) LDH. Chalcone and disuccinatoplatinum(IV) (DSCP) were synthesized as previously reported and characterized by ^{1}H and ^{13}C NMR on a Bruker Ultrashield 400 MHz NMR spectrometer (data not shown).[2]

2.2. Synthesis and characterization of Pt(IV)-chalcone/LDH

The solutions of LDH (4 mg/mL) and DSCP (10 mM) were independently adjusted to pH 8.5-9.0. LDH (5 mL) and DSCP (5 mL) were then mixed together slowly and stirred at room temperature for 24 h. A chalcone solution (5 mM, 10 mL, pH=9) was added to the mixture dropwise and continued to stir for further 2 h. The obtained suspension was washed 3 times with MilliQ water and centrifuged at 8,000 rpm for 10 min. The particles were then resuspended in MilliQ water and stored at 4 °C. Pt level was determined by ICP-OES and chalcone level was determined by UV-Vis absorption spectroscopy. The morphology of the LDH and Pt(IV)-chalcone/LDH nanoparticles was characterized by TEM (FEI / Philips Tecnai 12 BioTWIN) at 40 kV. The size distribution of LDH and Pt(IV)-chalcone/LDH were statistically analyzed using the TEM image by the Nanomeasure software.

2.3. Release Profile of Pt(IV)-chalcone/LDH

A 180 µL solution of Pt(IV)-chalcone/LDH was packed in a mini-dialysis cassette using 3.5 kDa semipermeable membrane. The cassette was put into a 50 mL PBS buffer (pH 7.4 or 6.0) at 37 °C with stir. A volume of 1 mL PBS was taken out at different time points to determine the released levels of Pt and chalcone. At each time point 1 mL of fresh PBS buffer was added to the system. Pt levels were determined by ICP-MS and chalcone concentrations were ascertained by UV-Vis absorption spectroscopy.

2.4. Protection of Pt(IV) by LDH

Calf thymus DNA was dissolved in 10 mM PBS, pH 7.4 at 665 mg/L. Stock solutions of cisplatin, DSCP, Pt(IV)/LDH, and Pt(IV)-chalcone/LDH were prepared in MilliQ water at a concentration of

125 μM. Ascorbic acid was prepared in PBS at 20 mM. A volume of 150 μL DNA solution was mixed with 120 μL Pt stocks, and a volume of 30 μL of PBS buffer or ascorbic acid was added. The mixture was incubated at 37 °C for 1, 3, 5, and 7 h. After reaction, the mixture was spun down for three times at 10,000 rpm, 4 °C to remove the nanoparticles, and the supernatants were collected. DNA was ethanol precipitated and the excess amount of Pt was removed by washing twice with ethanol. DNA levels were quantified by Nanodrop Spectrophotometer (Thermo Scientific ND-1000) and Pt levels were quantified by ICP-OES. The results were expressed as μg Pt per mg DNA.

2.5. Cell lines and cell culture

MCF-7, HeLa, U2OS, and A549 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% Fetal Bovine Serum (FBS) and 100 U/mL penicillin/streptomycin. A2780 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 supplemented with 10% FBS and 100 U/mL penicillin/streptomycin. Cells were maintained in a humidified incubator at 37 °C in the presence of 5% CO_2 and subcultured every other day.

2.6. Cell viability tests

Cell viability upon the treatment of cDDP and Pt(IV)-chalcone/LDH was tested by the MTT assay using MCF-7, HeLa, U2OS, A549, and A2780 cells. Cells were seeded in 96-well plates at a density of 2×10^3 cells/well and incubated for 24 h to reach 30% confluence. Then, the original medium was replaced with 200 μL fresh medium containing different concentrations of cDDP and Pt(IV)-chalcone/LDH. After incubation for 72 h, the drug-containing medium was replaced with medium containing 3 mg/mL MTT. After further incubation for 3 h, the MTT solution was removed and 200 μL DMSO was added in each well to dissolve the formazan. After shaken for 2 min, the absorbance was measured at 570 and 630 nm by a microplate reader (Powerwave XS MQX200R).

2.7. Cell cycle arrest

MCF-7 cells were seeded in 35 mm dishes at a density of 4×10^4 cells/well and incubated for 24 h. Then, cells were treated with 0, 1, 5, and 10 μM cDDP or Pt(IV)-chalcone/LDH. After 24 h of drug exposure, cells were collected and washed by ice-cold PBS (1×) for 3 times. After spinning down, 70% ethanol was added to fix the cells at 4 °C overnight. After washing another time with PBS (1×), cells were stained with a PI solution (0.1% Triton X-100, 200 μg/mL RNase A, and 20 μg/mL PI in PBS, pH 7.4) for 15 min at 37 °C in dark. Cell cycle distribution was measured by a flow cytometer (BD Bioscience FACS Calibur) and data were analyzed by ModFit 1.2.

2.8. Apoptosis assay

MCF-7 cells were seeded in 6-well plates at a density of 5×10^4 cells/well and incubated for 24 h. Then, cells were treated with 15 μM cDDP and Pt(IV)-chalcone/LDH. After exposure for 48 h, cells were collected by trypsinization and washed with ice-cold PBS (1×) twice, followed by washing in Annexin-V binding buffer (1×) once. Then the cell pellets were resuspended in Annexin-V binding buffer at a density of 1×10^6 cells/mL. A volume of 0.1 mL cell suspension was transfer to a 1.5 mL tube and stained with Annexin V and PI following manufacturer's instructions. The apoptosis events were analyzed by a flow cytometer (BD Bioscience FACS Calibur).

2.9. Whole cell Pt uptake

MCF-7 cells were seeded in 100 mm dishes. When the cells reached 80% confluency, medium containing 10 μM cDDP or Pt(IV)-chalcone/LDH was added. After incubation for 8 h, cells were washed twice with 5 mL PBS (1×), followed by trypsinization. Cells were then collected and washed another twice with ice-cold PBS (1×). Then cells were resuspended in PBS (0.1 M phosphate, 0.15 M NaCl, pH 7.2). After counting, cells were transferred to a 1.5 mL tube and spun down at 600×g

for 5 min. The supernatant was removed and 65% HNO3 was added to digest the cells at 55 °C overnight. Pt level was determined by ICP-MS.

2.10. Pt distribution in membrane, cytoplasm, and nucleus

The extraction of cytoplasmic, nuclear, and membranous fractions of MCF-7 cells was performed using a NE-PER Nuclear and Cytoplasmic Extraction Kit according to manufacturer's instructions. Briefly, cells were treated with test agents and washed with PBS as described above. Then, 1×10^6 cells were transferred to a pre-chilled 1.5 mL tube for cytoplasm/nuclear extraction. Cells were then spun down at 500×g for 3 min at 4 °C. The supernatant was removed as completely as possible. A volume of 4 µL complete mini EDTA-free protease inhibitor cocktail solution was added to 200 µL CERI reagent, and 2 µL of cocktail solution was added to 100 µL NER reagent. The extraction was performed following the manufacturer's instructions. The Pt level was determined by ICP-MS, and the protein level was determined by a BCA assay. All steps were performed on ice.

2.11. Pt level on genomic DNA

MCF-7 cells were seeded in 100 mm dishes. When cells reached 80% confluency, 10 µM cDDP or Pt(IV)-chalcone/LDH was added. After exposure for 12 h, cells were washed twice with 5 mL PBS (1×) followed by trypsinization. Cells were then collected and washed twice with ice-cold PBS (1×). Cell pellets was lysed in 2 mL lysis buffer containing 100 mM Tris-HCl, 5 mM EDTA, 200 mM NaCl, 0.2% SDS, and 100 µg/mL proteinase K, pH 8.5 at 55 °C for overnight. The lysate was then extracted with 2 mL phenol/chloroform/isoamyl alcohol twice, followed by two extractions with chloroform. Genomic DNA was precipitated with 0.7× volume isopropanol and spun down at 16,000×g for 20 min. After washing twice with ethanol, DNA was dried in a speed vacuum and dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, 50 µg/mL RNase A, pH 8.0). DNA level was measured by Nanodrop Spectrophotometer (Thermo Scientific ND-1000) and Pt level was determined by ICP-MS.

2.12. Statistical analysis

Data are expressed as mean ± SD. Student's t-test and one-way ANOVA test were used for statistical analyses of the data. All statistical analyses were conducted using Excel. Cases in which p values of < 0.05 were considered statistically significant.

References

1 Z. P. Xu, G. Stevenson, C.-Q. Lu and G. Q. Lu, *J. Phys. Chem. B*, 2006, **110**, 16923.

2 K. R. Barnes, A. Kutikov and S. J. Lippard, *Chem. Biol.*, 2004, **11**, 557; F. Bois, A. Boumendjel, A.-M. Mariotte, G. Conseil and A. Di Petro, *Bioorg. Med. Chem.*, 1999, **7**, 2691.

Tables and Figures

Table S1. IC_{50} of complexes in different cell lines (μM)

Complexes	MCF-7	HeLa	A2780
chalcone	>25	>25	N.D.
DSCP	>50	N.D.	N.D.
Pt(IV)/LDH	1.9 ± 0.6	N.D.	0.34 ± 0.17
chalcone/LDH	108 ± 5	N.D.	N.D.
Pt(IV)-chalcone/LDH(Pt:chalcone=1:2)	0.7 ± 0.5	N.D.	N.D.
Pt(IV)-chalcone/LDH(Pt:chalcone=1:3)	0.2 ± 0.1	N.D.	N.D.
Pt(IV)-chalcone/LDH(Pt:chalcone=1:8)	0.9 ± 0.3	N.D.	N.D.
Pt(IV)-chalcone/LDH(Pt:chalcone=1:10)	0.3 ± 0.1	N.D.	N.D.
Pt(IV)-chalcone/LDH(Pt:chalcone=1:30)	0.2 ± 0.1	0.3 ± 0.03	N.D.
Pt(IV)-chalcone/LDH(Pt:chalcone=1:40)	0.08 ± 0.01	0.08 ± 0.01	N.D.

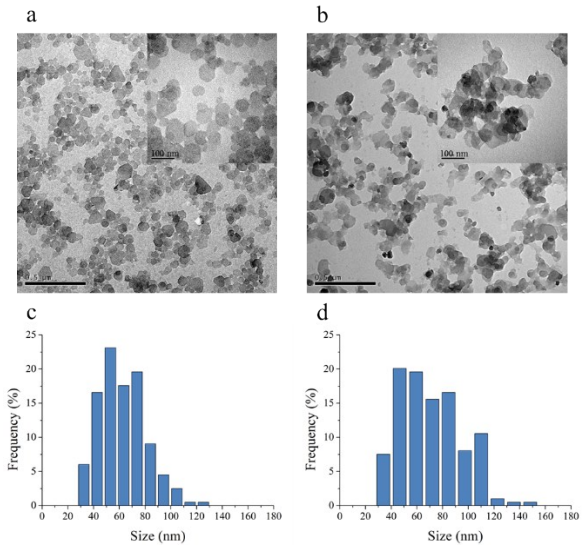

Figure S1. TEM image of LDH (a) and Pt(IV)-chalcone/LDH (b); Size distribution of (c) LDH and (d) Pt(IV)-chalcone/LDH.

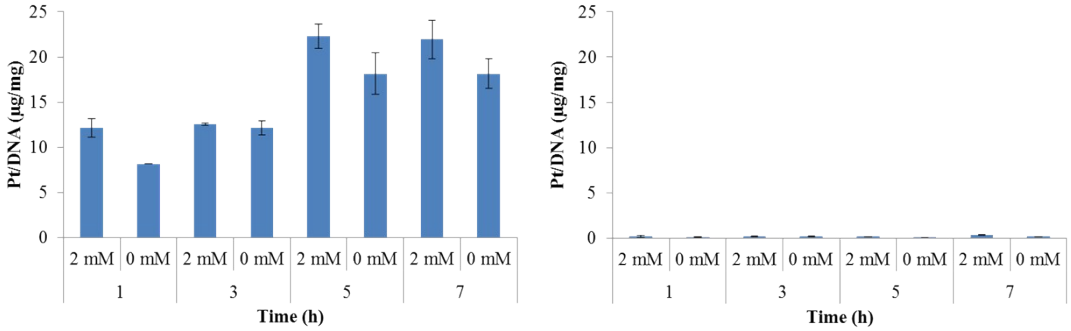

Figure S2. DNA binding ability of Pt in cDDP (left) and Pt(IV)/LDH (right) with or without 2 mM ascorbic acid.

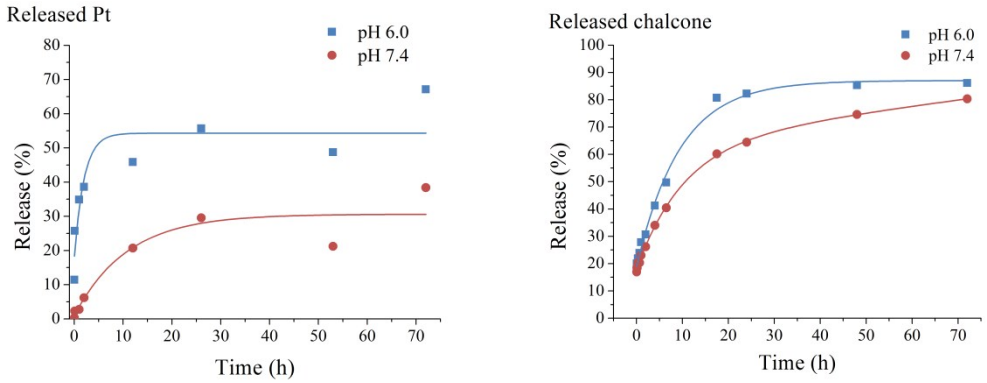

Figure S3. Release profile of Pt(IV)-chalcone/LDH at pH 6.0 and pH 7.4.

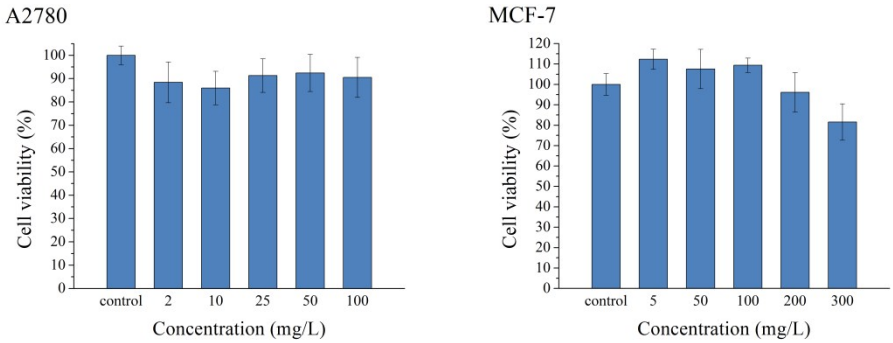

Figure S4. Cytotoxicity of LDH NPs in A2780 and MCF-7 cells at 72 h.

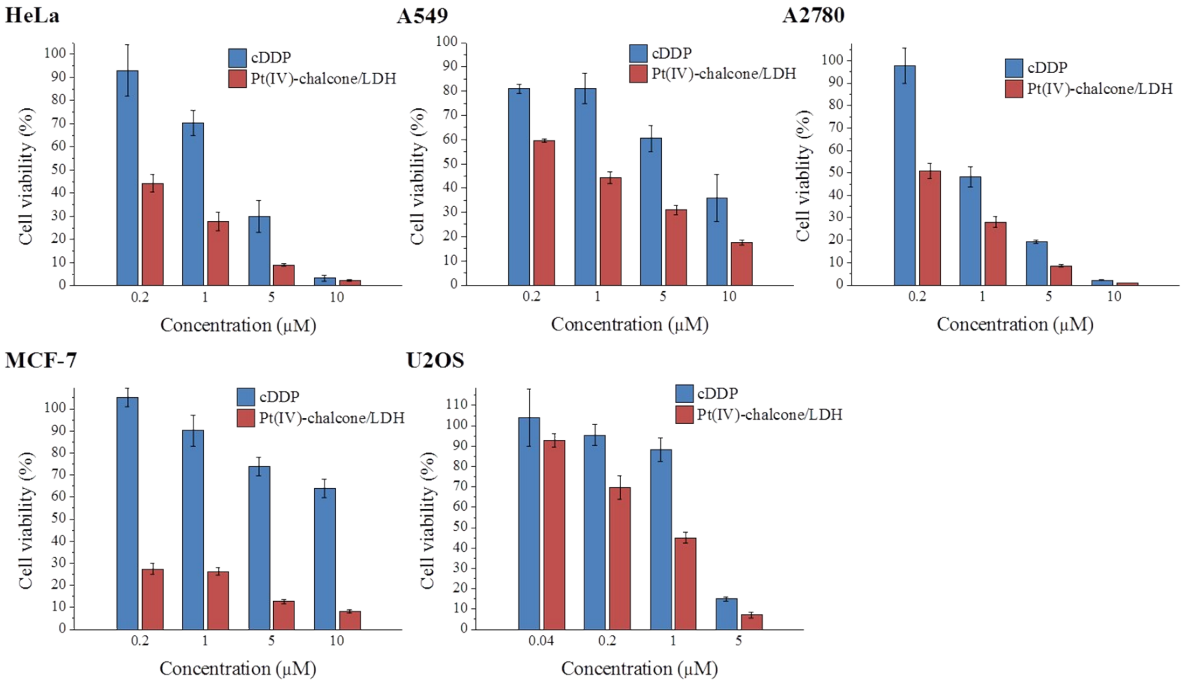

Figure S5. Cytotoxicity of cDDP and Pt(IV)-chalcone/LDH in different cell lines.

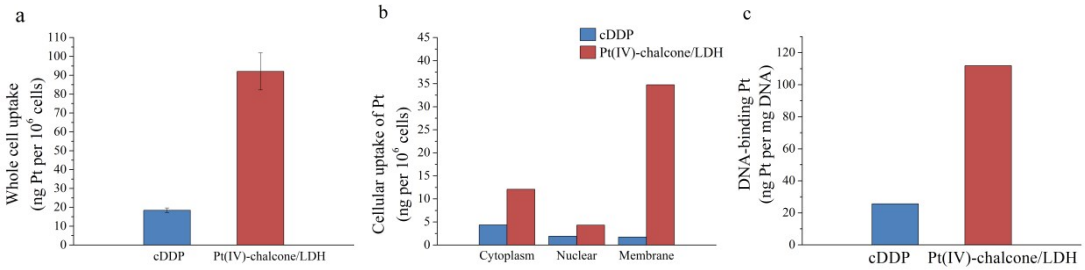

Figure S6. (a) Whole cell uptake of Pt in MCF-7 cells. (b) Cellular distribution of Pt for cDDP and Pt(IV)-chalcone/LDH in cytoplasm, nuclear, and membrane of MCF-7 cells. (c) the levels of Pt on the genomic DNA of MCF-7 cells.