Supporting Information

Sengupta et al. 10.1073/pnas.1203129109

SI Materials and Methods

All reactions were performed under inert conditions unless otherwise indicated. All commercially obtained compounds were used without further purification. Dichloromethane (DCM), dry DCM, methanol, cholesteryl chloroformate, cholesterol, ethylenediamine, succinic anhydrite, silver nitrate, sodium sulfate, pyridine, cisplatin, L-α-phosphatidylcholine (PC), sephadex G-25, FITC, and 1,2-phenylenediamine were bought from Sigma-Aldrich; 1,2-Distearoyl-*sn*-Glycero-3-Phosphoethanolamine-*N*-[Amino(Polythylene Glycol)2000] (DSPE-PEG) and the mini handheld Extruder kit (including 0.2 μm Whatman Nucleopore Track-Etch Membrane, Whatman filter supports, and 1.0 mL Hamiltonian syringes) were bought from Avanti Polar Lipids. Anhydrous solvent dimethylformamide (DMF) was supplied by Acros Organics. Phosphotungstic Acid was from Ted Pella. Analytical TLC was performed using precoated silica gel aluminum sheets 60 F_{254} bought from EMD Laboratories. Spots on the TLC plates were visualized using alkanine permanganate or 6% ninhydrin solution in acetone. ^{1}H NMR (300 MHz) and ^{13}C NMR (75 MHz) spectra were obtained on a Varian Mercury 300 spectrophotometer. The chemical shifts are expressed in parts per million (ppm) using suitable deuterated NMR solvents with reference to TMS at 0 ppm. MTS reagent was supplied by Promega. The cell viability assay and release kinetic data were plotted using GraphPad Prism software. Each sample was repeated at least in triplicate.

Synthesis of Cholesterol-Ethylenediamine Conjugate. For synthesis of cholesterol-ethylenediamine conjugate, 1,044 μL (15.58 mmol, 14 equiv) of ethylene diamine (compound **2**) was dissolved in 5.0 mL anhydrous DCM followed by cooling down to 0–5 °C with ice. Next, 500.0 mg (1.113 mmol, 1.0 equiv) of cholesteryl chloroformate was dissolved in 5.0 mL anhydrous DCM and was added to the reaction mixture drop-wise over a period 15 min with vigorous stirring, and was continued overnight until it came to room temperature. The reaction was worked up 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 evaporated with the help of a rotary evaporator. Light yellow colored clear oily product (compound **3**) was separated with 99.1% yield. ^{1}H-NMR (300 MHz) δ (ppm) = 5.37 (s, 1H), 5.06 (S, 1H), 4.49 (bs, 1H), 3.22–3.20 (m, 2H), 2.82–2.81 (m, 2H), 2.34–2.26 (m, 2H), 2.02–1.83 (m, 6H), 1.54–0.84 (m, 37H). ^{13}C-NMR (75 MHz) δ (ppm) = 156.7, 140.1, 122.7, 74.5, 56.9, 56.3, 50.2, 42.5, 42.0, 39.9, 39.7, 38.8, 37.2, 36.8, 36.4, 36.0, 32.1, 28.5, 28.4, 24.5, 24.1, 23.1, 22.8, 21.3, 19.6, 18.9, 12.1.

Synthesis of Cholesterol-Ethylenediamine-Succinic Acid Conjugate. For synthesis of cholesterol-ethylenediamine-succinic acid conjugate, 350 mg (0.74 mmol, 1 equiv) of compound **3** was dissolved in 5.0 mL anhydrous DCM. To this mixture, 370.0 mg (3.7 mmols, 5 equiv) of succinic anhydride and 2 mL of pyridine were added. The stirring was continued for 24 h followed by work up in 0.1 N HCl and DCM several times. The organic layer was dried over sodium sulfate and evaporated to get white amorphous solid compound (compound **5**). Yield: 95%. ^{1}H-NMR (300 MHz) δ (ppm) = 7.72–7.70 (m, 1H), 7.54–7.53 (m, 1H), 5.37 (s, 1H), 5.07 (s, 1H), 4.49 (bs, 1H), 4.22–4.19 (m, 2H), 3.36–3.30 (m, 4H), 2.68–2.33 (m, 4H), 2.02–1.83 (m, 6H), 1.54–0.84 (m, 37H). ^{13}C-NMR (75 MHz) δ (ppm) = 174.5, 174.2, 156.3, 140.4, 122.4, 74.5, 56.9, 56.4, 50.1, 42.5, 40.9, 39.3, 36.7, 36.9, 36.0, 30.6, 29.8, 29.4, 28.4, 28.1, 23.3, 23.0, 19.1, 12.0.

Synthesis of Aquated Cisplatin $[Pt(NH_3)_2(OH_2)_2]^{2+}$. For synthesis of aquated cisplatin $[Pt(NH_3)_2(OH_2)_2]^{2+}$, 50 mg (0.166 mmol, 1 equiv) of cisplatin was partially dissolved in 10.0 mL of H_2O. To this mixture, 28.0 mg (0.166 mmol, 1 equiv) of silver nitrate was added and the resulting reaction mixture was stirred at room temperature for 24 h. It looked milky white and silver chloride was removed by centrifuging at 25,000 × *g* for 1 h. Finally, the aquated cisplatin (compound **6**) was obtained by filtration through 0.2 μm filter.

Synthesis of Cholesterol-Cisplatin Conjugate. For synthesis of cholesterol-cisplatin conjugate (**7**), 200 mg (0.35 mmol, 1.0 equiv) of compound **5** was dissolved in 5.0 mL DMF. To this mixture, 20.0 mL of aquated cisplatin (compound **6**) (conc 5.0 mg/mL, 1.0 equiv) was added and stirred for 24 h. The solvent was evaporated using a lyophilizer. The dried product (compound **7**) was purified using a 500-Da molecular weight cutoff (MWCO) dialysis membrane for 24 h followed by lyophilization. ^{195}Pt-NMR :δ (ppm) = −1,621.497 (s).

General Procedure of Synthesizing Self-Assembling Cholesterol-Succinic Acid-Cisplatinum II-Based Nanoparticles. The general procedure of synthesizing self-assembled cholesterol-succinic acid-cisplatinum II-based nanoparticles (SACNs) is as follows: 10.0 mg of PC, 5.0 mg cholesterol-cisplatin conjugate (**7**), and 1.0 mg of DSPE-PEG were dissolved in 10.0 mL DCM. Solvent was evaporated into a thin and uniform lipid-drug film using a rotary evaporator. The lipid-drug film was then hydrated with 1.0 mL H_2O for 1 h at 60 °C. The hydrated nanoparticles looked light yellow to white with little viscous texture. This mixture was passed although Sephadex G-25 column and extruded at 65 °C to obtain sub-200 nm particles.

General Method of Pt(II) Quantification in SACNs. A measured amount of the SACNs was heated at 100 °C in 1.2 mg/mL concentration of 1,2-phenylenediamine in DMF for 2 h. Pt(II) amount was calculated by UV-VIS spectrophotometry by using standard absorbance vs. concentration curve drawn at wavelength λ = 706 nm (Shimadzu 2450). This result was validated using an inductively coupled plasma-atomic absorption spectroscopy (ICP-AAS)-based method.

Release Kinetics of Pt(II) from Nanoparticle at Different pH. Concentrated drug-loaded nanoparticles were suspended in buffer (pH = 5.5 and 8.5) and sealed in a dialysis membrane (MWCO = 500 Da; Spectrum Lab). The dialysis bags were incubated in 30 mL PBS buffer at room temperature with gentle shaking. A 500-μL portion of the aliquot was collected from the incubation medium at predetermined time intervals, and the released drug was quantified by UV-VIS spectrophotometer (Shimadzu 2450) and ICP-AAS.

Sample Preparation for Cryo-Transmission Electron Microscopy. The sample was preserved in vitrified ice supported by holey carbon films on 400 mesh copper grids. The sample was prepared by applying 3 μL of sample suspension to a cleaned grid, blotting away with filter paper and immediately proceeding with vitrification in liquid ethane. Grids were stored under liquid nitrogen until transferred to the electron microscope for imaging. Electron microscopy was performed using an FEI Tecnai T12 microscope, operating at 120 KeV equipped with an FEI Eagle 4K × 4K CCD camera. Vitreous ice grids were transferred into the electron microscope using a cryostage that maintains the

grids at a temperature below −170 °C. Images of the grid were acquired at multiple scales to assess the overall distribution of the specimen. After identifying potentially suitable target areas for imaging at lower magnification, high magnification images were acquired at nominal magnification of 52,000× (0.21 nm/pixel) and 21,000× (0.50 nm/pixel). Images were acquired at a nominal underfocus of −5 μm (21,000×) and −4 μm (52,000×) at electron doses of ∼10–15 e/A^2.

Cell Viability Assay. The Lewis lung carcinoma (LLC) cells, breast cancer cell line (4T1), and hepatocellular carcinoma cells (CP20) were purchased from American Type Culture Collection. LLC cells and CP20 cells were cultured in DMEM and 4T1 cells were cultured in RPMI medium 1640-supplemented with 10% FBS, 50 unit/mL penicillin, and 50 unit/mL streptomycin. Trypsinized cultured LLC, 4T1, and CP20 cells were washed twice with PBS and seeded into 96-well flat-bottomed plates (4×10^3 cells per well). Free drugs and SACNs were added at appropriate concentrations (0.01, 0.1, 1, 10, 20, 50 μM Pt concentration). The plates were then incubated for 48 h in a 5% CO_2 atmosphere at 37 °C. The cells were washed and incubated with 100 μL phenol-red free medium (without FBS) containing 20 μL of the CellTiter 96 Aqueous One Solution reagent (Promega). After 2 h incubation in 5% CO_2 atmosphere at 37 °C, the absorbance in each well was recorded at 490 nm using an Epoch (BioTek) plate reader. Results were quantified by subtracting the blank value from each value then normalizing against the control values and results were analyzed by using Prism software (GraphPad). Data shown are mean ± SE of $n = 3$.

Synthesis of FITC-Labeled SACNs. For synthesis of FITC-labeled SACNs, 10.0 mg of PC, 5.0 mg of cholesterol-cisplatin conjugate **7**, 1.0 mg of DSPE-PEG, and 1 mg of FITC were dissolved in 10.0 mL DCM. Solvent was evaporated into a thin and uniform lipid-drug film with the help of a rotary evaporator. The lipid-drug film was hydrated with 1.0 mL H_2O for 2 h at 60 °C. The hydrated nanoparticles appeared light yellow to white with little viscous texture. The mixture was passed although Sephadex G-25 column and extruded at 65 °C in dark to obtain sub 200-nm particles.

FITC-SACN Internalization Study. The 4T1 or 7404-CP20 cells were seeded on glass cover-slips in 24-well plates until subconfluent, and then treated with FITC-encapsulated SACNs for a time-course ranging from 30 min to 18 h. At the indicated times, cells were washed twice in PBS and incubated in LysoTracker Red (Ex: 577 nm; Em: 590 nm) for 30 min at 37 °C. Cells were then washed again, fixed in 4% paraformaldehyde, then treated with DAPI (Ex: 350 nm; Em: 470 nm) for 30 s, and mounted using Prolong Gold antifade reagent (Invitrogen). Images taken in three random fields were captured at 40× using an inverted microscope (Nikon) equipped with UV, blue, and green filters to visualize DAPI, FITC, and LysoTracker red fluorescence, respectively. Cells incubated either only FITC encapsulated CNP or LysoTracker red served as negative controls. Pharmacological inhibitors are often used to study endocytic pathways. We used Chlorpromazine (25 μM) as Clathrin-mediated endocytosis inhibitor, Nystatin (25 μg/mL) as caveolae-mediated endocytosis inhibitor, and Cytochalasin D (5 μg/mL) as macropinocytosis/phagocytosis inhibitor. Cells were pretreated with the inhibitors for 2 h before incubation with the FITC-SACNs for 4 h, following which they were imaged after being processed as described earlier. In other studies the cells were incubated at 4 °C to block energy-dependent endocytosis, as described by Drin et al. (1)

Quantification of Intracellular Pt in Vitro in 7404-CP20 Cells. For quantification of intracellular Pt in vitro in 7404-CP20 cells, 5×10^5 CP20 cells were plated in 100-mm cell culture dish. When cells became around 70% confluent, they were serum-deprived for 6 h before the addition of the drugs. Cells were incubated with either 20 μM of free cisplatin or SACN at 37 °C or same dose of SACN at 4 °C to inhibit energy-dependent internalization. After that, drug-containing media was removed and cells were washed three times using cold PBS. Cell numbers from each dish were counted following trypsinization, and then lysed overnight in 70% nitric acid. Nitric acid was then evaporated and amount of Pt was measured by ICP-MS after appropriate dilutions in 5% nitric acid.

FACS Analysis of Apoptosis. The 4T1 cells were grown in six-well plates incubated in the presence of SACNs or free cisplatin or free carboplatin at 1 μM concentration at 37 °C for 24 h. After 24 h, the cells were washed with PBS and collected at 0 °C. The cells were then treated with Annexin-V-Alexa Fluor 488 conjugate (Molecular Probes, Invitrogen) and incubated in the dark, at room temperature, for 15 min. The cells were then washed with PBS and incubated with propidium iodide (PI) solution (50 g/mL; Sigma) containing RNase (1 mg/mL; Sigma). The cell suspension were then transferred to FACS tubes and analyzed for Annexin-V/PI staining on a BD FACS Calibur instrument. Data were analyzed using a CellQuestPro software (BD Biosciences).

In Vivo Murine 4T1 Breast Cancer Model. The 4T1 breast cancer cells (3×10^5) were implanted subcutaneously in the flanks of 4-wk-old BALB/c mice (weighing 20 g; Charles River Laboratories). The drug therapy was started on day 9. The drug therapy consisted of administration of SACNs (1 mg/kg and 3 mg/kg), free cisplatin (1 mg/kg and 3 mg/kg), and free carboplatin (3 mg/kg) (administered by tail-vain injection). PBS (100 μL) administered by tail-vain injection was used as a control for drug treatment. The tumor volumes and body weights were monitored on a daily basis. The tumor volume was calculated by using the formula, $L \times B^2$, where the longest diameter was considered as L and the shortest diameter as measured using a vernier caliper as B. The animals were killed when the average tumor volume of the control exceeded 2,000 mm^3 in the control group. The tumors were harvested immediately following sacrifice and stored in 10% formalin for further analysis. All animal procedures were approved by the Harvard Institutional Use and Care of Animals Committee.

In Vivo Murine Ovarian Cancer Tumor Model. Ovarian adenocarcinomas were induced in genetically engineered K-$ras^{LSL/+}$/$Pten^{fl/fl}$ mice via intrabursal delivery of adenovirus-carrying Cre recombinase. Tumor cells were engineered to express luciferase once activated by Adeno-Cre, to make tumor imaging feasible before and after drug treatment. Once mice developed medium-to-large tumors they were placed into one of three treatment groups (control, cisplatin 3 mg/kg, and SACNs), with all drugs administered intravenously via tail vein. The dose of SACNs was selected to be equivalent in Pt content as 3 mg/kg of cisplatin. The animals were injected with the drugs three times over a 6-d period with a 1-d interval between the dosings. Tumor imaging in vivo was performed with the IVIS Lumina II Imaging System. Quantification of bioluminescence was achieved by using the Living Image Software 3.1 (Caliper Life Sciences). Mice received 150 mg/kg of D-luciferin firefly potassium salt via intraperitoneal injection before imaging. Five minutes postluciferin injection, animals were anesthetized in a 2.5% isoflurane induction chamber where they were kept under anesthesia by a manifold supplying isoflurane and their body temperature was maintained by a 37 °C temperature stage. Bioluminescent signal was collected 15 min after luciferin administration for an exposure time of 30 s. Images were taken a day before treatment (day 0, baseline), in the middle of the treatment cycle, and 1 d following the final treatment. Treatment efficacy was quantified by examining

the fold-increase in bioluminescence of the posttreatment signal compared with baseline. Statistical analysis of the toxicity data were analyzed using a one-way ANOVA test with the Prism 5 software.

Biodistribution of Cisplatin and SACNs. After the in vivo experiments, the animals were killed and the organs were harvested. The organs were then weighed and dissolved in concentrated HNO_3 (approximately 10 mL) by shaking for 24 h at room temperature and then heating at 100 °C for 12 h. To these mixtures 30% H_2O_2 was added, the resulting solutions were stirred for 24 h at room temperature, and then heated for another 12 h to evaporate the liquids. All solid residues were redissolved in 1 mL water and then amount of platinum was measured by inductively coupled plasma-spectrometry.

Platinum II can react with nitrogen, sulfur, and oxygen residues in other biomolecules, such as plasma proteins. As a result of these reactions, a variety of platinum species may be present in the body after treatment with cisplatin. This protocol does not distinguish platinum that may have become deactivated by reactions with plasma proteins from active drug, and only quantifies the total Pt concentrations at defined time point. We anticipate that such interactions will be normalized when we make comparisons between the SACN-treated groups and cisplatin-treated animals. However, there is a possibility that the preferential release of Pt (II) from SACNs in acidic vs. physiological pH might translate into lower deactivation by coordination with plasma proteins, and greater concentration of activated Pt (II) in the tumor.

Histopathology and TUNEL Assay (Apoptotic Assay). The tissues were fixed in 10% formalin, paraffin-embedded, and sectioned at the Harvard Medical School Core Facility. Tumor and kidney paraffin sections were deparaffinized and stained with standard TMR red fluorescent TUNEL kit following the manufacturer's protocol (In Situ Cell Death Detection Kit, TMR-Red; Roche). Images were obtained using a Nikon Eclipse TE2000 fluorescence microscope equipped with red filter.

Immunohistochemical Analysis of Kidney Injury Molecule-1. Immunohistochemical analysis was performed on formalin-fixed, paraffin-embedded tissue sections. Briefly, paraffin embedded tissue sections were deparaffinized, rehydrated, and antigen retrieval was carried out in 0.1 M citrate buffer (pH 6.0) for 20 min in a pressure cooker. The slides were then washed in PBS solution and incubated in 3% BSA solution for 30 min following which they were incubated with goat anti-mouse kidney injury molecule-1 (Kim-1) antibody (R&D Systems), at 1:500 dilution for 60 min. Slides were washed in PBS solution and incubated in anti-goat secondary antibody (1:200 dilution; R&D Systems) for another 30 min. Staining of the tissue sections were done using VECTASTAIN ABC kit (Vector Laboratories) for 30 min followed by counterstaining with hematoxylin. Pictures were taken using Nikon ECLIPSE 90i microscope.

1. Drin G, Cottin S, Blanc E, Rees AR, Temsamani J (2003) Studies on the internalization mechanism of cationic cell-penetrating peptides. *J Biol Chem* 278:31192–31201.

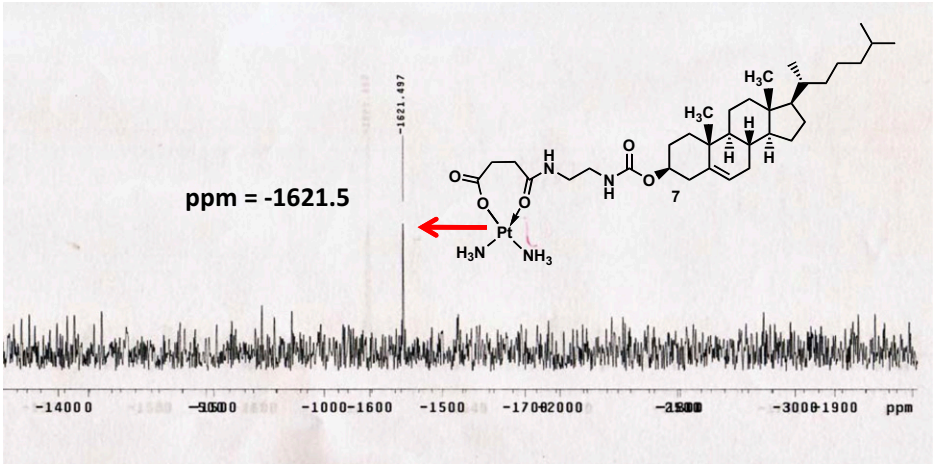

Fig. S1. ^{195}Pt NMR spectra of the cholesterol-ethylenediamine-succinic acid conjugate with Pt.

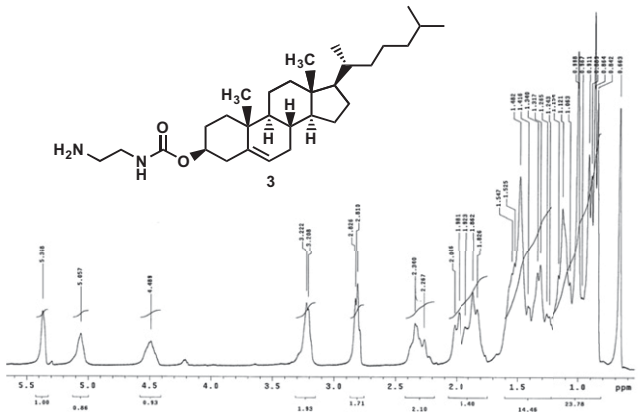

Fig. S2. ^{1}H NMR spectra of cholesterol-ethylenediamine conjugate.

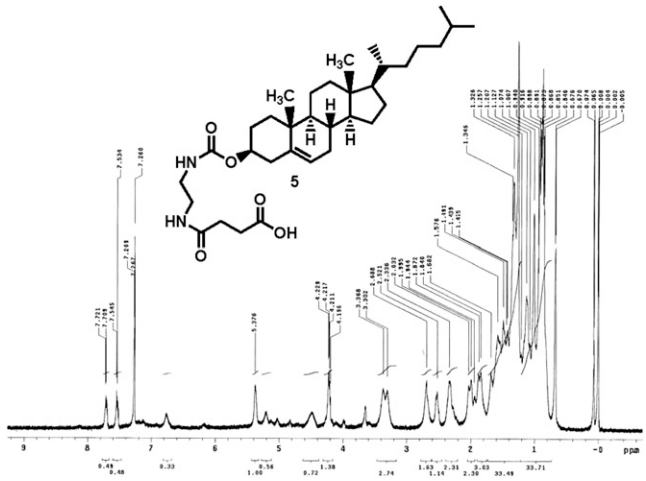

Fig. S3. ^{1}H NMR spectra of cholesterol-ethylenediamine-succinic acid conjugate.

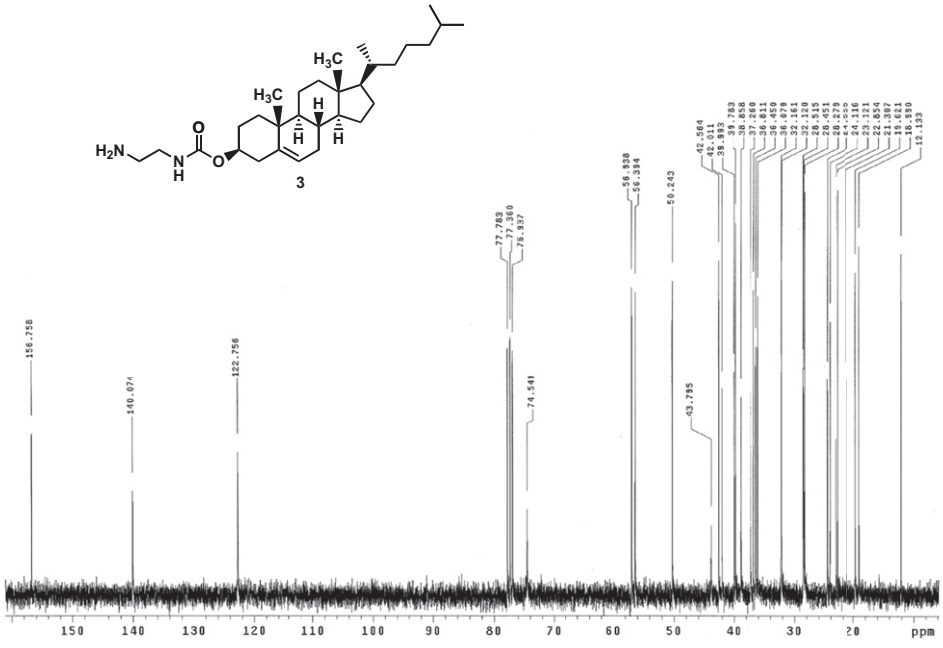

Fig. S4. The ^{13}C NMR spectra of cholesterol-ethylenediamine conjugate.

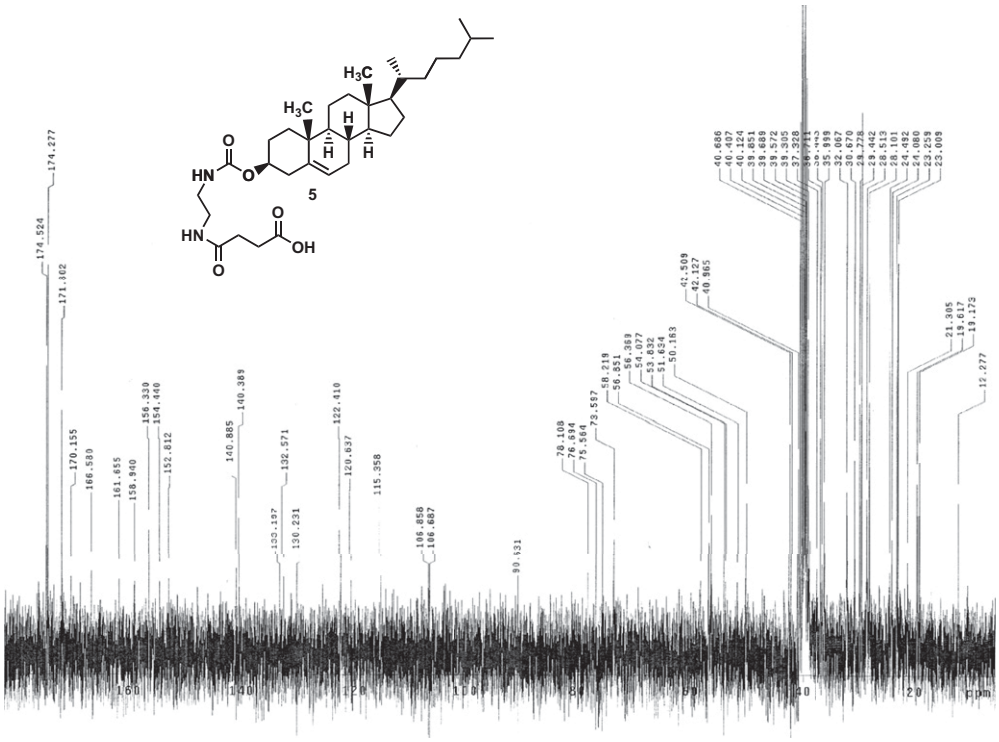

Fig. S5. The ^{13}C NMR spectra of cholesterol-ethylenediamine-succinic acid conjugate.

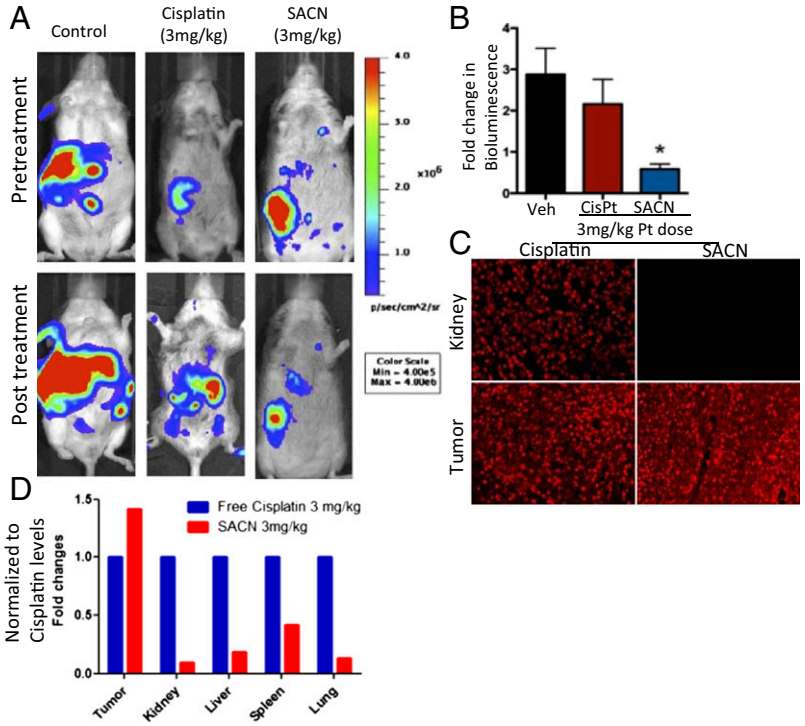

Fig. S6. In vivo anti-tumor activity of SACNs in a K-Ras$^{LSL/+}$/Pten$^{fl/fl}$ ovarian cancer model. (*A*) Representative images from treatment groups (cisplatin and SACNs, 3 mg/kg equivalent Pt dose) before and after treatments. Tumor images were obtained with the IVIS Lumina II Imaging System. (*B*) Bioluminescence quantification indicates a significantly decreased tumor luciferase signal in mice treated with SACNs compared with vehicle or cisplatin. Quantification of bioluminescence was achieved by using the Living Image Software 3.1. Data shown are mean ± SE of n = minimum of three animals per group. *$P < 0.05$ (ANOVA followed by Newman–Keuls post hoc test). (*C*) Representative images of kidney and tumor cross-sections with TUNEL as apoptosis marker. Images were captured using a Nikon Ti epifluorescence microscope at 20× magnification at 1,000 × 700 pixels. (*D*) Representative tissue distribution of platinum in different treatment groups as determined by ICP-MS shows preferential accumulation of SACNs in tumor vs. kidney.

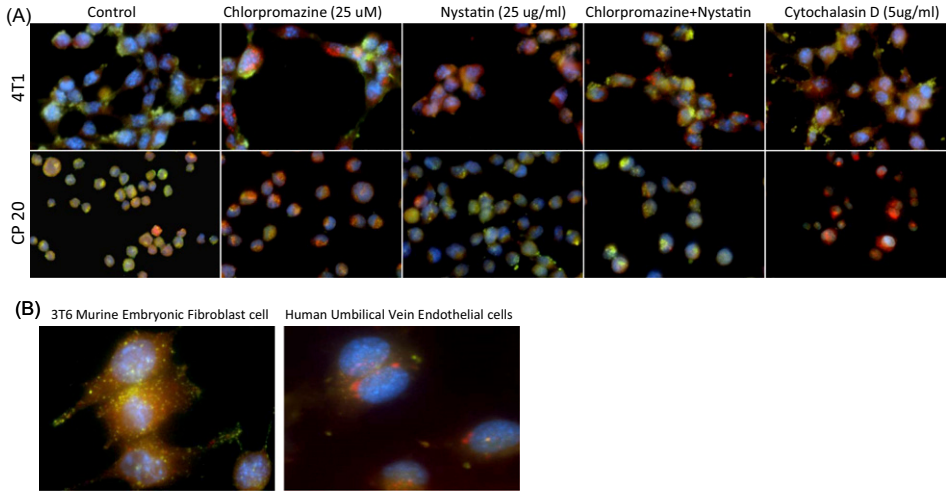

Fig. S7. (*A*) Internalization study of FITC-labeled SACNs in 4T1 and 7404-CP20 cells pretreated with different chemical inhibitors of endocytosis. Representative images were captured at 40× magnification. None of the inhibitors can block the endocytosis of SACN completely. (*B*) Representative images of human umbilical vein endothelial cells or fibroblasts treated with FITC-labeled SACNs. Pictures captured after 4 h of incubation (40× magnification). The cells were counterstained with DAPI to label the nuclei and with LysoTracker red to label the endosomes. The 4T1 images are 1,000 × 700 pixels; CP20 images are 900 × 600 pixels.