

Supporting Information

Micellar Nanoparticle Formation via Electrostatic Interactions for Delivering Multinuclear Platinum (II) Drugs

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Materials

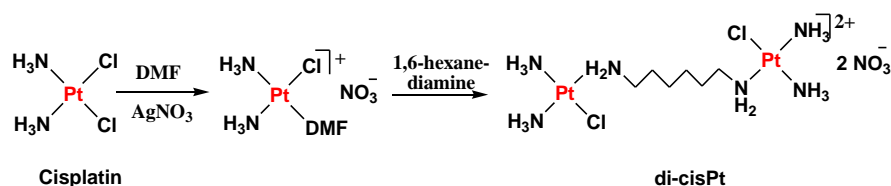
Cisplatin (purity 99%) was purchased from ChemiChem International Development Co., Ltd. MPEG_{5k}-b-PGA_{1.8k} was kindly provided by Dr. Xuesi Chen (Changchun Institute of Applied Chemistry, Chinese Academy of Sciences). All cell culture materials were purchased from Life Technologies.

Methods and Supplemental Results

¹H 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 ionization (ESI). Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES, iCAP 6300, Thermoscientific) was used to determine the total platinum contents in various nanostructures and samples obtained outside of the dialysis bags in drug release experiments. Inductively Coupled Plasma Mass Spectrometer (ICP-MS, Xseries II, Thermoscientific) was used for quantitative determination of trace levels of platinum. Size and size distribution of micelles were determined by DLS with a vertically polarized He-Ne laser (DAWN EOS, Wyatt Technology). The morphology of the nanostructures 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 Di-cisPt

Di-cisPt was synthesized as previously described [1]. Briefly, cisplatin (1 g, 3.33 mmol) was dissolved in DMF in a flask, to which AgNO₃ (0.566 g, 3.33 mmol) was added. The reaction mixture was kept in dark at room temperature for 24 h. AgCl precipitates were removed by filtration. 1,6-hexanediamine (0.194 g, 1.67 mmol) dissolved in 5 mL methanol was added into the filtrate and the solution was stirred at room temperature for 24 h in dark. After the reaction, the solvent was removed by rotate evaporation. The product was dissolved in methanol and then precipitated into diethyl ether. The white precipitates (di-cisPt) were filtered and dried in vacuum.



Scheme S1. Synthesis of di-cisPt

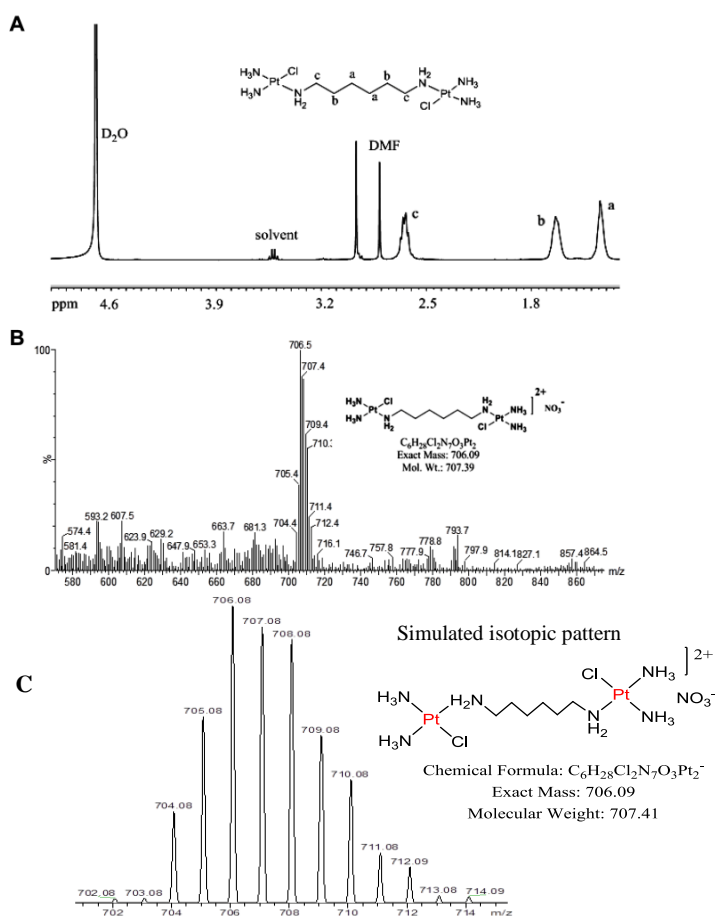


Figure S1. ¹H NMR of di-cisPt in D₂O (A), ESI-MS spectra of di-cisPt (B), and (C) simulated isotopic pattern and distribution of di-cisPt with one nitrate as counter ions (Formula: C₆H₂₈Cl₂N₇O₃Pt₂)

Micelle Formation with MPEG-PGA and di-cisPt

50 mg of MPEG_{5k}-PGA_{1.8k} was placed in 4 separate flasks. Then, they were neutralized by NaOH (the molar ratio of NaOH to the carboxyl group in the polymer chain, NaOH/COOH=1:1) in 50 mL deionized water under stirring. Due to NaOH neutralization, MPEG-PGA loses amphiphilic character and micelles do not form spontaneously. After that, different molar ratios of di-cisPt to carboxyl groups in the polymer chain (Pt/COOH) including 0:1, 0.1:1, 1:1 and 2:1 were added to the polymer solution and mixed through stirring for 12 h. At last, the mixed solutions were dialyzed and then lyophilized. Molar ratios of di-cisPt were selected to provide under, equal, and over saturation of positively charged Pt to complex with negatively charged MPEG-PGA.

Table S1. Parameters for nanostructures prepared

Sample	Pt/COOH ^a	Morphology	Pt content (w/w%) ^b	Zeta(mV)	DLS(nm) ^c	TEM(nm)
M1(di-cisPt)	0.1:1	micelles	5.26	-18.2		165nm
M2(di-cisPt)	1:1	micelles	22.5	-6.8	145	106
M3(di-cisPt)	2:1	micelles	23.1	-1.32	175	104

a: Pt/COOH is the molar ratio of Pt in di-cisPt and carboxyl groups in the polymer chain;

b: Platinum weight percentage in the lyophilized samples;

c: Size obtained by DLS is greater than TEM due to shrinking of micelle core during drying process.

Drug Release Studies from M2(di-cisPt) at Variable pH

10 mg of M2(di-cisPt) were dissolved in 5 mL of buffered solutions (PBS, pH=7.4; acetate buffered solution, pH=5.0). The solution was then placed into a pre-swollen dialysis bag with a molecular weight cutoff of 3.5 kDa and immersed into 50 ml buffered solution. The dialysis was conducted at 37 °C in a shaking incubator. 1.5 mL of sample solution was withdrawn from the incubation medium at specified time intervals and measured for Pt concentration by ICP-OES. After sampling, equal volume of fresh buffered solution was immediately added into the incubation medium. The platinum released from the micelles was expressed as the percentage of cumulative platinum outside the dialysis bag to the total platinum in the micelles.

Drug Release Studies from M2(di-cisPt) at Variable pH in the Presence of Fetal Bovine Serum (FBS)

10 mg of M2(di-cisPt) were dissolved in 5 mL of buffered solutions (PBS, pH=7.4; acetate buffered solution, pH=5.0). The solution was then placed into a pre-swollen dialysis bag with a molecular weight cutoff of 3.5 kDa and immersed into 100 mL buffered solution supplemented with 10% FBS (the pH values were regulated to pH=7.4 and pH=5.0). The dialysis was conducted at 37 °C in a shaking incubator. 1.5 mL of sample solution was withdrawn from the incubation medium at specified time intervals and digested by 60% nitric acid in H₂O₂ for 2 h. The samples were then measured for Pt concentration by ICP-OES. After sampling, equal volume of fresh buffered solution was immediately added into the incubation medium. The platinum released from the micelles was expressed as the percentage of cumulative platinum outside the dialysis bag to the total platinum in the micelles.

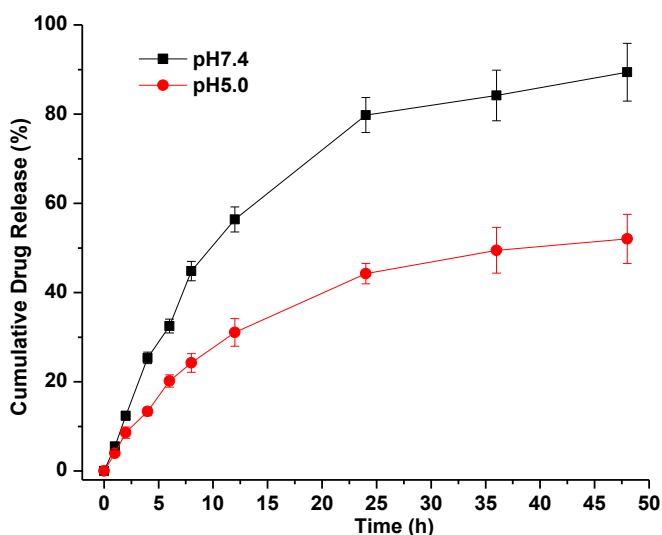


Figure S2. Drug release profiles of M2(di-CisPt) in 10% FBS at pH 5.0 and 7.4.

Released Drug Species in the Presence of 5'-GMP

50 mg M2(di-cisPt) was dissolved in 5 mL deionized water. Then, the solution was transferred to a dialysis bag (MW cut-off, 1000) and sealed. The dialysis bag was washed several times to remove any residual M2(di-cisPt) on the surface and was incubated in 5 mL of a freshly prepared solution containing 5'-GMP (5mM) at 37 °C for 12 h. The dialysate was collected for MALDI-TOF-MS analysis.

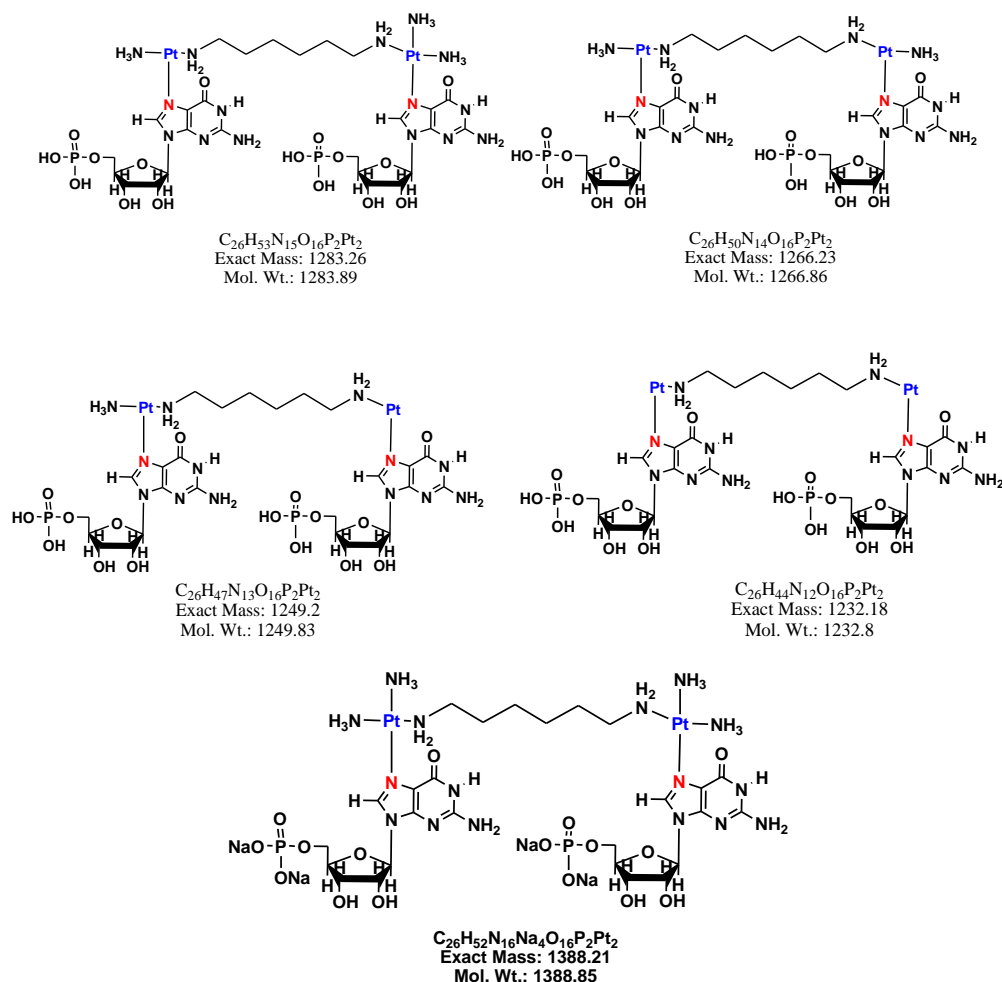


Figure S3. Possible structures of adducts formed by di-cisPt and 5'-GMP.

Cell Culture

HeLa (human cervical cancer cell line), A549 (human lung cancer cell line), HepG-2 (human lung cancer cell line), MCF-7 (human breast cancer cell line), and H22 cells (murine hepato carcinoma cells) were cultured in RPMI 1640 media containing 10% fetal bovine serum, 0.03% L-glutamine, 100 units/mL penicillin, and 100 μ g/mL streptomycin at 37 °C.

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

Cells lines were seeded in 96-well plates at a density of 10^5 cells/well and treated with increasing concentrations of di-cisPt and M2(di-cisPt) at various concentrations. At 48 h, 20 μ L 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. Then, the culture media containing MTT was removed and 150 μ L of DMSO was added to each well to dissolve the formazan crystals. Finally, the plates were shaken for 10 minutes, and the absorbance of

formazan product was measured at 492 nm by a microplate reader.

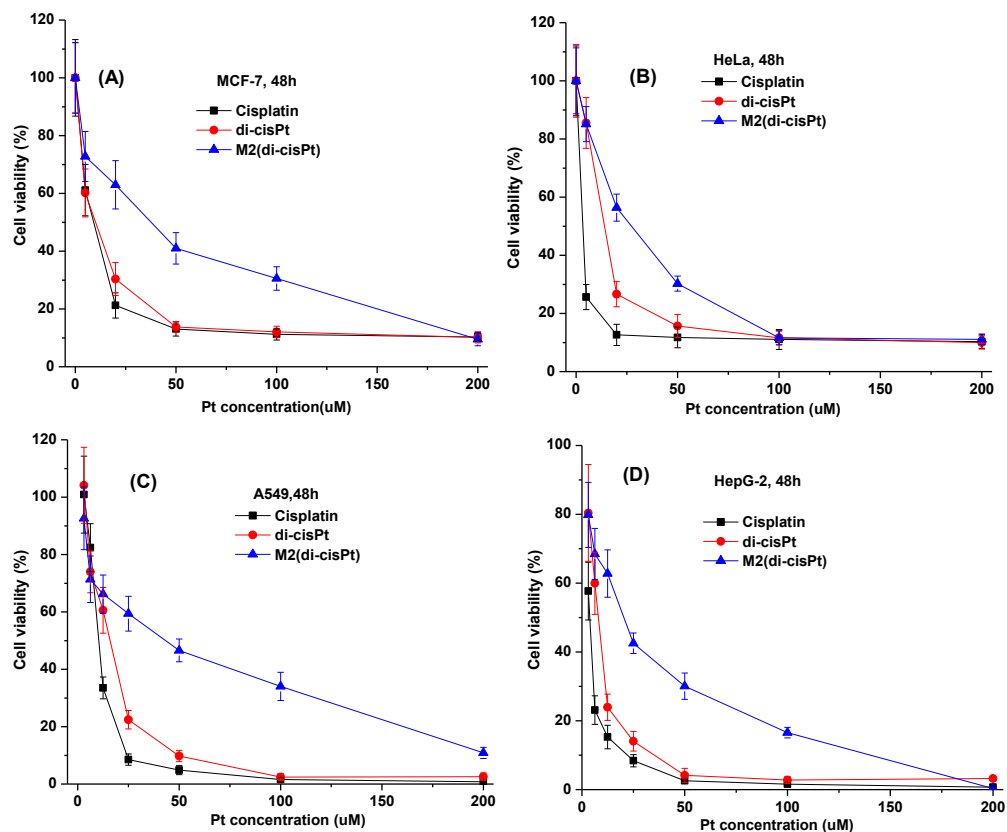


Figure S4. *In vitro* cytotoxicity of di-cisPt and M2(di-cisPt) against MCF-7(A), HeLa(B), A549(C) and HepG-2 (D) cancer cell lines at 48h.

Cellular Uptake Studies

HepG-2 cancer cells were seeded in 24-well plates and were treated with di-cisPt and M2(di-cisPt) at 10 μ M Pt equivalents at 37 °C for 1 h and 4 h. To quantitatively determine Pt uptake by cells, the drug-treated cells were washed with ice-cold PBS to remove surface-bound drug, 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, harvested by scraping in ice-cold PBS, and centrifuged. The cell pellet was lysed with 200 μ L cell lysis buffer (Promega) and 100 μ L of the cell lysate was used directly to measure the Pt content by ICP-MS. The remainder 100 μ L of the cell lysate was used to determine the total protein content in sample by using bicinchoninic acid (BCA) protein assay kit as described elsewhere [2,3]. The platinum content was expressed as ng Pt per mg of total proteins.

Animal Use

Chinese Kunming (KM) female mice were obtained from Jilin University, China (56–84 days old,

20–25 g in weight). All animal studies were conducted in accordance with the principles and procedures outlined in “The National Regulation of China for Care and Use of Laboratory Animals” approved by the National Science and Technology Commission of China. To develop the tumor xenografts, H22 cells were injected into the right flank of the mice (5×10^6 cells in 0.1 ml PBS). After the tumor volume reached 100–200 mm³, mice were separated into the groups for the studies described below.

Biodistribution

Nine KM mice bearing xenograft H22 tumors were randomly divided into three groups and were injected with cisplatin, di-cisPt and M2(di-cisPt) via tail vein at an equivalent Pt dose of 5 mg Pt/kg body weight. The animals were then sacrificed at 24 h. Major organs including heart, liver, spleen, lung, kidneys, and tumor were collected and washed with 0.9% saline. After weighing, the organs were dissolved in 65% (v/v) nitric acid at 60 °C for 2 h, and Pt concentrations were measured by ICP-MS (ICP-MS, Xseries II, ThermoScientific, USA). Results show that cisplatin accumulates significantly more in kidney than in other organs, while di-cisPt accumulates more in liver and kidney. This is in agreement with the widely reported high kidney toxicity of cisplatin and even higher toxicity of multinuclear platinum (II) drugs [4]. Importantly, M2(di-cisPt) accumulates 7.6 and 6.1 fold more in the tumor compared to cisplatin and di-cisPt, respectively, demonstrating enhanced tumor accumulation. Furthermore, M2(di-cisPt) retains high bioavailability, with ~11.7 and 14.4 fold greater levels detected in the blood relative to cisplatin and di-cisPt, respectively, at 24 h.

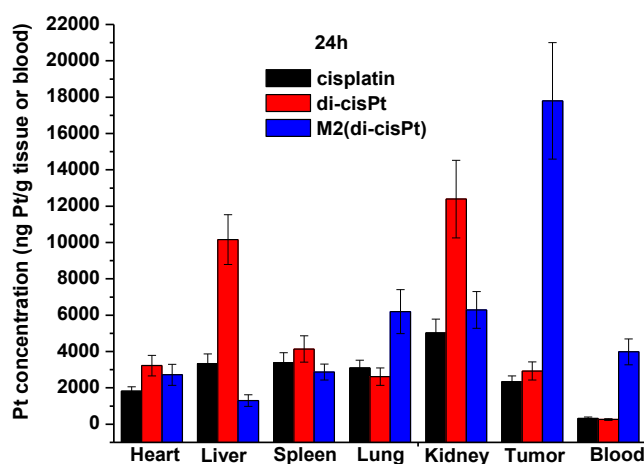


Figure S5. Biodistribution of cisplatin, di-cisPt and M2(di-cisPt) at a dose of 5 mg Pt/kg body weight after 24 h of one time intravenously injection using a murine hepatocarcinoma xenograft model (H22).

Anti-tumor efficacy

Fifty six KM mice bearing H22 tumors were randomly divided into seven groups (8 mice in each group) for (a) cisplatin, 3.25mg Pt/kg; (b) cisplatin, 5mg Pt/kg; (c) di-cisPt, 1.25mg Pt/kg; (d) di-cisPt, 2.5mg Pt/kg; (e) M2(di-cisPt), 2.5mg Pt/kg; (f) M2(di-cisPt), 5mg Pt/kg; (g) Blank control, PBS. Intravenous injections of the indicated drugs were performed on day 1, 3, and 5, with the first injection designated as day 1. The tumor size was measured every other day for 2 weeks with a caliper and the tumor volume (V) was calculated by the formula of $V = \frac{1}{2}ab^2$, where a and b were the length and width of tumor, respectively (Figure S6). Group b (cisplatin, 5mg Pt/kg) and group d (di-cisPt(II), 2.5mg Pt/kg), lost significant body weight and were sacrificed due to excessive toxicity on days 5 and 7, respectively (Figure S6B). As a result, further data of these two groups was not obtained. Cisplatin, at a lower dose of 3.25 mgPt/kg (group a), proved effective in maintaining a small tumor volume, but exhibited acute body loss in the mice. Similarly, di-cisPt was highly toxic and caused acute body weight loss even at a lower dose at 1.25mg Pt/kg (group c). Cisplatin and especially multinuclear platinum (II) drugs are notorious for their high toxicity [4], which is also observed in the studies reported here. These results indicate that cisplatin and di-cisPt(II) are too toxic to be used at these doses, in agreement with our earlier reports [3]. Importantly, nanoparticle formulations of M2(di-cisPt) at 2.5 mg Pt/kg (group e) and 5 mg Pt/kg (group f), showed dramatic tumor growth inhibition and reduced systemic toxicity. This indicates that the micellar formulation of di-cisPt has improved therapeutic index when compared to free di-cisPt as well as cisplatin.

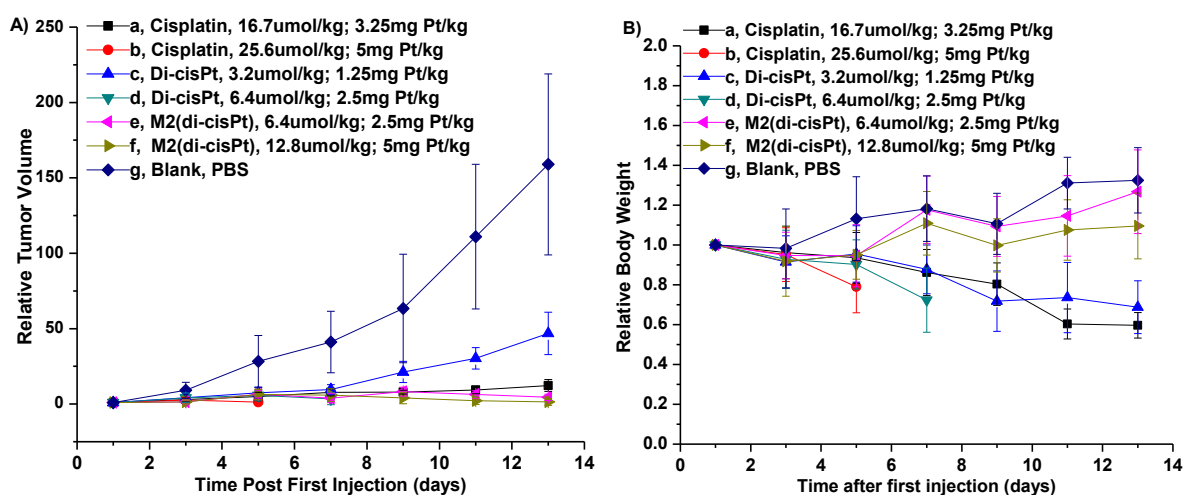


Figure S6. *In vivo* study of M2(di-cisPt) in a murine hepatocarcinoma xenograft model (H22). Mice were injected on day 1, 3, and 5, at the indicated doses of cisplatin, di-cisPt, and M2(di-cisPt). Tumor volume (A), and body weight loss (B) was plotted.

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