Electronic Supplementary Information

A dual-targeting hybrid platinum(IV) prodrug for enhancing efficacy

Haihua Xiao[a,b], Lesan Yan[a,b], Yu Zhang [c], Ruogu Qi[a,b] ,Wenliang Li[a,b], Rui Wang[a,b], Shi Liu[a,b], Yubin Huang[a], Yuxin Li[c]**, Xiabin Jing[a]*

[a] State Key Laboratory of Polymer Physics and Chemistry, Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, Changchun 130022, China

[b] Graduate School of Chinese Academy of Sciences, Beijing 100049, China

[c] *National Engineering Laboratory for Druggable Gene and Protein Screening, Northeast Normal University, Changchun 130024, People's Republic of China.*

Materials and Methods

Materials

Methoxyl-poly(ethylene glycol)-block-poly(ε-caprolactone)-block-poly(L-lysine) (MPEG-b-PCL-b-PLL, P) was synthesized as described in our previously published paper[1]. N-hydroxysuccinimide (NHS), 1-ethyl-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC·HCl), succinic anhydride and sodium dichloroacetate were purchased from Sigma-Aldrich. Cisplatin (purity 99%) was bought from Shandong Boyuan Pharmaceutical Co. Ltd., China. All other chemicals and solvents were used without further purification.

General Measurements

^{1}H NMR spectra were measured by a Unity-300MHz NMR spectrometer (Bruker) at room temperature. Mass Spectroscopy (ESI-MS) measurements were performed on a Quattro Premier XE system (Waters) equipped with an electrospray interface (ESI). Matrix-assisted laser-desorption ionization and time-of-flight mass spectroscopy (MALDI-TOF-MS, Waters, USA) was used to study the dialysate of P1 in the presence of 5'-GMP and sodium ascorbate. Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES, iCAP 6300, Thermoscientific, USA) was used to determine the total platinum contents in M(P1) and samples obtained outside of the dialysis bags in drug release experiments. Size and size distribution of micelles were determined by DLS with a vertically polarized He-Ne laser (DAWN EOS, Wyatt Technology, USA). The morphology of M(P1) was measured by TEM performed on a JEOL JEM-1011 electron microscope. Particle size and zeta potential measurements were conducted on a Malvern Zetasizer Nano ZS.

Synthesis of cisPt(IV)-OH and cisPt(IV)-COOH

CisPt(IV)-OH and cisPt(IV)-COOH were prepared as previously described in our published paper[1]

Synthesis of DCA-cisPt(IV)-COOH

DCA-cisPt(IV)-COOH was synthesized as depicted in Scheme 1(a). cisPt(IV)-COOH (0.434 g, 1 mM) was reacted with 1N DCA anhydride at 30 °C in 20 ml dried DMSO for 24 h to form a clear yellow solution. Then the solution was poured in to a large excess of diethyl ether and cooled down to remove the DMSO. This procedure was repeated for several times. Then the solid residues were dissolved in 5 ml methanol and precipitated with diethyl ether to get a yellow powder (0.3 g) to obtain DCA-cisPt(IV)-COOH. Its ESI spectrum was shown in Fig. S1.

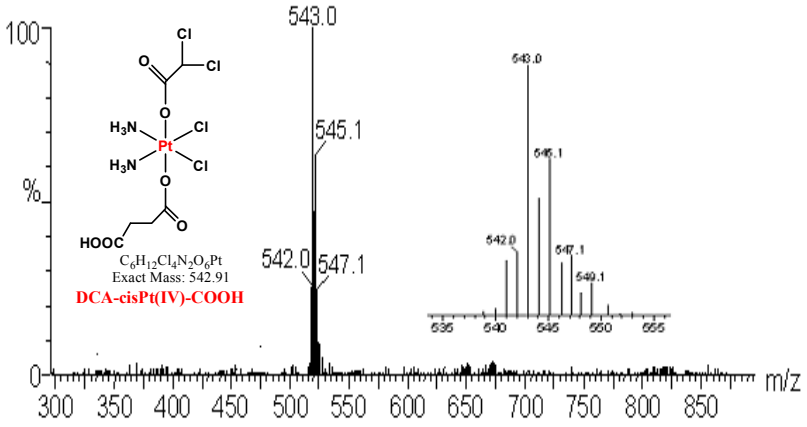


Fig. S1. Experimental ESI-MS spectra of DCA-cisPt(IV)-COOH and the calculated isotopic pattern for DCA-cisPt(IV)-COOH (inset).

^{1}H NMR Spectra of cisPt(IV)-OH, cisPt(IV)-COOH and DCA-cisPt(IV)-COOH

The Pt(IV) drugs cisPt(IV)-OH, cisPt(IV)-COOH were prepared and their characterization by ^{1}H NMR was previously described in our published paper[1]. The ^{1}H NMR of cisPt(IV)-OH, cisPt(IV)-COOH in DMSO-d_6 are shown in Fig. S2(A) and Fig. S2(B), respectively. CisPt(IV)-OH has the characteristic peak of NH_3 at 5.5 ppm. After it is reacted with succinic anhydride to form cisPt(IV)-COOH, the chemical shift of NH_3 moves to 6.0 ppm and a new chemical shift of CH_2 of succinic anhydride residue appears at 2.25–2.45 ppm, supporting the molecular formula of cisPt(IV)-COOH. The ^{1}H NMR of DCA-cisPt(IV)-COOH in DMSO-d_6 is shown in Fig. S2(C), attaching DCA to the Pt atom makes the chemical shift of protons in –NH_3 ligand of DCA-cisPt(IV)-COOH move to 6.5 ppm. Moreover, there is typical chemical shift at 7.8 ppm of protons in DCA (-OC-C**H**Cl_2) as well as typical chemical shift at 2.45 ppm of succinic acid (-C**H**$_2$-C**H**$_2$-COOH), denoting the co-existence of DCA and succinic acid in DCA-cisPt(IV)-COOH. The above results demonstrated that

DCA-cisPt(IV)-COOH was successfully synthesized.

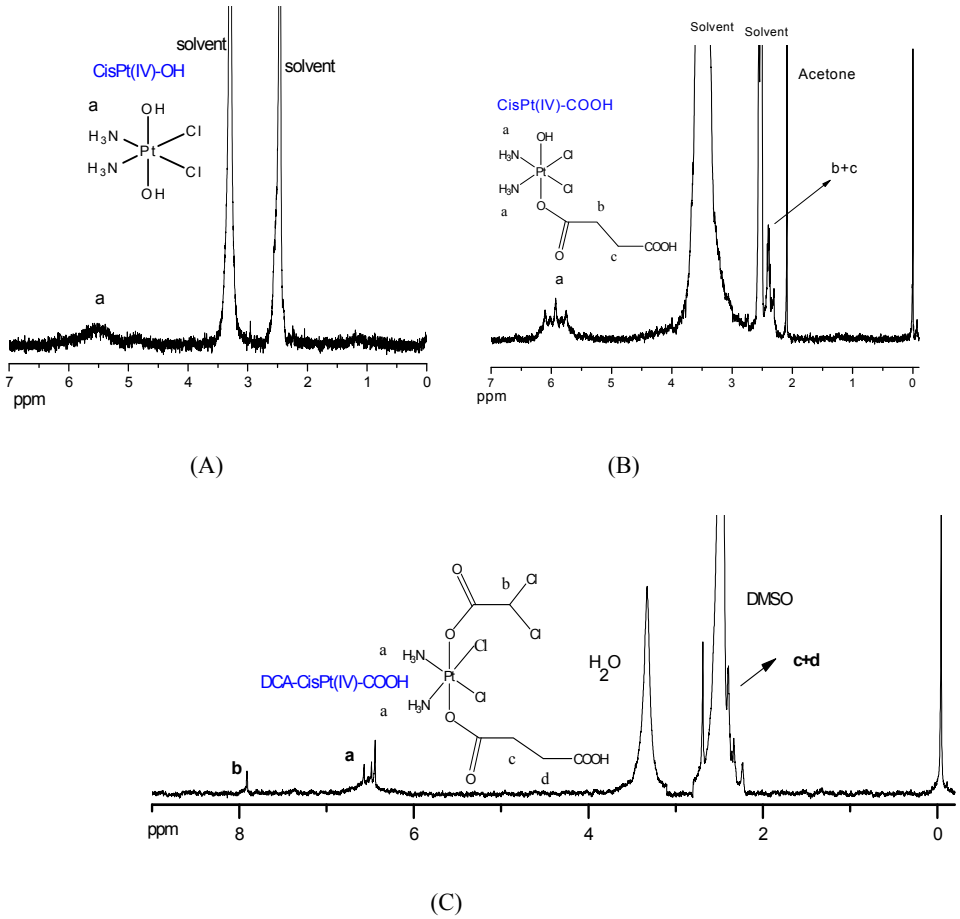


Fig. S2. ^{1}H NMR spectra of cisPt(IV)-OH (A), cisPt(IV)-COOH (B) and DCA-cisPt(IV)-COOH (C) in DMSO-d_6.

FTIR Spectra of Cisplatin, cisPt(IV)-OH, cisPt(IV)-COOH and DCA-cisPt(IV)-COOH

In Fig. S3 are collected FTIR spectra of Cisplatin, cisPt(IV)-OH, cisPt(IV)-COOH and DCA-cisPt(IV)-COOH. CisPt(IV)-OH and cisPt(IV)-COOH were prepared as previously described and their characterization was detailed in our published paper[1]. Briefly, cisPt(IV)-OH, which is obtained by oxidizing cisplatin with H_2O_2, displays a sharp and intense peak at 3460 cm^{-1} (OH stretching) and a new Pt-OH stretch at 540 cm^{-1}, respectively, compared with cisplatin. After reacting with succinic anhydride, the 3460 cm^{-1} band is weakened, and there appear two peaks (1702 cm^{-1} and 1645 cm^{-1}) characteristic of the coordinated carboxyl group (1645 cm^{-1}) and free carboxyl group (1702 cm^{-1}) in cisPt(IV)-COOH, respectively, confirming the structure of cisPt(IV)-COOH. The observed absorption at 1645 cm^{-1} of the coordinated carboxylate is in good agreement with previously published data for analogous complexes (1633-1669 cm^{-1}) [2]. Comparison of the three FTIR spectra of cisplatin, cisPt(IV)-OH and cisPt(IV)-COOH indicates that cisPt(IV)-OH has reacted with succinic anhydride to afford cisPt(IV)-COOH. Thereafter, cisPt(IV)-COOH was used to react with dichloroacetic anhydride

to afford DCA-cisPt(IV)-COOH with an axial DCA and a succinic acid. Because there is no Pt-OH in DCA-cisPt(IV)-COOH, the band of Pt-OH stretching at 540 cm^{-1} disappears. Moreover, due to the deficiency of -OH in DCA-cisPt(IV)-COOH, the band at 3460 cm^{-1} (OH stretching) does not appear. The above IR results proved that DCA-cisPt(IV)-COOH is successfully synthesized.

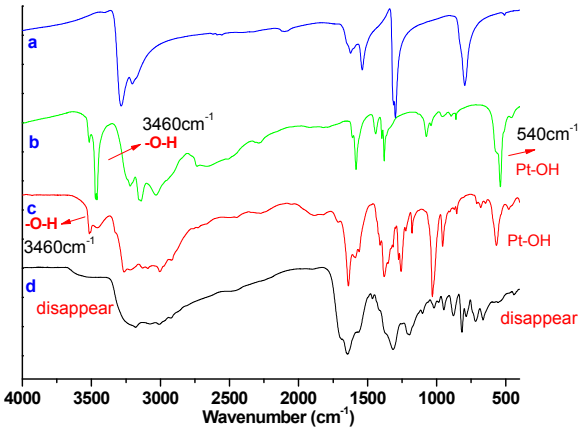


Fig. S3. IR spectra of cisplatin (a), cisPt(IV)-OH (b), cisPt(IV)-COOH (c) and DCA-cisPt(IV)-COOH (d).

Preparation of P1

DCA-cisPt(IV)-COOH was conjugated to the polymer MPEG-b-PCL-b-PLL using EDC/NHS method in aqueous solution to prepare P1. Briefly, EDC·HCl (0.191 g, 1 mmol) and NHS (0.115 g, 1 mmol) were dissolved in de-ionized water under stirring. DCA-cisPt(IV)-COOH (0.49 g, 0.8 mmol) was added into the aqueous solution. After the mixture became clear, 0.5 g of MPEG-b-PCL-b-PLL with pendant amino groups in 100 ml water was added and the reaction mixture was kept stirring at room temperature for 24 hours, then it was dialyzed against water for 12 hours and lyophilized to obtain P1. After that, 1 mg P1 was dissolved in 10 ml water and then the aqueous solution of P1 was subjected to ICP-OES measurement to determine the Pt content in P1.

Preparation of M(P1)

The micelles of P1 were defined as M(P1). They were prepared by nano-precipitation method. In brief, 50 mg P1 conjugate was dissolved in 5 ml DMF in a flask, and then 50 ml water was added drop-wise into the flask under stirring to form a micellar solution. The solution was dialyzed against water to remove DMF and then freeze-dried.

Drug Release from M(P1)

10 mg of M(P1) (Pt content: 14.1%w/w) was dissolved in 5 ml of phosphate buffered saline (PBS, 0.1 M, pH=7.4). The

solution was then placed into a pre-swelled dialysis bag (molecular weight cutoff of 3.5 kDa) and immersed into 140 ml of 0.1 mol/L PBS (pH=7.4). The dialysis was conducted at 37 °C in a shaking culture incubator. 1.5 milliliters of sample was withdrawn from the incubation medium at specified time intervals and measured for Pt concentration by ICP-OES. After sampling, equal volume of fresh PBS was immediately added into the incubation medium. The concentration of platinum released from the micelles was expressed as a percentage of the total platinum in the micelles. The same drug release procedure was performed in the presence of 5 mM sodium ascorbate.

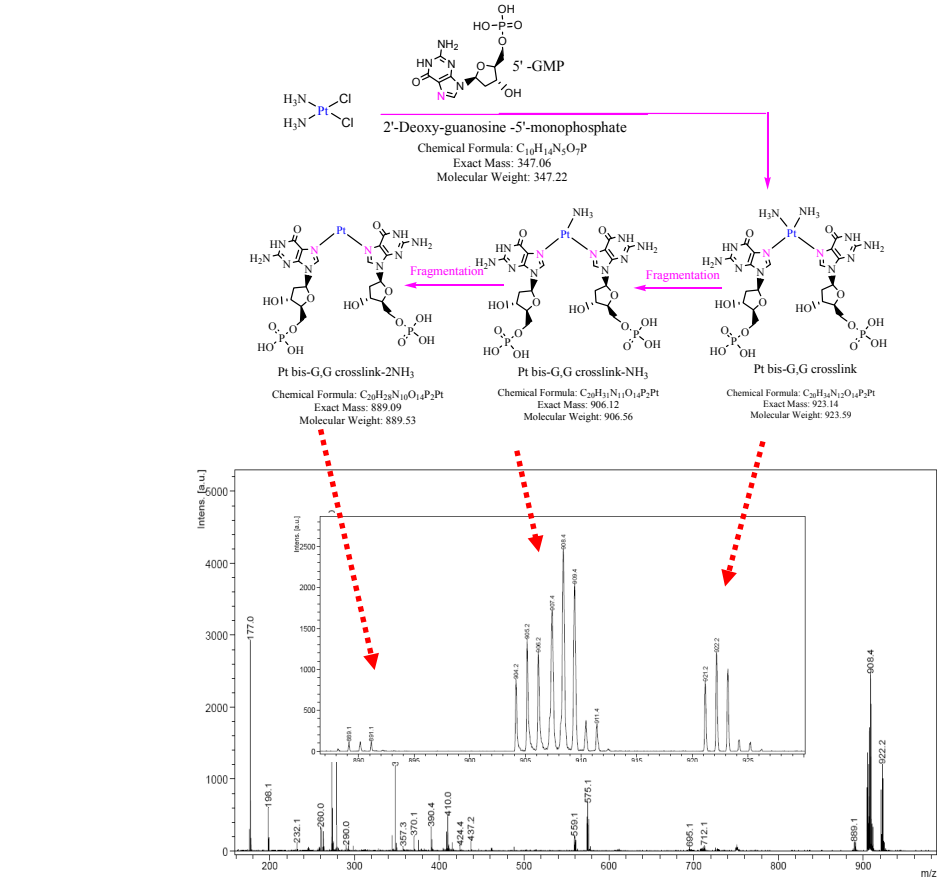
Possible released Pt species and chelation with 5'-GMP studied by MALDI-TOF-MS

50 mg M(P1) was dissolved in 5 ml of freshly prepared sodium ascorbate aqueous solution (5 mM, prepared by simply dissolving sodium ascorbate in de-ionized water). Then the solution (5 ml) was totally transferred to a dialysis bag (molecular cut-off, 1000), which was sealed firmly. The sealed dialysis bag was washed several times to remove any residual M(P1) on the surface of dialysis bag during the process and then the dialysis-bag-sealed M(P1) was incubated in another 5 ml sodium ascorbate solution (5 mM) containing 5'-GMP (5 mM) at 37 °C for 12 h. The dialysate was collected for MALDI-TOF-MS study.

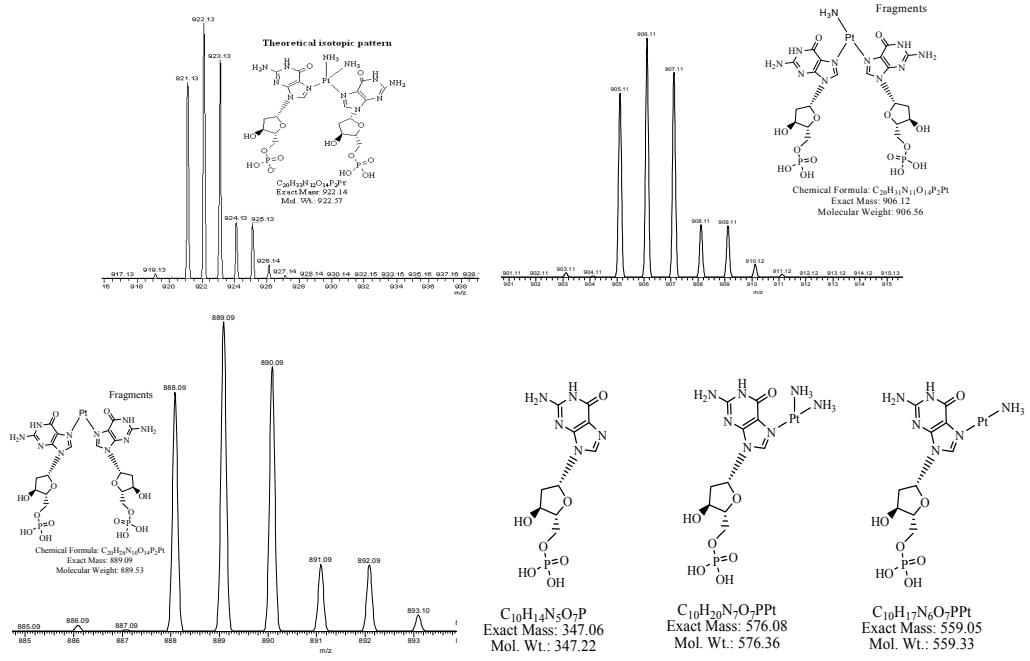
Cisplatin chelation with 5'-GMP

As for the chelation of cisplatin with 5'-GMP, general procedures were below: a solution of cisplatin plus 5'-GMP at final concentration of 1 mM and 5 mM, respectively, was prepared and incubated at 37 °C for 12 h. The solution was then collected directly and diluted to suitable concentration for MALDI-TOF-MS study.

The mechanistic pathway by which platinum drugs act as a chemotherapeutic agent involves the formation of adducts with DNA nucleobases. The preferred binding site with Pt(II) drugs such as cisplatin is N7 of guanine and the most probable cross-link formed is that between adjacent guanine units in the same DNA strand. Thus, 5'-GMP is often used to simulate the interaction of Pt(II) drugs with DNA in vitro and is chosen here. The possible chelation process of cisplatin with 5'-GMP and possible fragmentation in MALDI-TOF-MS are shown in Fig. S4(A). To be specific, cisplatin was hydrolyzed first and then chelated with 5'-GMP to form $Pt(NH_3)_2$-bis-GMP adducts (m/z=922). Fragmentation of this adduct by loss of one or two NH_3 groups leads to $Pt(NH_3)$-bis-GMP (m/z =906) and Pt-bis-GMP (m/z=889) (Fig. S4(A)). The above analysis indicated that cisplatin can chelate with 5'-GMP efficiently. Other possible peaks were also proposed in Fig. S4(B).



(A)



(B)

Fig. S4. MALDI-TOF-MS spectrum of the products between cisplatin and 5'-GMP and expanded view from m/z=886 to m/z=930 (insets) (A) and possible Pt species and theoretical simulations (B).

MTT (3-(4,5-dmethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) Assay

SKOV-3 cells were purchased from Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, Shanghai, China, and grown in RPMI 1640 (Life Technologies) supplemented with 10% fetal bovine serum, 0.03% L-glutamine and 1% penicillin/streptomycin in 5% CO_2 at 37 °C. SKOV-3 cells harvested in a logarithmic growth phase were seeded in 96-well plates at a density of 10^5 cells/well and incubated in RPMI 1640 for 24 h. The medium was then replaced by cisplatin, DCA-cisPt(IV)-COOH, M(P1) solutions. Moreover, DCA and cisPt(IV)-COOH were used as controls. All the drugs containing Pt were set at a final equivalent Pt concentration from 0.2 to 108 μM; the concentration gradient of DCA was set twice as much as Pt (ranging from 0.4 to 216 μM). As the free polymer MPEG-b-PCL-b-PLL was proved safe in our previous published works [1], there is no need to test its biocompatibility again. The incubation time for all the drugs was 48 h. After incubation, 20 μL of MTT solution in PBS with the concentration of 5 mg/mL was added and the plates were incubated for another 4 h at 37 °C, followed by removal of the culture medium containing MTT and addition of 150 μL of DMSO to each well to dissolve the formazan crystals formed. Finally, the plates were shaken for 10 minutes, and the absorbance of formazan product was measured at 492 nm by a microplate reader.

Quantitative determination of platinum contents in the cancer cells

SKOV-3 cells were seeded in 6-well plates at a density of 10^6 cells per well. At their logarithmic phase of growth, the cells were treated with cisplatin, DCA-cisPt(IV)-COOH and M(P1) with the final platinum concentration in the culture medium regulated to 5 μM and incubated at 37 °C for 2 h. As previously described [1,3,4], to remove surface-bound drugs, cells were washed three times with ice-cold PBS, incubated with 1.5 mL of 0.15 M sodium chloride (pH 3.0 was adjusted by acetic acid) for 3 min at 4 °C, then rinsed with 2 mL of cold PBS, harvested by scraping in ice-cold PBS, centrifuged. Thereafter, the cell pellet were lysed by adding 200 μL cell lysis buffer (Promega lysis buffer) and then the cell lysis solution was freezed at −20°C for 20 min and thawed at room temperature. 100 μL of the cell lysis solution for each sample was used directly to measure the Pt content by ICP-MS. The other 100 μL of the cell lysis solution was used to determine the protein content in each cell sample by using bicinchoninic acid (BCA) protein assay kit (Beyotime Institute of Biotechnology, Shanghai, China) according to previously published data[1,3,4,5]. The platinum content was expressed as nano-grams of Pt per milligram of total proteins. Results were shown in Fig. S5.

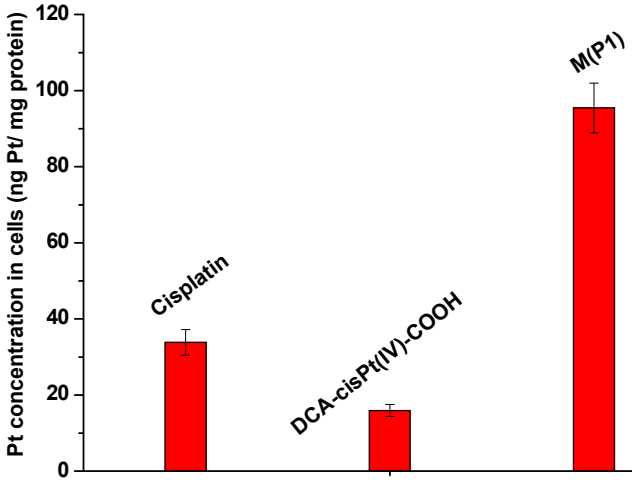
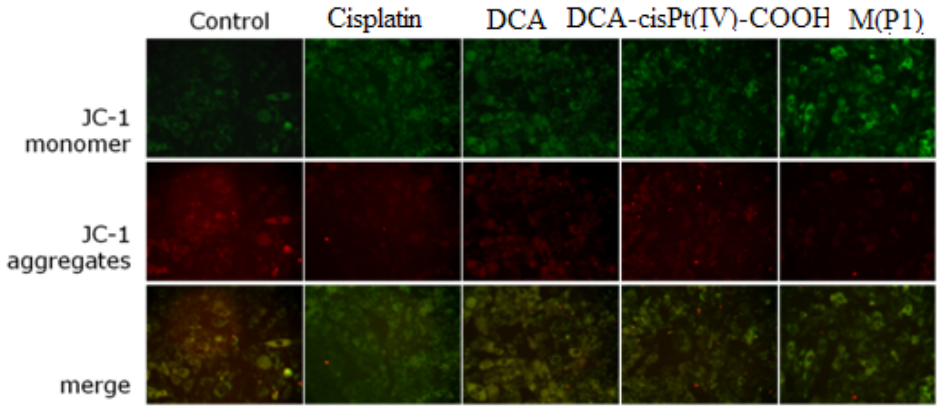


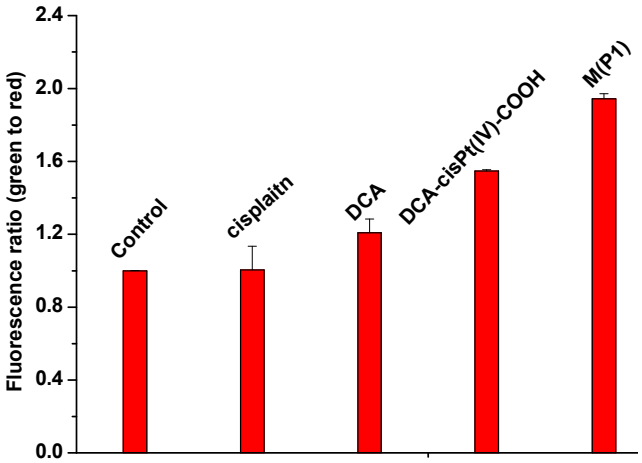
Fig. S5. Cellular uptake of various drugs by SKOV-3 cancer cells at 2 h determined by ICP-MS. The initial platinum concentration of all drugs was 5 μM for all drugs, and cellular uptake was expressed in the unit of (ng Pt/mg proteins).

Mitochondrial membrane potential (MMP) analysis

JC-1 (Beyotime Biotech, China) is cationic dye that exhibits potential-dependent accumulation in mitochondria by fluorescence emission shift from green (~520 nm) to red (~590 nm). Green fluorescence of JC-1 monomers is presented in the cell areas with high mitochondrial membrane potential, while red fluorescence of JC-1 aggregate is prevalent in the cell areas with low mitochondrial membrane potential. Consequently, mitochondrial depolarization is indicated by a increase in the green to red fluorescence intensity ratio. SKOV-3 cells were seeded into black 96-well microplates. After 24 h, drugs (cisplatin, DCA-cisPt(IV)-COOH and M(P1) at 50μM and DCA at 100μM) were added to the wells for 1 h incubation, respectively, and cells without treatment of drugs were used as controls. At the end of drug treatment, the medium was removed and the cells were incubated at 37 °C for 1 h with 5mg/L of JC-1, then washed twice with PBS and placed in fresh medium without serum. Then, images were viewed by high-throughput fluorescence confocal microscopy (BD Pathway Bioimager 855) at excitation wavelength of 488nm and emission of 530nm for green, and at excitation of 540nm and emission of 590nm for red. The fluorescence of both green fluorescence (JC-1 monomers) and red fluorescence (JC-1 aggregates) were measured with a FLUOstar optima plate reader (BMG LABTECH, Germany) with the filters set to 485 nm excitation/520 nm emission (green) and 544 nm excitation/590 nm emission (red), and data were presented as the ratio of green to red signals (520 nm/590 nm) with the ratio of green to red signals of non-treat cells as normalized unity of 1. Results were shown in Fig. S6.



(A)



(B)

Fig. S6. Changes in the mitochondrial membrane potential in SKOV-3 cells as revealed by the JC-1 assay. (A) JC-1 assay of SKOV-3 cells treated with various drugs (cisplatin, DCA-cisPt(IV)-COOH and M(P1) at 50uM and DCA at 100 uM) for1 h. The JC-1 stained cells images were viewed by high-throughput fluorescence confocal microscopy (BD Pathway Bioimager 855)(A) and the fluorescence intensity ratio (green to red) was measured with FLUOstar optima plate reader and compared with that of non-treated cells for 1 h as unity (control) (B).

References

[1] H. Xiao, R. Qi, S. Liu, X. Hu, T. Duan, Y. Zheng, Y. Huang, X. Jing. Biomaterials, 2011, **32**, 7732–7739.

[2] M. Reithofer, M. Galanski, A. Roller, B. K. Keppler. Eur. J. Inorg. Chem., 2006, **13**, 2612–2617.

[3] I. H. L. Hamelers, R.W. H. M. Staffhorst, J. Voortman, B. Kruijff, J. Reedijk, P. M. P. Henegouwen, et al. Clin. Cancer Res., 2009, **15**, 1259–1268.

[4] H. T. T. Duong, V. T. Huynh, P. Souza, M. H. Stenze. Biomacromolecules, 2010, **11**, 2290–2299.

[5] M. M. Bradford. Anal. Biochem., 1976, **72**, 248–254.