Electronic Supplementary Information (ESI)

Nanoparticle delivery of a triple-action Pt(IV) prodrug to overcome cisplatin resistance *via* synergistic effect

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1. Experimental materials and instruments

Materials and Reagents

PEG-PLGA was purchased from Xi'an ruixi Biological Technology *Co.,Ltd*. (Xi'an, China). Cisplatin, artesunate, (3aR,4S,7R,7aS)-3a,7a-dimethylhexahydro-4,7-epoxyisobenzofuran-1,3-dione, glutathione (GSH), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) and sodium dodecyl sulfate (SDS) were purchased from Aladdin (Shanghai, China). Hexadecyl isocyanate was purchased from Sigma-Aldrich (China). Hydrogen peroxide, N, N-dimethylformamide (DMF), and acetonitrile were purchased from Beijing Chemical Works (Beijing, China). 2-(4-amidinophenyl)-1H-indole-6-carboxamidine (DAPI) was purchased from Sigma-Aldrich (Shanghai, China). Annexin-V-FITC apoptosis detection kit was purchased from Solarbio (Beijing, China). DMEM medium, RPMI 1640 medium, fetal bovine serum (FBS), penicillin-streptomycin solution and trypsin were purchased from Gibco (Gran Island, NY, USA). Cell culture vessels were purchased from Corning (Corning, NY, USA). All the other solvents and reagents were sourced from Aladdin (Shanghai, P. R. China) and used as received.

Instruments

Dynamic light scattering (DLS) was performed by Malvern Zetasizer NanoZS90. Flow cytometry was conducted by Cytomics FC500 Flow Cytometry (Beckman Coulter *Ltd.*). Confocal laser scanning microscopy (CLSM) was performed with ZEISS LSM880. ^{1}H NMR spectra was measured by a 400 MHz NMR spectrometer (Bruker, USA). Inductively Coupled Plasma Mass Spectrometer (ICP-MS, Agilent technologies 7700 series, USA) was used for quantitative analysis of the total platinum (Pt). The morphology and size were measured by transmission electron microscope (TEM) (Titan G260-300, USA).

Synthesis of CisPt(IV)-OH

Pt(IV)-OH was synthesized as previously described.[1]

Synthesis of *CanPt(IV)*

CisPt(IV)-OH (1.2 mmol) was suspended in 200 mL anhydrous DMF. Then Artesunate anhydride (1.2 mmol) was added to the above mixture, which was stirred at room temperature for 48 h. The filtrate was evaporated under reduced pressure. The crude product was dispersed in methanol precipitated in ether, then dried under the vacuum to obtain CanPt(IV).

Synthesis of TriPt

CanPt(IV) (0.46 mmol) was suspended in 10 mL anhydrous DMF. Then (3aR,4S,7R,7aS)-3a,7a-dimethylhexahydro-4,7-epoxyisobenzofuran-1,3-dione (0.46 mmol) was added. The reaction mixture was stirred overnight at 75 ^{o}C until the solution became clear. The solvent was removed under reduced pressure. Art-CanPt(IV) was purified by column chromatography to give a solid.

Preparation process of nanoparticles

As the following description, TriPt (1.5 mg) and mPEG-*b*-PLGA (6 mg) were dissolved in DMF (1 mL) and H_2O (1 mL) respectively, mixed and stirred for 15 min, and then the de-ionized water (10 mL) was added dropwise to the mixture subsequently. Finally, the mixture was collected and dialyzed against a dialysis bag (MWCO: 3500 Da) for 48 h, and the supernatant was collected by centrifugation.

Cell lines and cell incubation conditions

7404, 7404DDP (cisplatin resistant), A2780, and A2780DDP (cisplatin resistant) cells were used in the following experiments. Culture medium were supplemented with 10% (v/v) fetal bovine serum, 1% penicillin and streptomycin. The cell lines were cultured in 37 °C with 5% (v/v) CO_2 atmosphere.

Confocal laser confocal scanning microscopy (CLSM)

The glass slides were used to put on the bottom of the twenty four-well plates in advance. 1 mL 7404 and 7404DDP cells at a density of 5×10^4 were added to each well

and incubated in 37 °C overnight. Then NP@Cy5.5 was added to each well at a final concentration of 100 ng/ml of Cy5.5 for 1, 4, and 7 hr. Then the cell culture slides were washed with PBS for three times, the cells were fixed with paraformaldehyde and the nuclei were stained with DAPI. At last, images were performed with CLSM.

Intracellular uptake studies of flow cytometry

7404DDP cells were seeded in six-well plates at a density of 3×10^5 per well and then incubated at 37 °C overnight. Subsequently, the cells were treated with NP@Cy5.5 at a final concentration of 100 ng/ml of Cy5.5 for 1, 4 and 7 hr. Finally, the cells were harvested to test by flow cytometry.

Platinum uptake in the cells

7404 and 7404DDP cells were seeded in six-well plates at a density of 1×10^6 per well and then incubated at 37 °C overnight. CisPt, TriPt and TriPt NPs were added to each well at a final concentration of 10 μM of Pt. The cells were collected after treating with drugs for 1, 4, and 7 hr. ICP-MS was performed to detect the Pt concentrations.

In vitro evaluation of ROS generation

7404DDP cells were seeded on 24 wells plate with cell slides covered and 12 wells plate at a density of 3×10^5 and 5×10^4 respectively. Cells were treated at the Pt concentration of 50 μM for 4 hr before adding the DCFH-DA probing. The samples were detected by CLSM and flow cytometry, respectively.

Cell relative viability studies

MTT assay was used to examine the relatively cell viability. 7404, 7404DDP, A2780, and A2780DDP cells were seeded in 96-well plates at a density of 5×10^3 per well and then incubated at 37 °C overnight, and they were treated with different drugs for 48 h, respectively. Then, 0.5 mg/ml MTT diluted with DMEM was added into each well. After incubation in 37 °C for 4 hr, 10% SDS was added to each well and incubated for 12 hr in the dark. The results were tested by Molecular Devices (SpectraMax). Cell

viability was expressed as the ratio of the absorbance of the test wells and control wells.

3D multicellular tumor spheroids (MCS) culture

50 μL agarose solution（1%, w/v）was added to each well of a 96-well plate. UV irradiation after planking for 30 minutes to sterilize. 1600 cells (200 μL) were added to each well and cultured for seven days for follow-up tests.

Colony formation assay

7404DDP cells (1.5×10^3 cells/well) were treated with various drug combinations for 24 h and at a fixed drug concentration; PBS served as a control treatment and was incubated with the cells at equal volumes. Following the 7 days of incubation with each experimental group, tumor cell colonies were fixed and stained with 0.5% crystal violet PBS solution and photographs were taken under illumination.

Apoptosis studies

7404DDP cells were seeded in twelve-well plates at a density of 3×10^5 per well and then incubated at 37 °C overnight. CisPt, CTD, CisPt+CTD+ART, TriPt and TriPt NPs were added to each well at the final concentration of 10 μM of drug. The media was removed after incubation for 24 h, then the cells were washed thrice with cold PBS. Then the cells were harvested and strained with Annexin V-FITC/PE Apoptosis Detection Kit for 10 min in the dark at room temperature. Finally, all the samples were detected by flow cytometry.

In vivo anticancer treatment

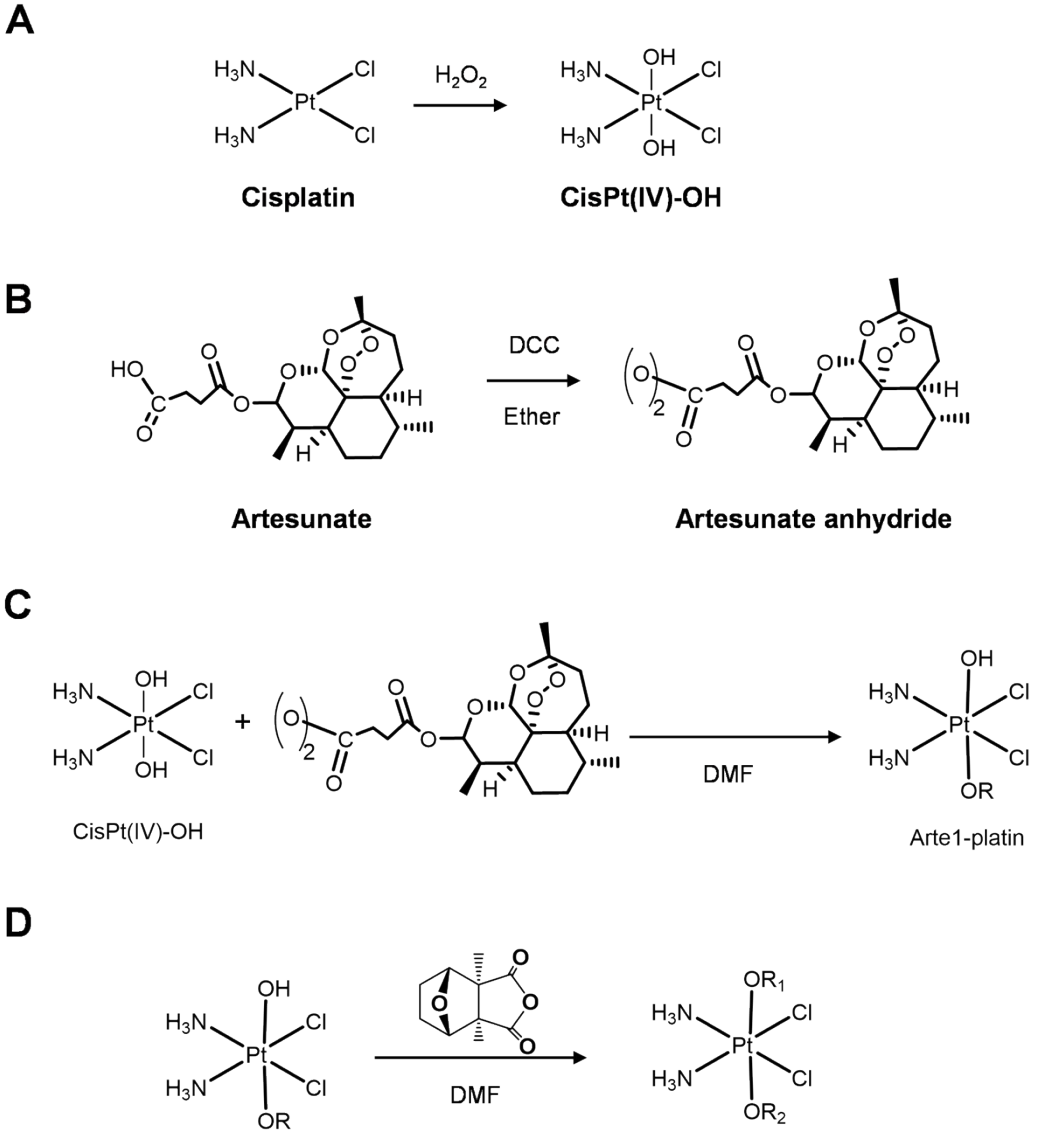
Female BALB/c nude mice were purchased from SPF Biotechnology (Beijing, China). 5×10^6 7404DDP cells were subcutaneous injected for tumor establishment. The drugs were *i.v.* injected at a dose of 3 mg Pt / kg body weight for three times. The tumor size was recorded every two days. Tumor suppression rate was calculated by the ratio to saline group. Tumor volume was calculated by the following formula:

$$v = \frac{1}{2} \times length \times width^2$$

References

1 T. Johnstone and S. Lippard, *J. Biol. Inorg. Chem.*, 2014, **19**, 667.

2. Supplementary figures



Scheme S1. Synthetic route of TriPt.

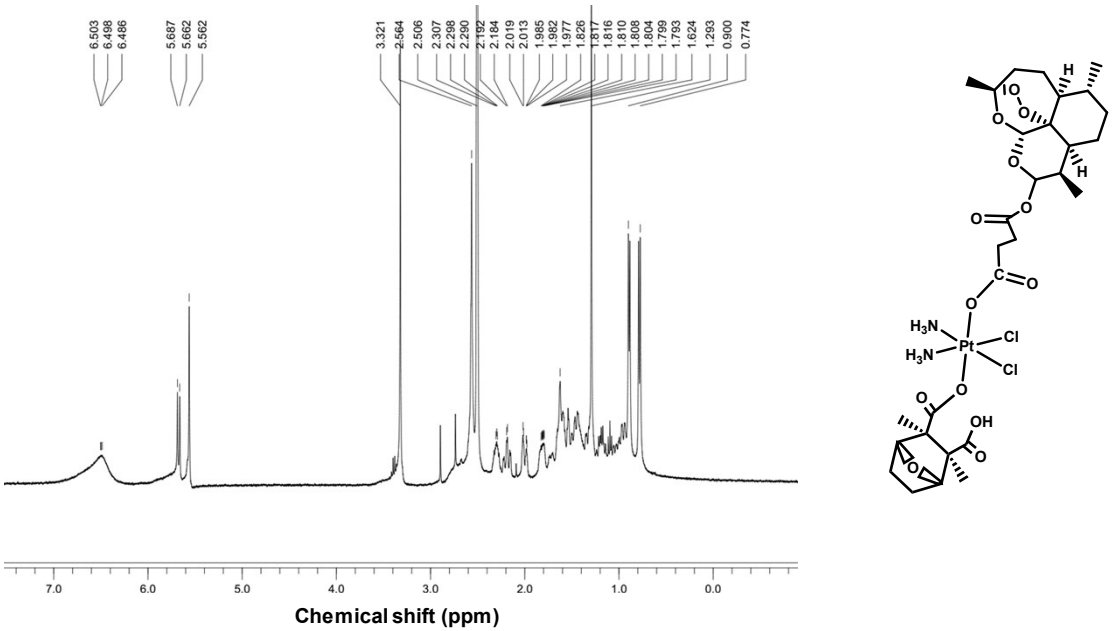


Figure S1. ^{1}H NMR spectrum of TriPt in DMSO-*d6*.

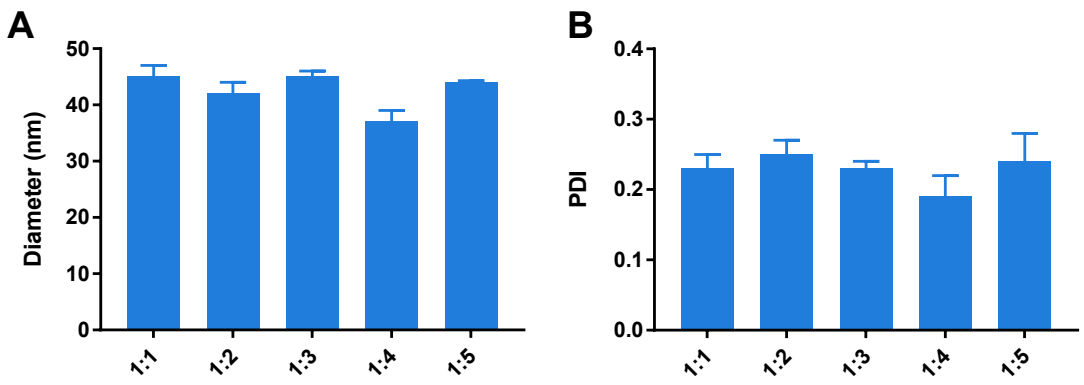


Figure S2. Formulation optimization of the nanoparticles. Diameter (A) and PDI (B) were shown according to various Pt to polymer mass ratios.

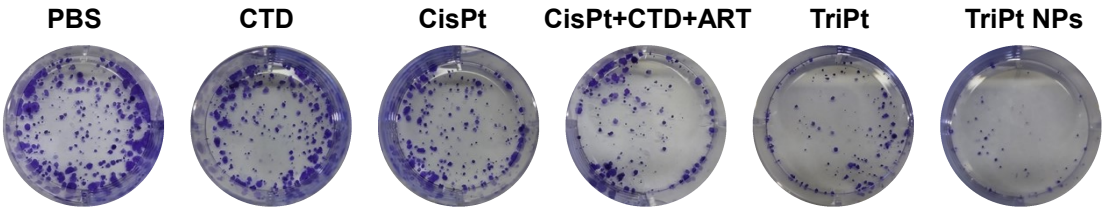


Figure S3. Colony formation assay for proliferation performed on 7404DDP cells after various treatments. 7404DDP cells (2×10^3 cells/well) were treated with a fixed drug concentration (0.1 μM); PBS served as control treatments. Following the 7 days of incubation with each treatment group, tumor cell colonies were fixed and stained with 0.5% crystal violet PBS solution and photographs were taken.

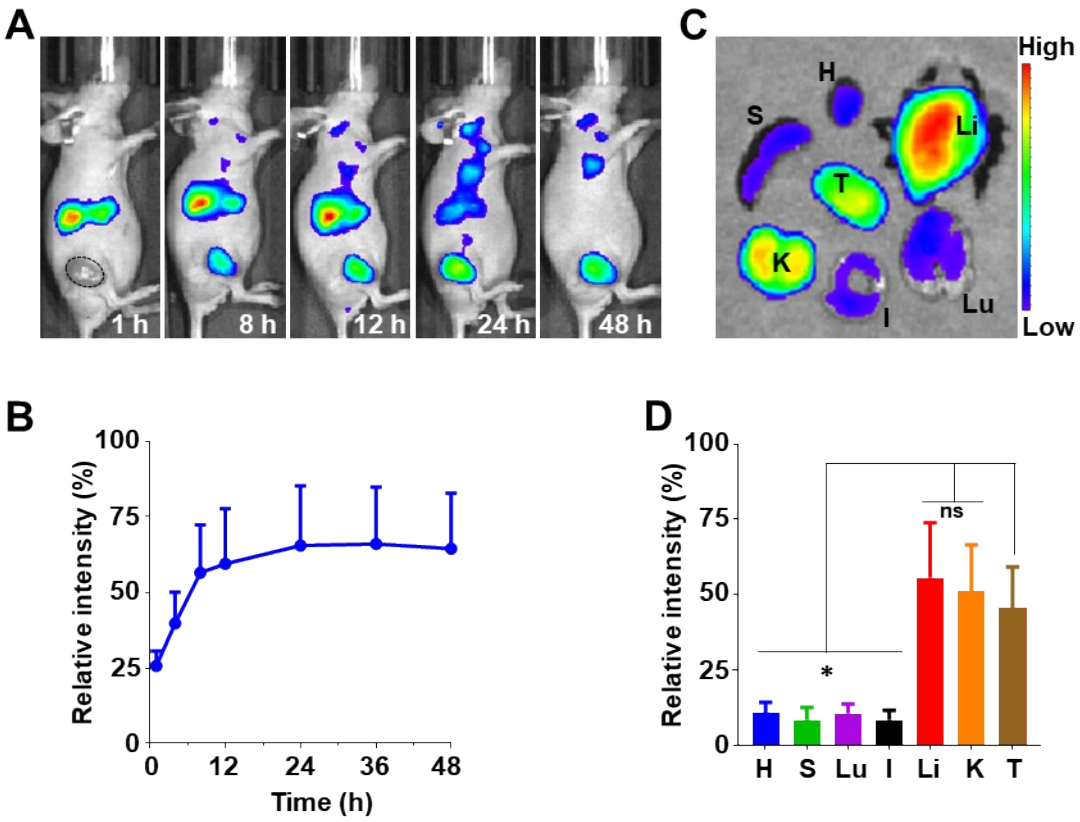


Figure S4. The biodistribution of Cy7.5-labeled NPs. (A) The *in vivo* fluorescence imaging and (B) fluorescent quantification of Cy7.5-labeled NPs in 7404DDP-bearing mice. (C) The *ex vivo* fluorescence imaging and (D) fluorescent quantitation of the major organs and tumor. Abbreviation: H, heart; Li, liver; S, spleen; Lu, lung; K, kidney; I, intestine; T, tumor.

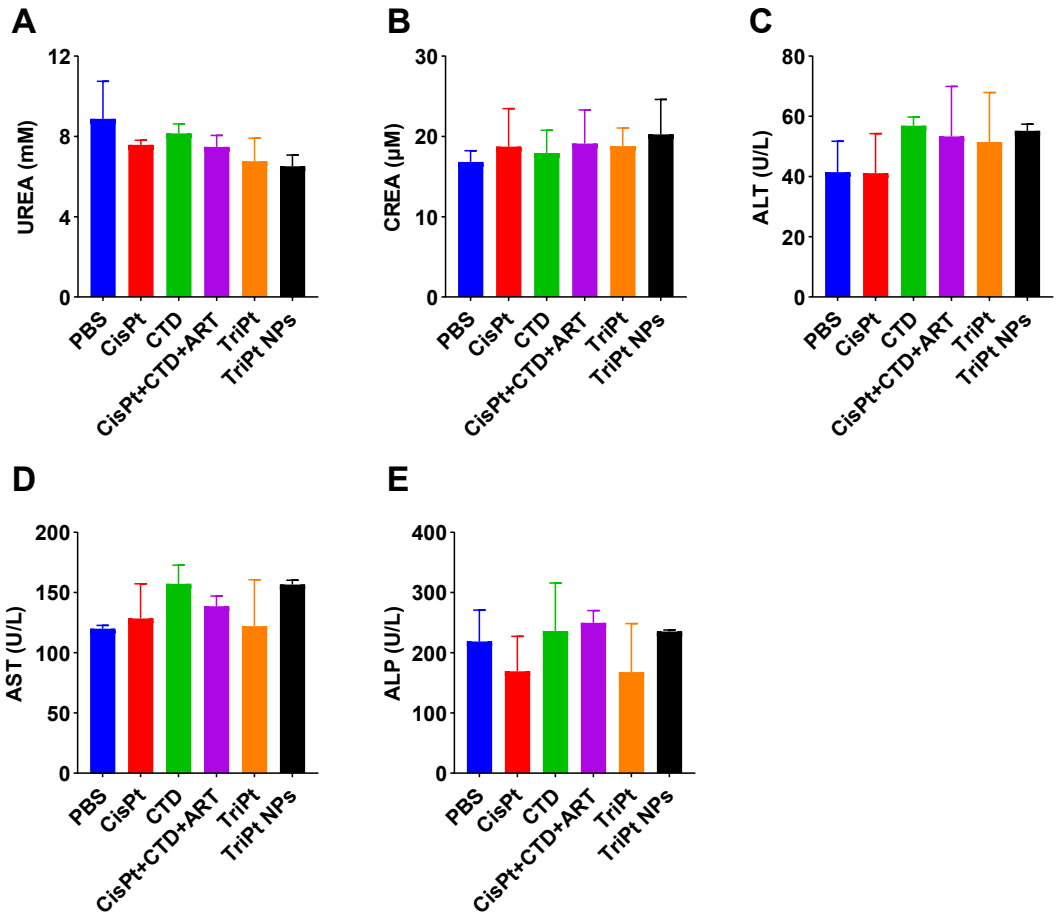


Figure S5. The blood biochemical index after different treatments. (A) UREA. (B) Creatinine (CREA). (C) Alanine aminotransferase (ALT). (D) Aspartate aminotransferase (AST). (E) Alkaline phosphatase (ALP).

Table S1. IC_{50} (μM) values of CisPt, CTD, CisPt+CTD+ART, TriPt and TriPt NPs on different cell lines.

Cell lines	A2780	A2780DDP	7404	7404DDP
CisPt	4.338	6.145	5.364	>20
CTD	4.564	5.981	7.34	5.867
CisPt+CTD+ART	0.6097	1.616	1.842	4.909
TriPt	0.7021	3.017	1.318	4.777
TriPt NPs	0.3216	1.893	0.6448	2.385