Electronic Supporting Information

for

Oral Delivery of Platinum Anticancer Drug Using Lipid Assisted Polymeric Nanoparticles

22 August 2024

Note added after first publication: This supplementary information file replaces that originally published on 12 October 2015. The original version of the supplementary information contained incorrect data in Fig. S11. This has been corrected in this updated version. Please see the separate correction notice for details. The correction of the figure does not affect the conclusions of the article.

Content

Experimental details

Materials. Methoxy-poly(ethylene glycol)-poly(lactide-co-glycolide) copolymer (mPEG5k-PLGA8k) was purchased from Jinan Daigang Biomaterial Co., Ltd. Cisplatin, cholesteryl chloroformate, ethylenediamine, succinic anhydrite, DAPI and Alexa Fluor 488, were purchased from sigma. 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetra-zolium bromide (MTT), ascorbic acid (AsA), hydrogen hydroxide (30%), were purchased from Sangon Biotech (Shanghai) Co., Ltd. Ultrapure water (18.2 MΩ) was obtained from Millipore Milli-Q Biocel purification system with a 0.22 μm filter. All other solvents and reagents were used as received.

Cell culture. The human cancer cells, including cervical HeLa, breast carcinoma MDA-MB-231, hepatocellular carcinoma HepG2, lung carcinoma A549 cells were obtained from the American Type Culture Collection (ATCC). The cisplatin-resistant lung cancer cell line A549R was purchased from Shanghai Fumengjiyin biotechnology (FMGbio Co., Ltd.). The cells were maintained in either DMEM (for HeLa, MDA-MB-231 and HepG2 cells) or RPMI1640 (for A549 and A549R cells) medium containing 10% fetal bovine serums in a humidified atmosphere containing 5% CO_2 at 37°C. A549R cells were maintained with 2 μg/mL cisplatin.

Animals. Balb/c nude mice (5 weeks old) and ICR mice (5 weeks old) were purchased from the Shanghai slack laboratory animal co., LTD (Shanghai, China). All animals received care in compliance with the guidelines outlined in the Guide for the Care and Use of Laboratory Animals. The procedures were approved by the University of Science and Technology of China Animal Care and Use Committee.

Synthesis of asplatin. Oxoplatin was synthesized as described in the literature.[1] H_2O_2 (3.5 mL 30% w/v, 0.03 mol) was added to the suspension of 100 mg cisplatin (0.33 mmol) in 5.0 mL water. The mixture was stirred at 50ºC for 1 h and then at room temperature for another 12 h. The solution was lyophilized. The resultant was washed with cold water, ethanol and ether, and dried again in vacuum.

For the preparation of asplatin, 204 mg acetylsalicylic anhydride (0.6 mmol) was added to 10 mL dimethylsulfoxide (DMSO) solution of oxoplatin (100.0 mg, 0.3 mmol). The mixture was stirred for 24 h at room temperature. The solution was lyophilized and the resultant was washed with acetone and diethyl ether to yield a pale yellow solid. The product was dried in vacuum and was characterized by NMR and ESI-MS.

Synthesis of asplatin-succinic acid. For synthesis of asplatin-succinic acid conjugate, 350.0 mg of asplatin (0.704 mmol, 1 equiv) was dissolved in 5.0 mL anhydrous dichloromethane (DCM). To this mixture, 352.2 mg of succinic anhydride (3.52 mmol, 5 equiv.) and 2.0 mL of pyridine were added. The resultant mixture was stirred for 24 h and then extracted by 0.1 M HCl and DCM several times. The organic phase was collected and dried with sodium sulfate. The product (asplatin-succinic acid) was obtained as white amorphous solid powder using rotary evaporator.

Synthesis of cholesterol-ethylenediamine. Cholesterol-ethylenediamine conjugate was synthesized according to literature.[2] 1.0 mL of ethylene diamine (14.9 mmol, 14 equiv) was dissolved in 5.0 mL anhydrous DCM in ice-bath. Next, 478.4 mg of cholesteryl chloroformate (1.065 mmol, 1.0 equiv) dissolved in 5.0 mL anhydrous DCM was added to the reaction mixture drop-wise over a period 15 min with vigorous stirring. The mixture was stirred for another 24 h and

then extracted using water (50 mL × 3) and DCM (50 mL), followed by saturated brine water wash. The organic layer was dried over anhydrous sodium sulfate and then evaporated with a rotary evaporator**.**

Synthesis of cholesterol-asplatin. For the synthesis of cholesterol-asplatin conjugate, 100.0 mg of asplatin-succinic acid (0.168 mmol, 1.0 equiv) was dissolved in 10.0 mL DMF/THF (6/1) mixture solution. To this mixture, 161.0 mg of EDC·HCl (0.84 mmol, 5 equiv), 38.7 mg of N-hydroxysuccinimide (NHS) (0.336 mmol, 2 equiv) and 136.0 mg of triethylamine (1.344 mmol, 8 equiv) were added. The resulting mixture was stirred at room temperature for 30 min. Then, 79.5 mg of cholesterol-ethylenediamine (0.168 mmol, 1 equiv) in 5 mL DMF was drop-wise added to the asplatin-succinic acid solution and the resulting mixture was stirred for 24 h at 25 ºC. The solvent was evaporated using a lyophilizer and the resultant was extracted with DCM for several times. The organic solvent DCM was removed using a rotary vacuum evaporator. The resultant was washed with water and then lyophilized. The lyophilized power was further recrystallization in DCM/diethyl ether (1/8) mixture solution. The product (cholesterol-asplatin) was obtained as pale yellow solid using rotary evaporator. The product was characterized by NMR and ESI-MS.

Synthesis of cholesterol-rhodamine. Synthesis of cholesterol-rhodamine conjugate was using the same method as for the synthesis of cholesterol-asplatin conjugate by replacing the asplatin-succinic acid with rhodamine B.

Preparation and characterizations of SCANs. SCANs were prepared by a single-emulsion technique.[1] A mixture solution of cholesterol-asplatin (5 mg) in 50 μL chloroform and mPEG-PLGA (25 mg) in 400 μL ethyl acetate was emulsified in 4.5 mL of ultra-purified water by sonication at 80 W for 2 min over an ice bath to form an oil-in-water emulsion. After the emulsion, the organic solvents (chloroform and ethyl acetate) were removed using a rotary vacuum evaporator, and the free cholesterol-asplatin was removed with centrifugation at 3000 g for 10 min.

Nanoparticles were analyzed using a Malvern Zetasizer Nano ZS90 with a He-Ne laser (633 nm) at 90i collecting optics. The data were analyzed by Malvern Dispersion Technology Program 4.20. Transmission electron microscopy (TEM) measurements were performed on a JEOL 2010 transmission electron microscope (JE OL Co., Ltd., Tokyo, Japan) with an accelerating voltage of 200 kV.

The stability of SCANs was evaluated in the simulated gastric fluid (SGF), the simulated intestinal fluid (SIF) and DMEM medium containing 10% fetal bovine serum (FBS) at 37°C for a period of 24 or 48 h. The size distribution of SCANs were monitored using a Malvern Zetasizer Nano ZS90 at various time intervals.

For the drug release kinetic study, SCANs were sealed in a dialysis tubing (molecular weight cutoff = 3000 Da). The dialysis bag was incubated in 20 mL simulated gastric fluid (SGF) for 1 h, followed by 20 mL simulated intestinal fluid (SIF) for 3 h and finally 20 mL PBS buffer for the total 120 h, at 37°C with gentle shaking. The platinum concentrations in the dialysis buffer were measured using ICP-MS at the time 0.5, 1, 2, 4, 8, 12, 24, 36, 48, 72, 96 and 120 h, respectively.

Cellular uptake of SCANs. The HepG2 cells were seeded in 12-well plates with 1×10^5 cells per well in 1 mL of complete medium, and incubated in a 5% CO_2 atmosphere at 37°C for 24 h. Then the cells were treated with rhodamine or SCRNs at a final concentration of 8 μg/mL rhodamine for

4 h. After removal of the media, cells were washed twice with cold PBS three times. After then the cells were harvested and the fluorescence of the rhodamine in cells was analyzed by flow cytometry (BD FACS Calibur Flow Cytometer).

For quantification of the cellular platinum accumulation, HepG2 cells were seeded in 6-well plates overnight and then incubated with cisplatin, asplatin, satraplatin or SCANs at a final concentration of 50 μM Pt at 37°C in the standard culture condition for 3 h. Then the cells were washed with PBS there times, and harvested by trypsinization. The harvested cells were digested with nitric acid for the ICP-MS measurement. The cell numbers were counted before the digested.

Intracellular distribution of SCRNs was investigated with confocal laser scanning microscope. The HepG2 cells were seeded on glass cover-slips and treated with SCRNs at 4 μg/mL rhodamine for 4 h. Then the cells were washed with PBS there times, and fixed in 4% paraformaldehyde. The nuclei were stained with DAPI and the cytoskeletons were stained with Alexa Fluor 488. After then the cells were mounted onto glass slides using mounting solution for imaging under a LSM 710 CLSM (Carl Zeiss, Jena, Germany) using a × 40 objective.

In vitro cellular cytotoxicity assays. Cells were seeded in 96-well plates at 4000 cells per well in 100 μL of complete medium, and incubated in a 5% CO_2 atmosphere at 37°C for 24 h. The culture medium was then replaced with 100 μL of freshly prepared culture medium containing drugs at different concentrations. The cells were further incubated for 72 h, and then the medium was replaced with fresh culture medium and the MTT solution was added. The cells were incubated for another 3 h to allow viable cells to reduce the yellow tetrazolium salt (MTT) into dark blue formazan crystals. Finally, 100 μL of lysis buffer was added to wells and incubated for another 4 h at 37°C. The absorbance was measured at 570 nm using a Bio-Rad 680 microplate reader. The IC_{50} values were calculated using GraphPad Prism software (version 5.01) based on data from three parallel experiments.

Apoptosis analysis with annexinV/PI assay. HepG2 cells cultured in 12-well plates were treated with drugs for 48 or 72 h. Apoptotic cells were detected by flow cytometry (BD FACS Calibur Flow Cytometer) after staining with Annexin V and Propidium Iodide (PI) using the AnnexinV-FITC apoptosis detection kit I (BD Biosciences, San Jose, USA). The data were analyzed using FlowJo software.

Cell cycle analyses. HepG2 cells cultured in 12-well plates were treated with drugs for 48 or 72 h. After then the cells were harvested and the DNA content was analyzed using the CycleTEST™ PLUS DNA Reagent Kit (BD Biosciences, San Jose, USA) according to the manufacturer's instructions. Cell cycle distributions and DNA contents were determined using BD FACS Calibur Flow Cytometer. The results were analyzed using FlowJo software. Percentages of cells in G_1, S, G_2/M phases were recorded.

In Vivo Pharmacokinetics studies. Pharmacokinetics studies of platinum formulations were performed in ICR mice. The mice were randomly assigned into four groups (four mice per group): cisplatin (iv, 1.5 mg/kg) and SCAN (iv, 1.5 mg/kg), asplatin (po, 20 mg/kg) and SCAN (po, 20 mg/kg) at equivalent platinum doses. At the predetermined time point 5 min, 30 min, 1 h, 2 h, 4 h, 6 h, 8 h, 12 h, 24 h, 48 h, 200 μL blood samples were collected from the retro-orbital plexus of the mouse into 1000 U/mL heparin sodium in PBS solution (10 μL). The blood was then centrifuged at

4°C (3000 g, 10 min) to collect the plasma. The content of platinum in plasma was measured with ICP-MS. The pharmacokinetic parameters were calculated using a non-compartmental model by the Drug and Statistics (DAS) software (version 3.2.6, Mathematical Pharmacology Professional Committee, China).

In vivo antitumor assay. The HepG2 single-cell suspension in PBS (5×10^6 per mouse) was injected subcutaneously into the buttock of mice. When the tumor grew to a size of 80-150 mm^3 at 18 days after cell implantation, the 45 mice were randomly divided into nine treatment groups (PBS, vehicle, cisplatin (iv, 1.5 mg Pt/kg), SCAN(iv, 1.5 mg Pt/kg), satraplatin (oral, 20 mg Pt/kg), asplatin (oral, 20 mg Pt/kg), SCAN(oral, 1.5 mg Pt/kg), SCAN(oral, 8 mg Pt/kg) and SCAN(oral, 20 mg Pt/kg). The drugs were given 5 times at 2-day intervals. Tumor growth was monitored by measuring the perpendicular diameter of the tumor using calipers every two days and calculated according to the formula: tumor volume (mm^3) = 0.5 × length × $width^2$. Two weeks after the last treatment, animals were sacrificed and kidneys and tumors were excised for immunohistochemistry analysis.

H&E and TUNEL assay. After the *in vivo* antitumor assay, the tissues were collected and fixed in 4% paraformaldehyde, paraffin-embedded, sectioned into 5 μm sections and mounted onto poly-L-lysine-coated glass slides, at the First Affiliated Hospital of Anhui Medical University. The slides were stained with haematoxylin/eosin (H&E). Apoptotic levels in tumor or kidney cells following the various treatments were determined using the transferase-mediated dUTP nick end-labeling (TUNEL) method according to the manufacturer's instructions (In Situ Cell Death Detection Kit, POD; Roche, Basel, Switzerland). All slides were examined under an Olympus IX81 microscope (Tokyo Prefecture, Japan).

Statistical Analysis. The statistical significance of treatment was assessed using the Prism software (GraphPad); The statistical differences were determined by ANOVA followed by Newman Keuls Post Hoctest or Student's t test. Values $p < 0.05$ indicate significant differences.

References:

[1] M. D. Hall, C. T. Dillon, M. Zhang, P. Beale, Z. Cai, B. Lai, A. P. Stampfl, T. W. Hambley, *Journal of biological inorganic chemistry : JBIC : a publication of the Society of Biological Inorganic Chemistry* **2003**, *8*, 726-732.

[2] P. Sengupta, S. Basu, S. Soni, A. Pandey, B. Roy, M. S. Oh, K. T. Chin, A. S. Paraskar, S. Sarangi, Y. Connor, V. S. Sabbisetti, J. Kopparam, A. Kulkarni, K. Muto, C. Amarasiriwardena, I. Jayawardene, N. Lupoli, D. M. Dinulescu, J. V. Bonventre, R. A. Mashelkar, S. Sengupta, *Proceedings of the National Academy of Sciences of the United States of America* **2012**, *109*, 11294-11299.

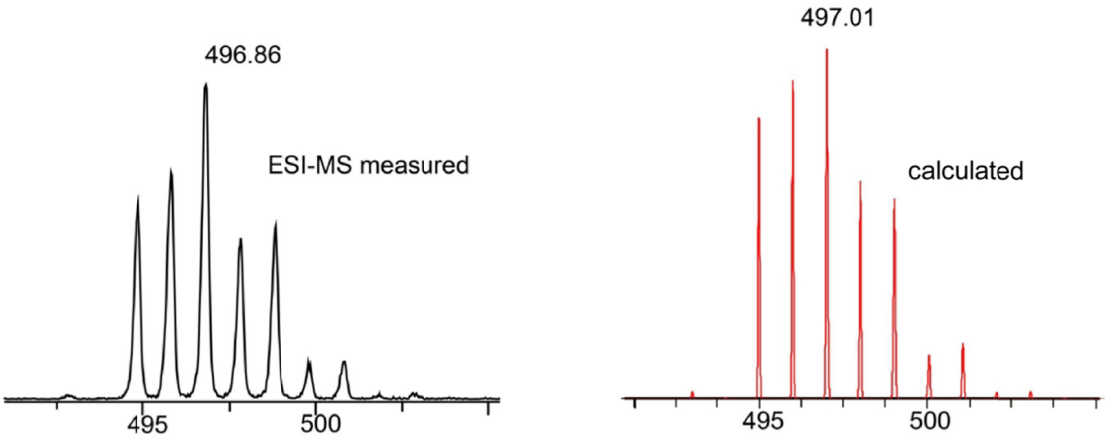

Figure S1. ESI-MS of asplatin measured in the positive mode. Composition: $[Pt(NH_3)_2Cl_2(C_9H_7O_4)OH+H]^+$. The right portion shows the theoretically simulated spectrum of asplatin. The measured m/z is 496.86 and the calculated m/z is 497.01.

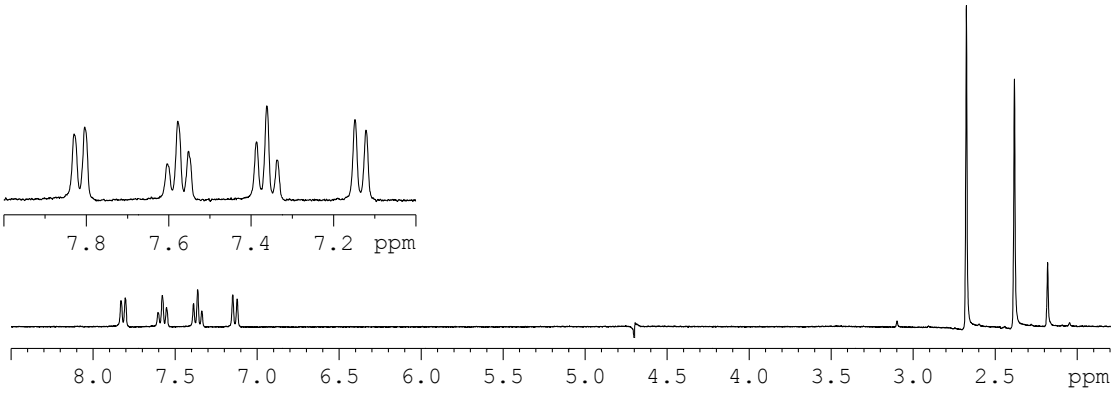

Figure S2. ^1H NMR spectrum of asplatin, *c,c,t*-$[Pt(NH_3)_2Cl_2(C_9H_7O_4)OH]$ in D_2O. The spectrum was recorded using presaturation pulse sequence to suppress the residual HDO signal. The inset shows the expansion of the aromatic proton signals.

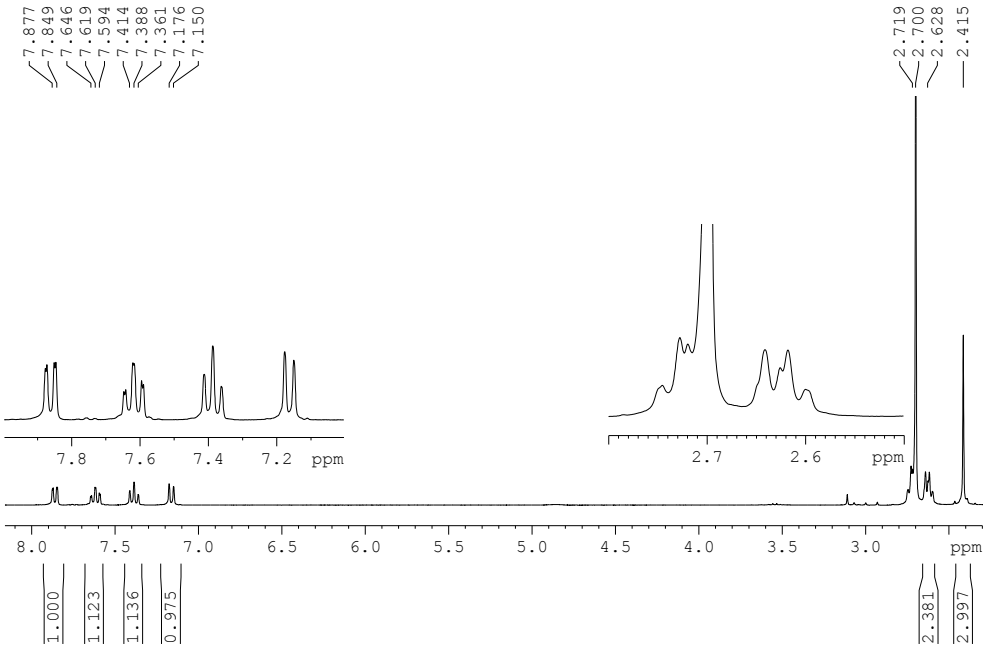

Figure S3. ^{1}H NMR spectrum of asplatin-succinic acid in D_2O. The spectrum was recorded using presaturation pulse sequence to suppress the residual HDO signal. The insets show the expanded regions of interest.

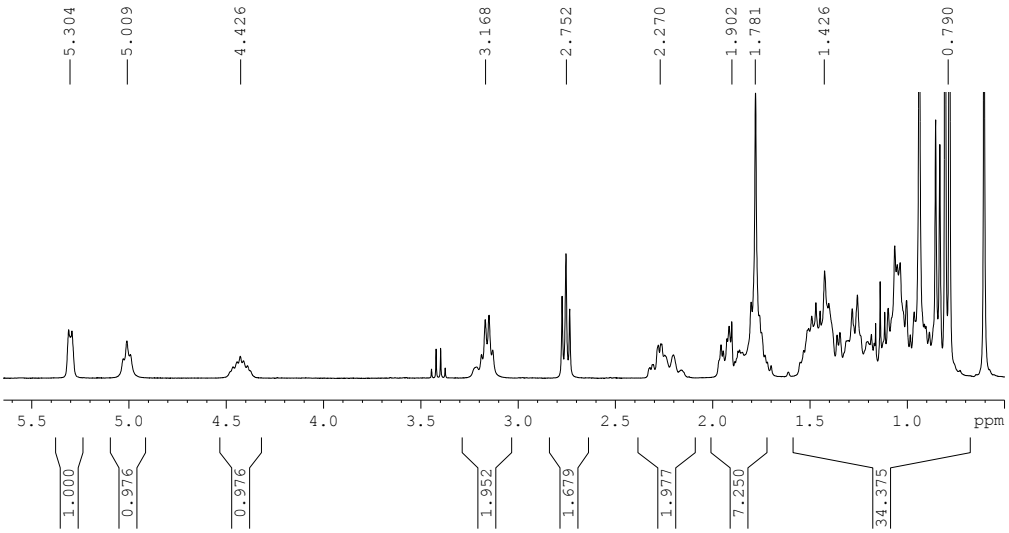

Figure S4. ^{1}H NMR spectrum of cholesterol-ethylenediamine in $CDCl_3$.

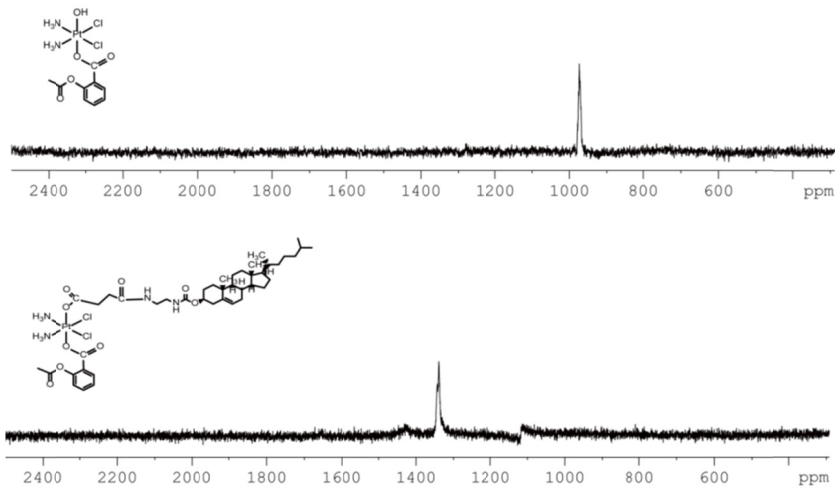

Figure S5. ^{195}Pt NMR spectrum of asplatin and cholesterol-asplatin in $CDCl_3$.

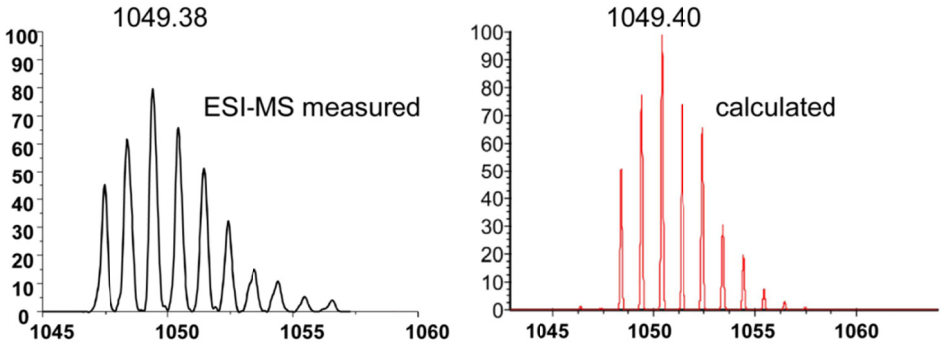

Figure S6. ESI-MS of cholesterol-asplatin, $[Pt(NH_3)_2Cl_2$(asplatin-succinic acid)(cholesterol-ethylenediamine)$]^-$ measured in the negative mode. Composition: $C_{43}H_{68}Cl_2N_4O_9Pt$. The right portion shows the theoretically simulated spectrum of cholesterol-asplatin. The measured m/z is 1049.38 and the calculated m/z is 1049.40.

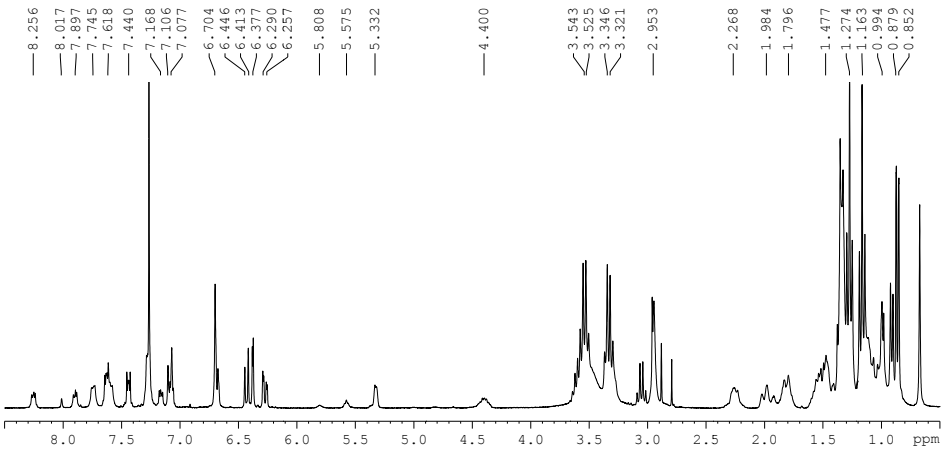

Figure S7. ^{1}H NMR spectrum of cholesterol-rhodamine in $CDCl_3$.

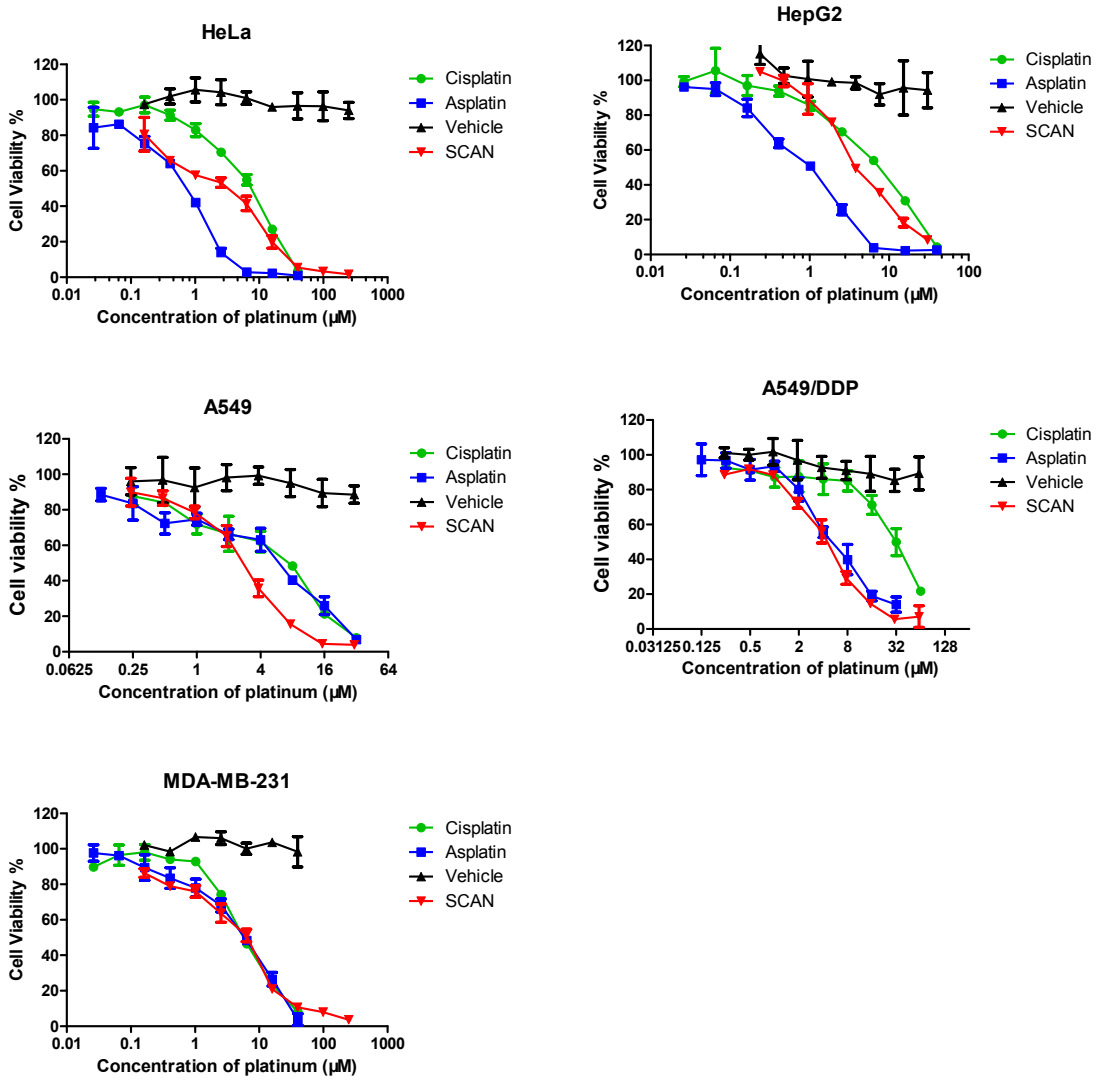

Figure S8. Cytotoxicity assay of cisplatin, asplatin and SCANs on different tumor cells. Vehicle was prepared in the same method as SCANs in the absence of the platinum complex.

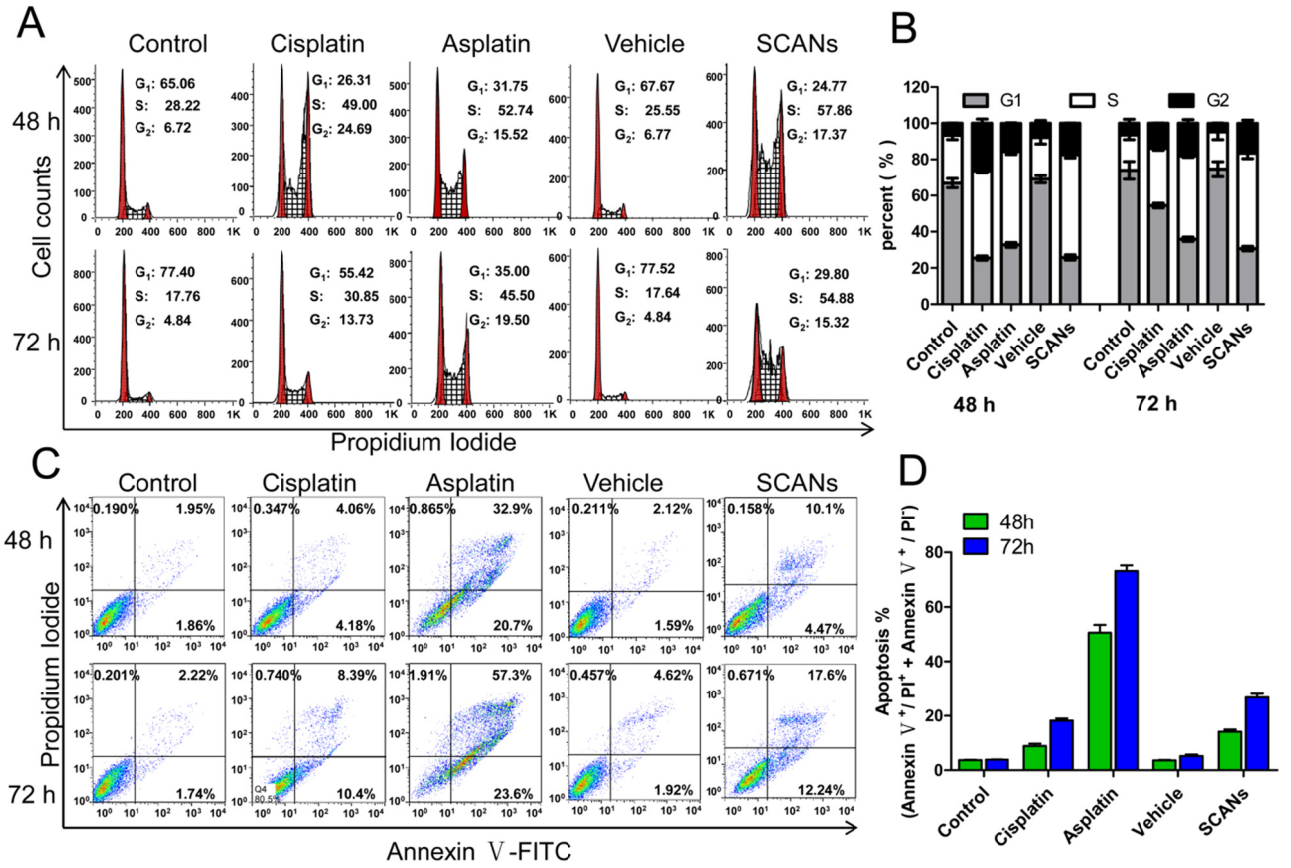

Figure S9. Cell cycle and apoptosis analysis. (A) Cell cycle analyses on HepG2 cells with the treatment of 4 μM cisplatin, asplatin and SCANs, respectively. (B) Quantification of the cells arrested in the different phases of cell cycle. (C) Apoptosis induced by 4 μM cisplatin, asplatin, or SCANs using an annexin V/PI assay. (D) Quantification of the apoptotic cells.

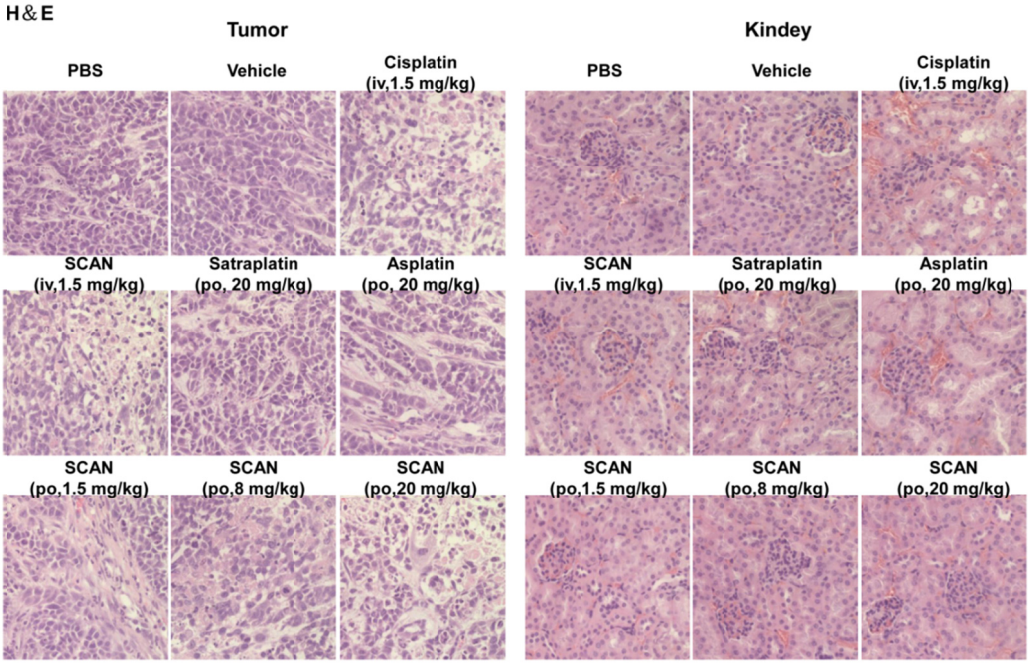

Figure S10. H&E analyses of tumor and kidney tissues. Hemalum stains nuclei in blue and eosin Y stains eosinophilic cytoplasm in various shades of red, pink and orange.

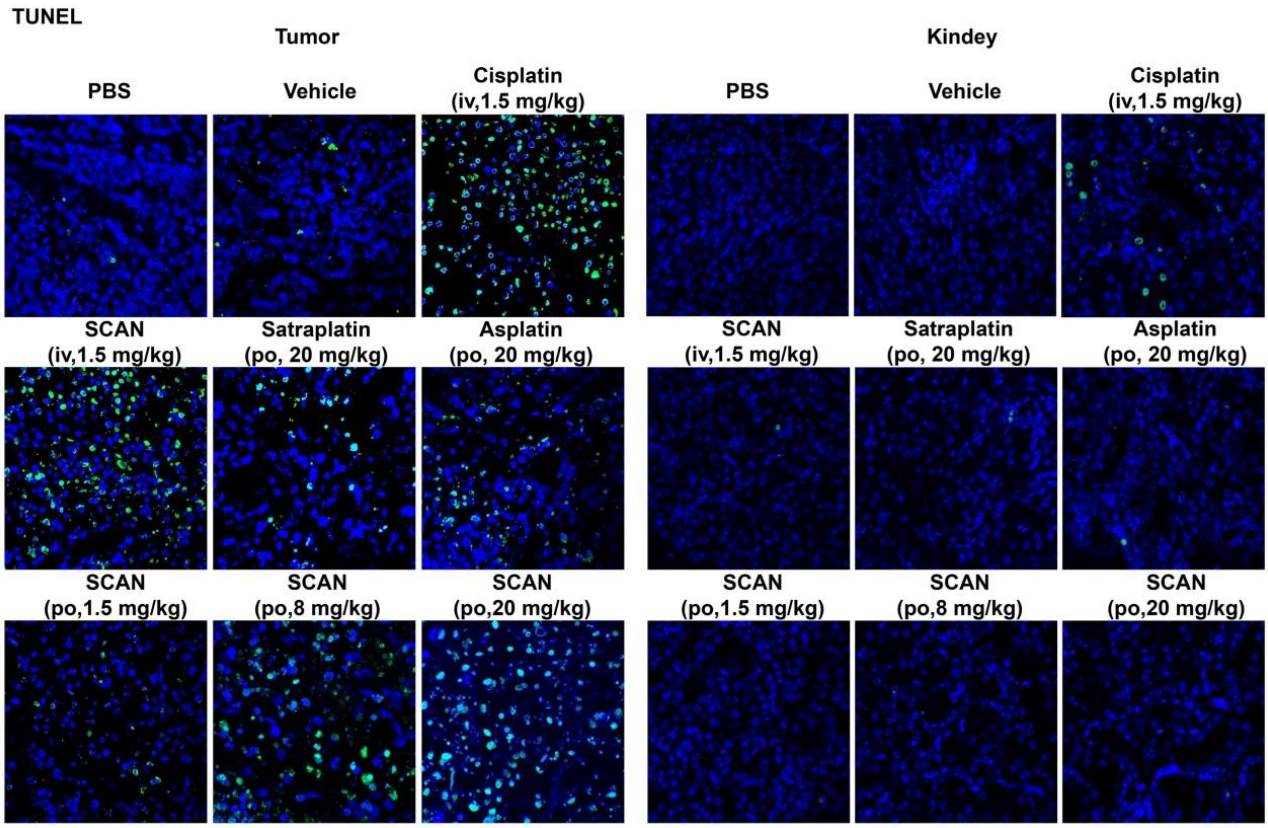

Figure S11. TUNEL analyses of tumor and kidney tissues. Nuclei are stained by DAPI in blue; the TUNEL-positive apoptotic cells are stained green.

Table S1. In vivo pharmacokinetic data with the treatment of platinum complexes.

PK parameters	Cisplatin (iv, 1.5 mg/kg)	SCANs (iv, 1.5 mg/kg)	Asplatin (po, 20 mg/kg)	SCANs (po, 20 mg/kg)
C_{max} (mg/L)	--	--	0.66±0.08	1.06±0.09
T_{max} (h)	--	--	0.36±0.24	7.33±1.16
AUC (mg h/L)	8.12±1.32	35.0±12.1	4.53±0.12	19.6±2.1
$t_{1/2}$ (h)	16.1±7.5	33.9±19.4	14.8±1.6	24.9±12.4
CL (L/h/kg)	0.19±0.03	0.05±0.02	4.42±0.12	1.03±0.12

C_{max}: peak concentration;
T_{max}: time to reach peak concentration;
AUC: area under the plasma concentration-time curve;
$t_{1/2}$: half-life of drug in plasma, at which the drug concentration drops to 1/2 C_{max};
CL: plasma clearance, the rate of drug clearance from plasma.