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Supplementary Information

Fighting against Drug‐Resistant Tumors by Inhibition of γ-Glutamyl Transferase with Supramolecular Platinum Prodrug Nano-Assemblies

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TABLE OF CONTENTS

Materials.

Dextran with an average molecular weight of 10,000 Da ($dextran_{10k}$), (99%), N-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (EDC•HCL), n-hydroxysuccinimide (NHS), N,N-dicyclohexylcarbodiimide (DCC) and 51 mg 4-(dimethylamino)pyridine (DMAP), succinic anhydride, β-CD-NH_2, 1-adamantaneacetic acid (Ad-COOH) and sodium ascorbate (NaVc) were purchased from Sigma-Aldrich. Cisplatin (purity 99%) was bought from Shandong Boyuan Chemical Company, China. 2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from SigmaAldrich. Rabbit Bax, Bcl-2, Caspase-3, Cyto-3, and GGT antibody were purchased from Bioss Biotechnology Co., Ltd. Beijing, China. The mitochondrial membrane potential assay kit (with JC-1) and Annexin V-FITC/PI kit were purchased from Beyotime. (Shanghai, China) and used according to the protocols provided by the manufacturers. Dimethyl sulfoxide (DMSO) and chloroform were dried over calcium hydride for 7 days before distillation.

Cell Lines and Animals.

A549 (Human non-small cell lung cancer cell line) and A549/DDP (cisplatin resistance human non-small cell lung cancer cell line), A2780 (Human ovarian cancer cell) and A2780/DDP (cisplatin resistance human ovarian cancer cell) cells were bought from Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. A549 and A549/DDP cells were cultured in 1640 medium containing 10% FBS (50 ng Pt/mL for A549/DDP cells) A2780 and A2780/DDP cells were cultured in DMEM medium containing 10% FBS (50 ng Pt/mL for A2780/DDP cells). Female BALB/c nude mice (6-8 week old, 16-18 g) were purchased from Southern Medical University (Guangzhou, China) and were housed in an SPF room. All mice received required care conditions throughout the experiments. All animal experiments were approved by the local institution review board and performed according to the Guidelines of the Committee on Animal Use and Care of Southern Medical University.

Instrumentation.

NMR spectra (^{1}H NMR) were detected with a Bruker NMR spectrometer (400 MHz) at 25 ℃. Fourier transform infrared spectra (FT-IR) were measured on Bruker Vertex 70 spectrameter. Mass spectroscopy (ESI-MS) was detected by a Quattro Premier XE system (Waters) with an electrospray interface (ESI) equipment. The platinum contents were calculated with an inductively coupled plasma massspectrometry (ICP-MS, Thermoscientific, USA). Transmission electron microscopy (TEM) imaging was taken on an electron microscope (JEOL JEM-1011). Diameters were recorded by a Brookhaven 90Plus size analyzer. UV-visible electronic absorption spectra were measured on a Varian Cary 300 UV-visible spectrophotometer in 1 cm

path-length cuvettes. Cellular and histological fluorescence images were observed on confocal laser scanning microscope (CLSM) imaging system (Olympus FV1000, Zeiss, Japan). Flow cytometry analysis was analyzed by a BD FACS-CaliburTM flow cytometer. Histological section imaging was observed with an optical microscope (Nikon TE2000U).

Synthesis of Pt-CD.

c,c,t-[$Pt(NH_3)_2Cl_2(OH)_2$] [Pt(IV)] was synthesized according to the reported procedures (Scheme S1, Figure S1). Pt(IV) (546 mg, 1.634 mmol) was stirred with succinic anhydride (163.5 mg, 1.634 mmol) in DMF at 65 ℃ in the dark for 24 h. Then the solution was precipitated in ethyl ether, and the light yellow precipitate c,c,t-[$Pt(NH_3)_2Cl_2(OH)(OOCCH_2CH_2COOH)$] (Pt-COOH) was dried.

Pt conjugated β-CD (CD-Pt) was synthesized by amidation reaction between Pt-COOH and β-CD-NH_2. 8 mL N,N-dimethylformamide (DMF) and 3 mL water were added in a single neck flask. 0.1 mmol (126 mg) of β-CD-NH_2, 0.15 mmol (65 mg) of Pt-COOH, 0.15 mmol (30 mg) of 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC•HCl), and 0.15 mmol (18 mg) of n-hydroxysuccinimide (NHS) were then added. The mixed solution was stirred at 35 ℃ for three days. The solution was then concentrated by a rotary evaporator and then precipitated in acetonitrile. The solid was dissolved in DMSO and dialyzed (MWCO 1,000 Da) against DMSO for 48 h and water for 24 h. The solution was then freeze-dried to obtain white solid. ^{1}H NMR (DMSO-d6, 400 MHz, ppm): 2.30-2.40 (m, -C*H*$_2$-C*H*$_2$-COO-), 4.82 (m, -CH-C*H*-(O)$_2$-), 3.25-3.75 (CD ring) (Figure S1)..

Synthesis of Dextran (Dex)-Adamantine (Ad) Conjugate.

Dex-Ad was synthesized by the reaction of Dex and 1-adamantaneacetic acid (Ad-COOH). Briefly, 0.1 g of Dex and 85 mg of 1-adamantaneacetic acid were dissolved in 10 mL anhydrous DMSO. 88 mg of N, N-dicyclohexylcarbodiimide (DCC) and 51 mg 4-(dimethylamino)pyridine (DMAP) were then added. The mixed solution was stirred at room temperature for 24 h. The solution was then dialyzed (MWCO 3,500 Da) against water for 48 h. After that, the solution was freeze dried to obtain Dex-Ad.

Isothermal titration calorimetry measurements.

The host-guest interaction between Pt-CD and Dex-Ad in water was characterized by using a NanoITC apparatus (TA Instruments) at room temperature at a stirring speed of 250 rpm. The sample cell (1.0 mL) was filled with degassed Pt-CD aqueous solution (0.2 mM). The syringe was filled with Dex-Ad solution (4.48 mM, in DMSO/water, 1:19, v/v). The titration experiment was set to 25 injections of 10 μL each with 600 s intervals. The heats of dilution were determined in blank experiments in which the DMSO/water (1:19) solutions were injected into the sample cell containing Pt-CD. The dilution heats were then subtracted to obtain the binding heats. The data was processed using ITCAnalyze software and fitting in an “independent” binding model.

Resistance factor (RF) and Combination index analysis of Pt-CD/Dex-Ad@OU nano-assemblies.

Resistance factor (RF) and Combination index (CI) was calculated with the equation as below[1]:

Resistance factor (RF) was calculated with the equation as below:

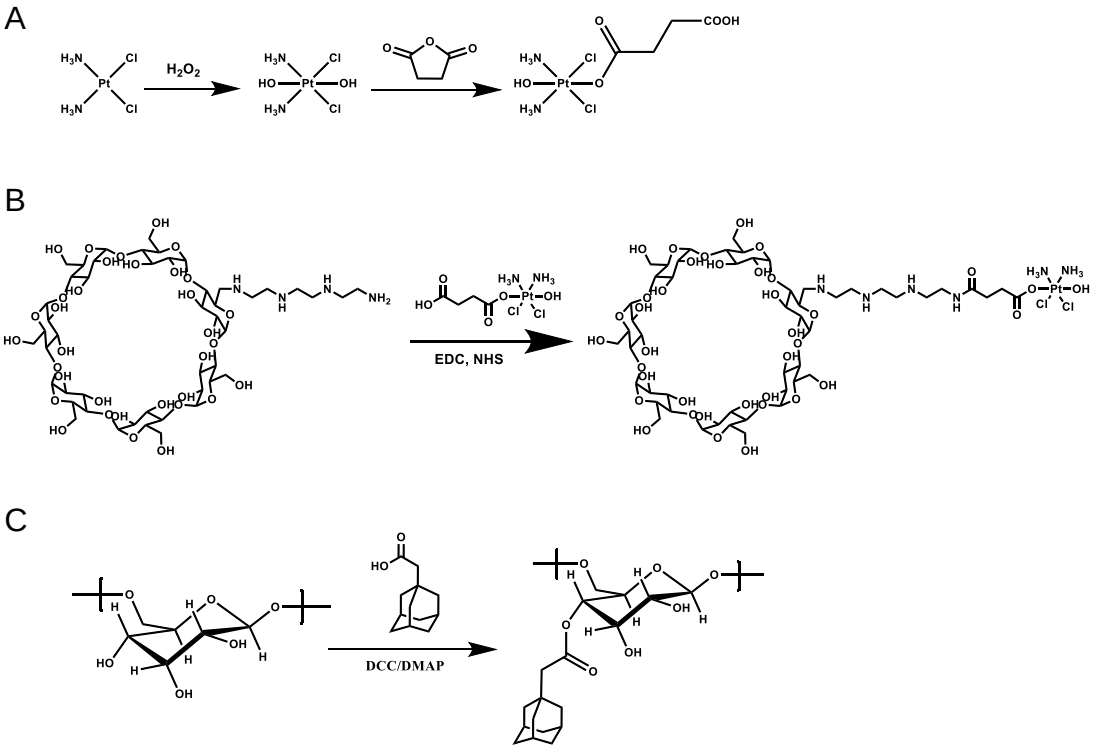
$$RF = \frac{IC_{50,A}}{IC_{50,B}}$$

$IC_{50,A}$ and $IC_{50,B}$ are the concentrations the concentrations for Pt-CD/Dex-Ad@OU nano-assemblies to achieve 50% drug effect for A549 cells and cisplatin-resistant A549 cells.

Combination index (CI) analysis provides qualitative information on the nature of drug interaction. CI, a numerical value calculated as described in equation S1 below, also provides a quantitative measure of the extent of drug interaction.

$$CI = \frac{C_{A,x}}{IC_{x,A}} + \frac{C_{B,x}}{IC_{x,B}}$$

$C_{A,x}$ and $C_{B,x}$ are the concentrations of drug A (Pt) and drug B (OU749) used in combination (Pt-CD/Dex-Ad@OU nano-assemblies) to achieve x% (eg, 50%) cell inhibiting effect. $IC_{x,A}$ and $IC_{x,B}$ are the concentrations for single agents (Pt-CD/Dex-Ad nano-assemblies and OU749) to achieve the same effect. A CI of less than, equal to, or more than 1 indicates synergy, additivity, or antagonism, respectively.



Scheme S1. Synthetic procedures of (A) Pt(IV)-COOH, (B) Pt-CD and (C) Dex-Ad.

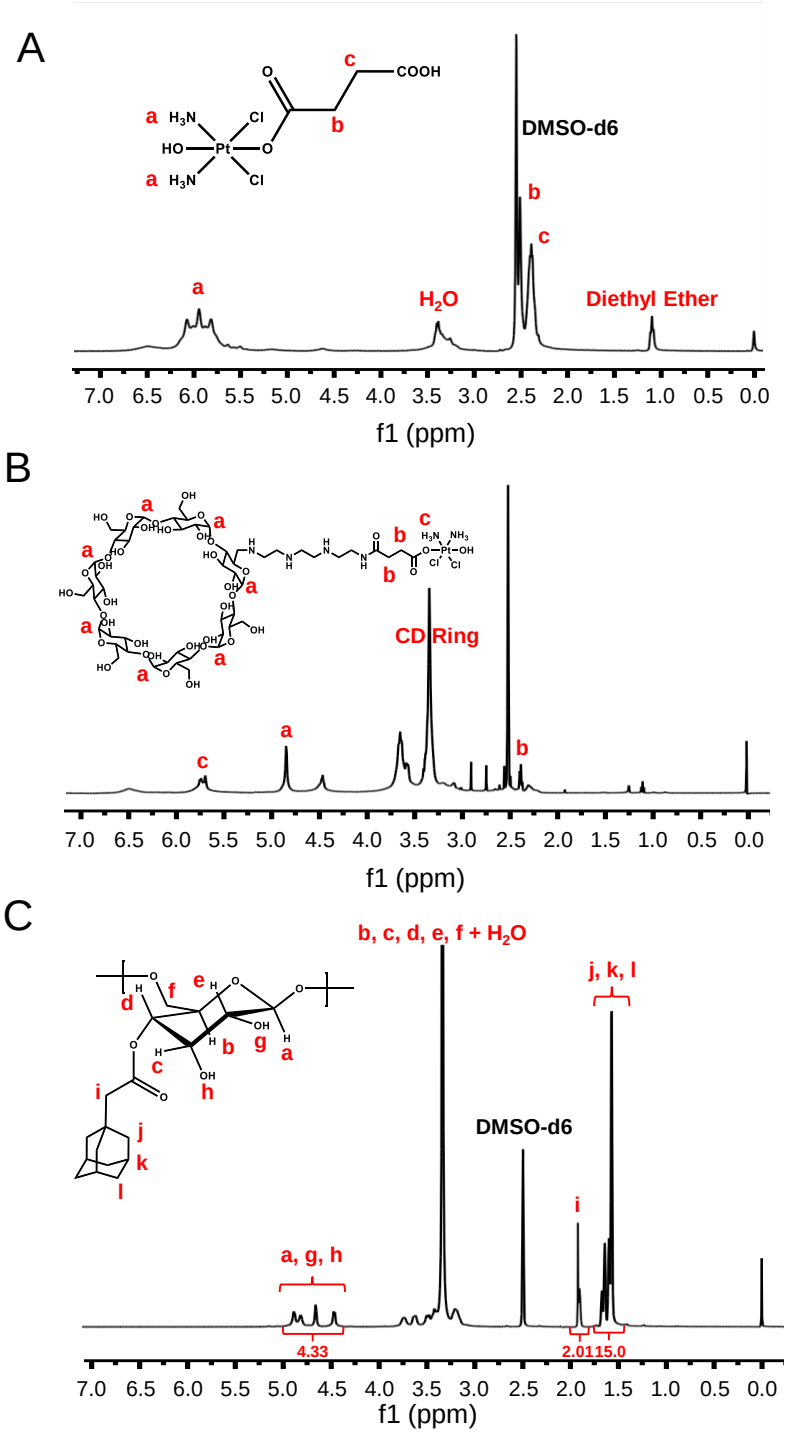


Figure S1. The ^{1}H NMR spectra (400 MHz, room temperature) of (A) Pt(IV)-COOH, (B) CD-Pt and (C) Dex-Ad.

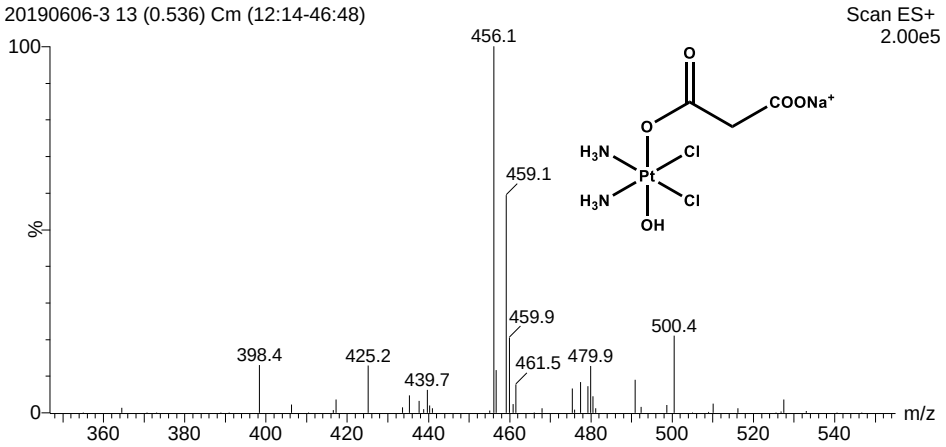


Figure S2. ESI-MS of c,c,t-[Pt(NH_3)$_2$$Cl_2$(OH)($OOCCH_2CH_2COOH$)] (Pt-COOH) a measured by (positive mode).

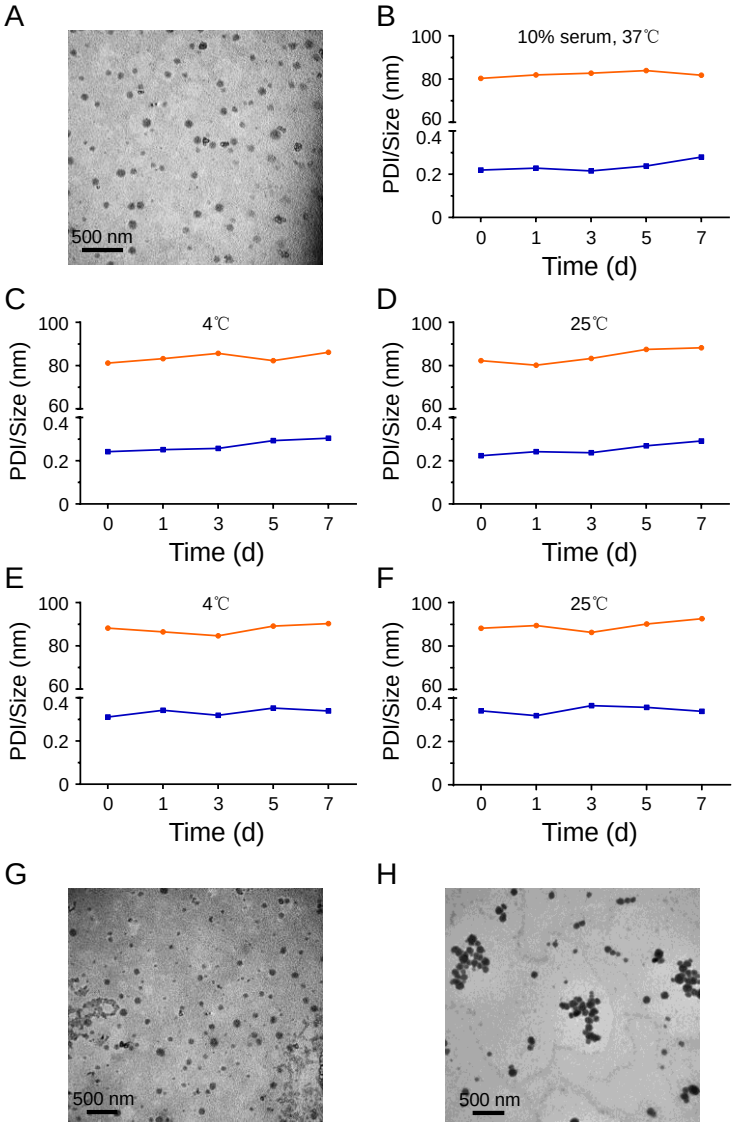


Figure S3. (A) TEM image of Pt-CD/Dex-Ad nano-assemblies. The particle size and PDI changes of Pt-CD/Dex-Ad nano-assemblies in the presence of (B) PBS (10% FBS, pH 7.4, 0.01 M) at 37 ℃, PBS (pH 7.4, 0.01 M) at (C) 4 ℃ and (D) 25 ℃ over a week. The particle size and PDI changes of Pt-CD/Dex-Ad@OU nano-assemblies in the presence of PBS (pH 7.4, 0.01 M) at (E) 4 ℃and (F) 25 ℃ over a week. TEM images of (G) Pt-CD/Dex-Ad nano-assemblies and (H) Pt-CD/Dex-Ad@OU nano-assemblies with time in the presence of PBS (10% FBS, 0.01 M) at pH 7.4 at 37℃. No morphological change of Pt-CD/Dex-Ad nano-assemblies and Pt-CD/Dex-Ad@OU nano-assemblies was observed by TEM images over 24 h.

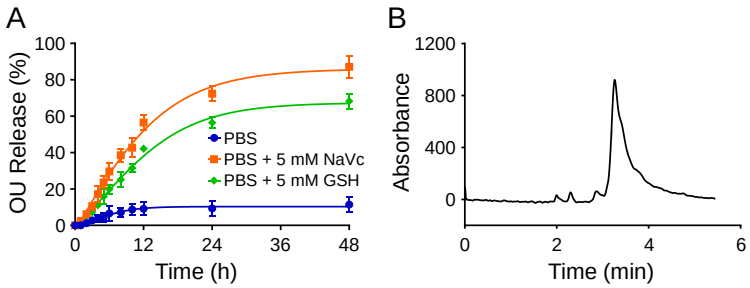


Figure S4. (A) OU749 release profiles of Pt-CD/Dex-Ad@OU nano-assemblies in PBS with or without sodium ascorbate (5 μM) or GSH (5 μM). (B) HPLC spectrum of the solution of OU749 extracted from Pt-CD/Dex-Ad@OU nano-assemblies.

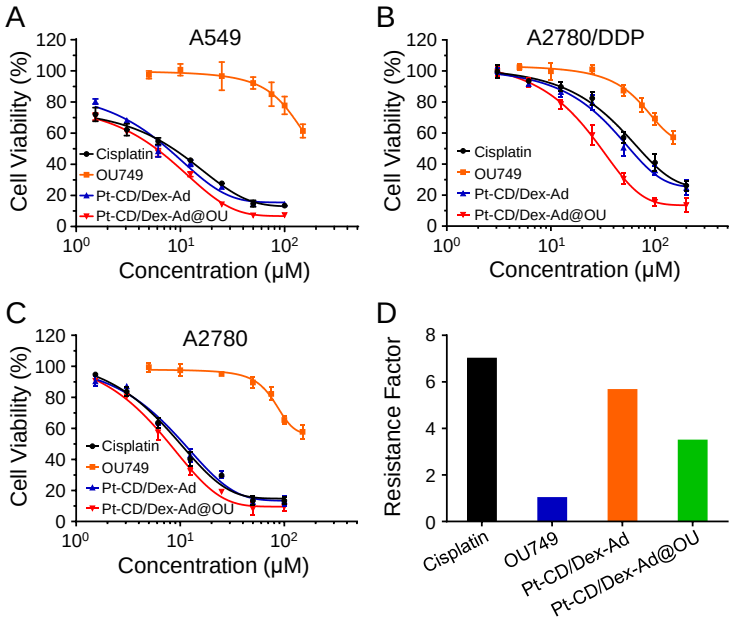


Figure S5. Viability curves of (A) A549 cells, (B) A2780 cells and (C) A2780/DDP cells after treatment cisplatin, OU749, Pt-CD/Dex-Ad nano-assemblies and Pt-CD/Dex-Ad@OU nano-assemblies for 72 h. (D) Resistance factor of cisplatin, OU749, Pt-CD/Dex-Ad nano-assemblies and Pt-CD/Dex-Ad@OU nano-assemblies against A549/DDP cells.

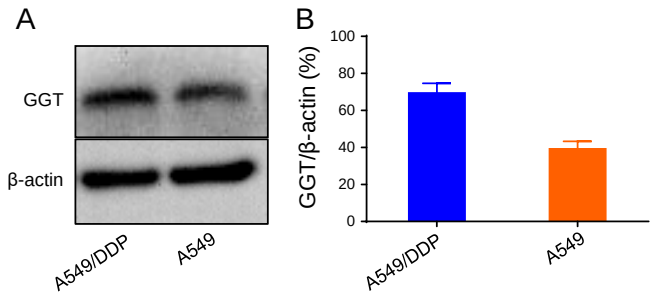


Figure S6. Quantified GGT levels of A549 and A549/DDP cells according to western blot images using ImageJ software.

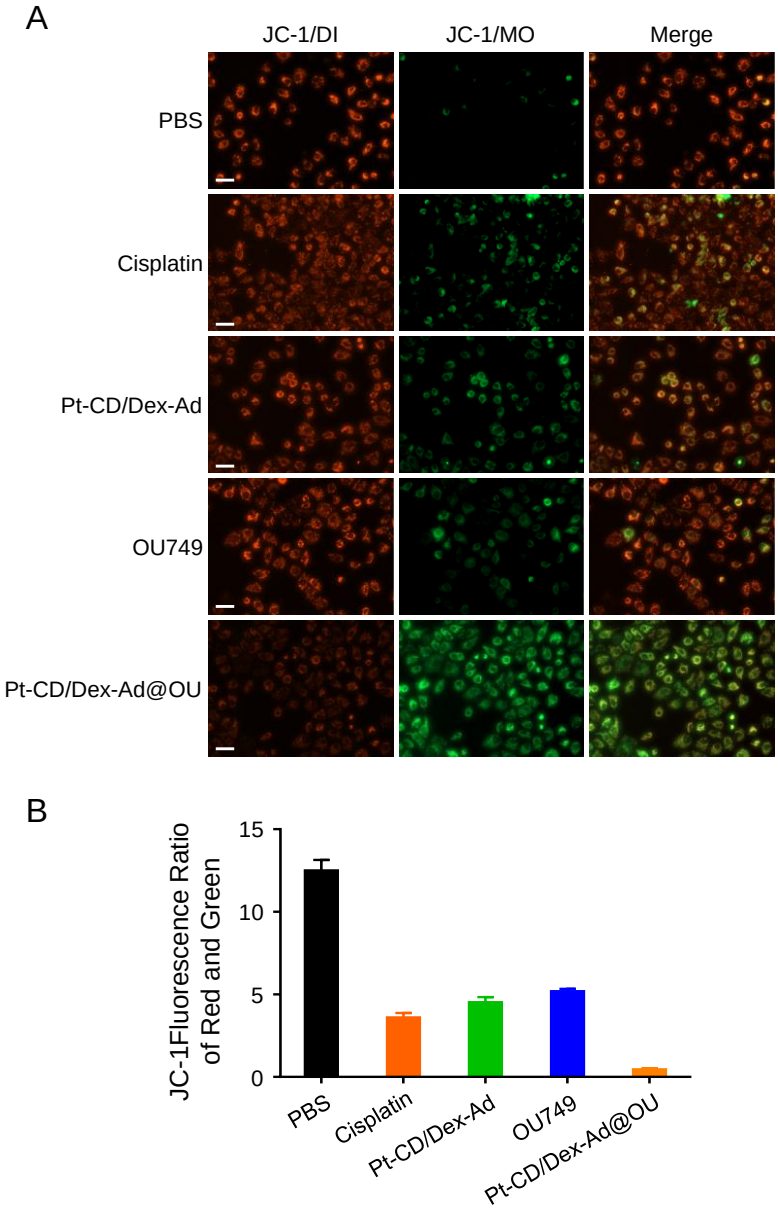


Figure S7. (A) CLSM of mitochondrial membrane hyperpolarization as detected by the JC-1 probe. Scale bar = 20 μm. (B) Fluorescence semi-quantitative analysis of JC-1fluorescence ratio of red and green.

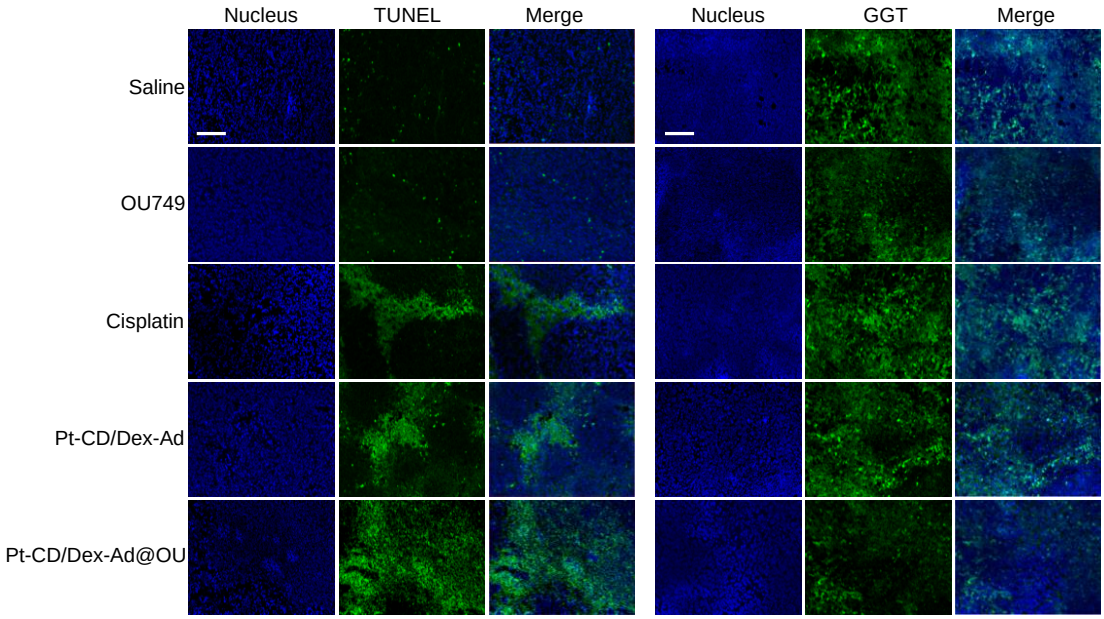


Figure S8. Immunohistochemical analysis of GGT expression and TUNEL in tumor sections isolated from mice on day 18, Scale bar = 200 μm.

Table S1. The mole ratio of Pt in the obtained Pt-CD/Dex-Ad under different feed ratio of Pt and Dex.

Pt:Dex in feed (n/n)	20:1	30:1	40:1	50:1
Pt:Dex in Pt-CD/Dex-Ad (n/n)	19.41±0.72	27.49±1.45	36.81±2.45	46.17±2.18

Table S2. IC_{50} values of cisplatin, OU749, Pt-CD/Dex-Ad nano-assemblies, and Pt-CD/Dex-Ad@OU nano-assemblies against A549, A549/DDP, A2780 and A2780/DDP cells for 72 h.

		Cisplatin	OU749	Pt-CD/Dex-Ad	Pt-CD/Dex-Ad@OU
IC_{50}	A549	6.78	165.10	7.11	4.854
	A549/DDP	67.15	195.30	53.67	21.92
	A2780	10.62	171.5	11.05	7.948
	A2780/DDP	74.42	173.09	62.45	27.71

Table S3. Survival number of mice after treated with cisplatin, Pt-CD/Dex-Ad nano-assemblies, and Pt-CD/Dex-Ad@OU nano-assemblies for 7 days.

	Cisplatin	Pt-CD/Dex-Ad	Pt-CD/Dex-Ad@OU
Pt: 3 mg/Kg	5/5	5/5	5/5
Pt: 6 mg/Kg	2/5	4/5	5/5
Pt: 9 mg/Kg	0/5	2/5	4/5