Supporting information

for

Modular Design of Nanobody-drug Conjugates for Targeted-delivery of Platinum Anticancer Drug with MRI Contrast Agent

Experimental Procedures

Materials

cis-$[PtCl_2(NH_3)_2]$ (cisplatin) was purchased from Shandong Boyuan Pharmaceutical Co., Ltd. (Sandong, China). Fluorescein isothiocyanate isomer I (FITC) was purchased from Aladdin (Shanghai, China). 6-Maleimidocaproic acid was purchased from Bide Pharmatech Ltd. (Shanghai, China.). (1-Cyano-2-ethoxy-2-oxoethylidenaminooxy)dimethylamino-morpholinocarbenium hexafluorophosphate (COMU) was purchased from Alfa Aesar (Tewksbury, US.). Gadopentetate dimeglumine (Gd-DTPA) was purchased from Shen Zhen Reagent Biotechnology Co., Ltd. (Shenzhen, China.). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra-zolium bromide (MTT) and Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). Dulbecco's Modified Eagle's medium (DMEM), trypsin-EDTA and fetal bovine serum (FBS) were obtained from Biological Industries (Kibbutz Beit-Haemek, Israel). Anti-EGFR were obtained from Abcam (Cambridge, US). Anti-GAPDH antibody was purchased from Proteintech (Wuhan, China). Apoptosis detection kit was purchased from Solarbio Science & Technology Co., Ltd. (Beijing, China). Ultra-purified water was prepared using a Milli-Q Synthesis System (Millipore, Bedford, MA). All other solvents and reagents were used as received.

Cell culture

The human cutaneum carcinoma cells A375 and A431 cells were obtained from the American Type Culture Collection (ATCC) and maintained in DMEM containing 10% FBS in a humidified atmosphere containing 5% CO_2 at 37°C.

Animals

Balb/c nude mice (5 weeks old) and 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 on animal were approved by the 'Animal Care and Use Committee' of University of Science and Technology of China.

Synthesis of maleimide-functionalised platinum(IV) prodrug (compound 2)

2 mL of H_2O_2 (30% w/v) was added to the suspension of 300 mg of cisplatin in 60 mL of acetic acid. The mixture was stirred at 60ºC for 4 h yielding a bright yellow solution. The solution was reduced to near dryness on a rotary evaporator. After that, 5 mL of acetone and, subsequently, a lot of cool diethyl ether led to the precipitation of a yellow solid which was dried in vacuo to afford 420 mg of hydroxyl(acetoxy)cisplatin acetic acid complex (compound **1**) as a yellow solid (96% yeild). ^{1}H NMR

(300 MHz, DMSO-d_6, ppm): δ 6.13-5.87 (m, 6H), 2.09 (s, 3H), 1.87 (s, 3H). ESI-MS (m/z): 374.96 [M-H^+].

For the preparation maleimide-functionalised platinum(IV) prodrug (compound **2**), compound **1** (300 mg, 0.688 mmol, 1 eq.) suspended in anhydrous DMF (15 mL) was added 6-Maleimidocaproic acid (174.4 mg, 0.826 mmol, 1.2 eq.) followed by 4-methylmorpholine (NMM) (84 mg, 0.826 mmol, 1.2 eq.) and 1-Cyano-2-ethoxy-2-oxoethylidenaminooxy)dimethylamino-morpholinocarbenium hexafluorophosphate (COMU) (353.8 mg, 0.826 mmol, 1.2 eq.). The mixture was stirred at 40ºC for 12 h yielding a yellow solution. The solution was reduced to near dryness on a rotary evaporator. The resulting residue was diluted in water and washed twice with tert-butyl methyl ether. The aqueous layer was concentrated to about 4 mL on a rotary evaporator. This concentrated sample was injected onto a pre-packed RediSep Rf Gold C18 colum (50g) for and eluted on a reverse phase system with a 15-50% (CH_3CN/water) 15 CV gradient. Pure collected fractions were combined and concentrated on a rotary evaporator and dried in vacuo to afford 280 mg of maleimide-functionalised platinum(IV) prodrug (compound **2**) as a white solid (71.5% yeild). ^{1}H NMR (300 MHz, DMSO-d_6, ppm): δ 7.00 (s, 2H), 6.68-6.39 (m, 6H), 3.37 (t, 2H), 2.19 (t, 2H), 1.52-1.40 (m, 4H), 1.9 (s, 3H), 1.27-1.17 (m, 2H). ESI-MS (m/z): 568.02 [M-H^+].

Protein expression and purification

All proteins were expressed in pET22b with a His_6 and Trx tag in *E. coli* Rosetta-gami 2. The proteins were purified using Ni-NTA affinity chromatography and the His_6 and Trx tag was removed by TEV protease. The proteins were further purified through Ni-NTA affinity chromatography and gel filtration chromatography on a Superdex 200 PG column (GE Healthcare). The protein concentration was determined through UV absorption.

Fluorescent labeling of NGCA with FITC

Fluorescent labeled NGCA was prepared by addition of 15-fold molar excess of FITC to NGCA in PBS (pH 8.0~8.5). After 12 h incubation at 4°C, the excess of FITC was removed by ultrafiltration (Amicon Ultra-15, 10 KDa). The FITC-labeled NGCA was further purified using desalting columns (GE Healthcare) in PBS (pH 7.2~7.4).

Loading maleimide-functionalised platinum(IV) prodrug and Gd^{3+} ions to proteins

NGC or NGCA was reduced by addition of 5-fold molar excess of TCEP in PBS (pH 7.2~7.4) with 10 mM EDTA. After incubation for 2 h at room temperature, the reduced protein solution was buffer exchanged to PBS (pH 8.0) using desalting columns (GE Healthcare) at 4°C. For site-specific conjugation a 30-fold molar excess of maleimide-functionalised platinum(IV) prodrug was added to the reduced protein and incubated for 12 h at room temperature. The conjugated NGC or NGCA solution was dialyzed against a buffer contain 0.5 mM $GdCl_3$ to bind Gd^{3+}. After dialysis for 12 h at 4°C, the protein solution was concentrated and purified with desalting columns (GE Healthcare).

The stability of Mal-Pt conjugated peptide

A Cys_3-containing model peptide (GSGCCCGSG) was synthesized by a standard solid-phase synthesis method, and the product was verified by ESI-MS (m/z: 752.18 [M + Na^+]). Mal-Pt was reacted with 6 eq. model peptide in water at room temperature for 4 hours, The reaction was analyzed using a high-performance liquid chromatography (HPLC) on an Agilent 1200 system equipped with a ZORBAX SB-Aq C18 column (250×4.6 mm, 5 μm). HPLC profiles were recorded on UV detection at

220 nm. 0.1% CF_3COOH in H_2O (A) and CH_3CN (B) were used as eluents in a flow rate of 1.0 mL/min. Linear gradient was used from 5% B (0-5 min) to 100% B in 5-17 min. The product of Mal-Pt/peptide conjugate was verified by ESI-MS and its stability was analyzed by HPLC in 48 h.

The interaction between Pt-NGCA and DNA

Interactions were performed on Herring sperm DNA (100 ng/μL) with Pt agents a [Pt]/[nucleotide] ratio of 1:2 in 10 mM phosphate buffer (pH 7.4) containing 10 mM $NaClO_4$ at 25°C for 24 h. The fluorescence probe EtBr (0.04 mg) was added to DNA before fluorescence measurements. The fluorescence spectra were recorded with the excitation at 530 nm.

Western Blot

200×10^4 of A375 or A431 cells were harvested and lysed in RIPA lysis buffer. Cell lysates with equal amounts of protein were resolved on 8% SDS-PAGE and transferred to a polyvinylidene fluoride membrane, then detected with anti-EGFR antibodies. The proteins were visualized with Chemiluminescent (Thermo Fisher).

Specific affinity assay

Specific affinity assay was conducted by FACS and Confocal laser scanning microscopy. For flow cytometry, 10 μM FITC-NGCA were incubated with $2x10^5$ of A431 and A375 cells individually at 4°C for 30 min. Then cells were washed three times with PBS and suspended with 200 μL PBS. The fluorescence was determined by a BD FACS Calibur Flow Cytometer. For confocal laser scanning microscopy analysis, The A431 and A375 cells were seeded in 24-well plates with cover glass for 12 h before experiment. Cells were incubated with 10 μM of FITC-NGCA at 4°C for 1 h or 37°C for 2 h. After washed three times with fresh PBS, cells were fixed with 4% paraformaldehyde for 15min, followed by stained with DAPI. The images were taken by a LSM 710 CLSM (Carl Zeiss, Jena, Germany).

Cellular Platinum Uptake

5×10^6 of A375 or A431 cells were seeded in 6-well plates overnight and then incubated with Cisplatin, Pt-NGC or Pt-NGCA at a concentration of 200 μM Pt at 37°C for 4 h in standard culture conditions. Then the cells were washed with PBS for three times, and harvested by trypsinization. The harvested cells were concentrated and digested by nitric acid for the ICP-MS. The cell numbers were counted before the digested.

Cell cytotoxicity assays

A375 or A431 cells were seeded in 96-well plates at 4000 cells/well in 100 μL of DMEM containing 10% FBS and incubated in a 5% CO_2 atmosphere at 37°C for 12 h. The culture medium was then replaced with 100 μL of freshly prepared culture medium containing drugs at a concentration of 100 μM Pt. The cells were further incubated for 72 h, and then the medium was replaced with fresh culture medium and the MTT solution at a concentration of 0.5 mg/mL was added. The cells were incubated for another 4 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 cells were incubated for another 4 h at 37°C. The absorbance was measured at 490 nm using a Bio-Rad 680 microplate reader.

Apoptosis analysis

A375 or A431 cells cultured in 24-well plates at a density of 2×10^5 cells per well were treated with various drugs at a concentration of 20 μM Pt for 24 hours. Apoptotic cells were detected by flow cytometry after staining with Annexin V and Propidium Iodide (PI) using the AnnexinV-FITC apoptosis detection kit and determined using BD FACS Calibur Flow Cytometer. The data were analyzed using FlowJo software.

Albumin binding assays

20 μM NGC or NGCA were incubated with 2 eq. of HSA, MSA or MSA individually at 4°C for 1 h. After that, the protein solutions were analyzed using gel filtration chromatography on a Superdex 200 column (GE Healthcare) and native-PAGE.

***In Vivo* Pharmacokinetics studies**

Pharmacokinetics studies of platinum formulations were performed in Balb/c nude mice. The mice were randomly assigned into three groups (nine mice per group). Cisplatin, Pt-NGC or Pt-NGCA were administered intravenously *via* tail vein at a doses of 2 mg/kg Pt. At the predetermined time point 5 min, 30 min, 1 h, 2 h, 4 h, 6 h, 8 h, 12 h, 24 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.

***In vivo* biodistribution**

Balb/c nude mice were inoculated subcutaneously A375 cells at the left buttock and A431 cells at the right buttock. When the tumor grew to a size of 200-400 mm^3, Cisplatin, Pt-NGC or Pt-NGCA was administered *via* tail vein intravenously at a dose of 2 mg/kg Pt. The mice were sacrificed at 12 h after injection. The tumor, kidney, liver, blood and spleen were excised. The blood and organs were decomposed with heat using nitric acid. The platinum concentration in the solution was measured by ICP-MS.

***In vivo* antitumor assay**

Balb/c nude mice were inoculated subcutaneously A375 cells at the left buttock and A431 cells at the right buttock. When the tumor grew to a size of 50-100 mm^3, Cisplatin, Pt-NGC or Pt-NGCA was administered *via* tail vein intravenously at a dose of 2 mg/kg Pt for 6 times at 4-day intervals. Tumor growth was monitored by measuring the perpendicular diameter of the tumor using calipers every four days and calculated according to the formula: tumor volume (mm^3) = 0.5 × length × $width^2$. After 24 days, animals were sacrificed and the blood samples were collected from the retro-orbital plexus of the mouse for biochemical parameters and toxicity markers assay. The livers, kidneys and tumors were excised for immunofluorescence TUNEL analysis.

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. Apoptotic levels were determined using the transferase-mediated dUTP nick end-labeling (TUNEL) method according to the manufacturer's instructions. All slides were examined under an Olympus IX81 microscope (Tokyo Prefecture, Japan).

Biochemical parameters and toxicity markers assay

ALT, AST, BUN and CR in serum were analyzed using a commercial Kit according to the manufacturer's instructions (Changchun Huili Biotech Co., Ltd. China.) by Auto Chemistry Chemray 240 (Rayto Life and Analytical Sciences Co., Ltd., China.).

Determination of longitudinal relaxivity (r1) values

The relaxation times of T_1 were measured using a 9.4 T MR scanner at room temperature. The contrast agents at different concentrations were prepared in 50 mM Tris, 200 mM NaCl, pH 8.0. Longitudinal relaxivity (r1) were obtained from the slopes of linear fits of experimental data.

T_1-Weighted MRI

Balb/c nude mice were inoculated A431 cells. MRI images were recorded after the injection of Gd-NGC or Gd-NGCA on a 9.4 T MR scanner. Gd-agents were injected in a dose of 7 μmol/kg Gd, in which the protein concentration is equivalent to tumor inhibition assay. The quantify assay of the MR signal was analyzed using the software ImageJ.

Supporting Figures

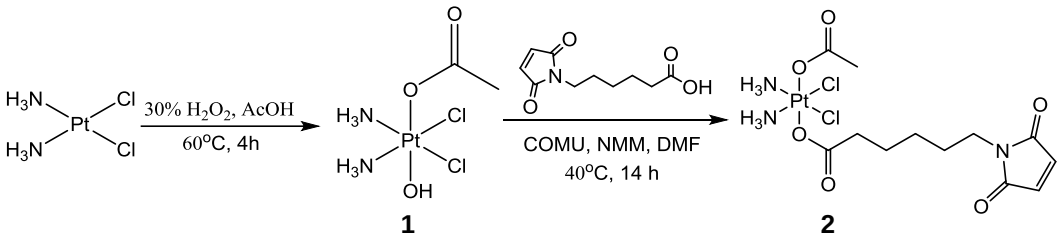

Fig. S1 The synthesis of Mal-Pt prodrug.

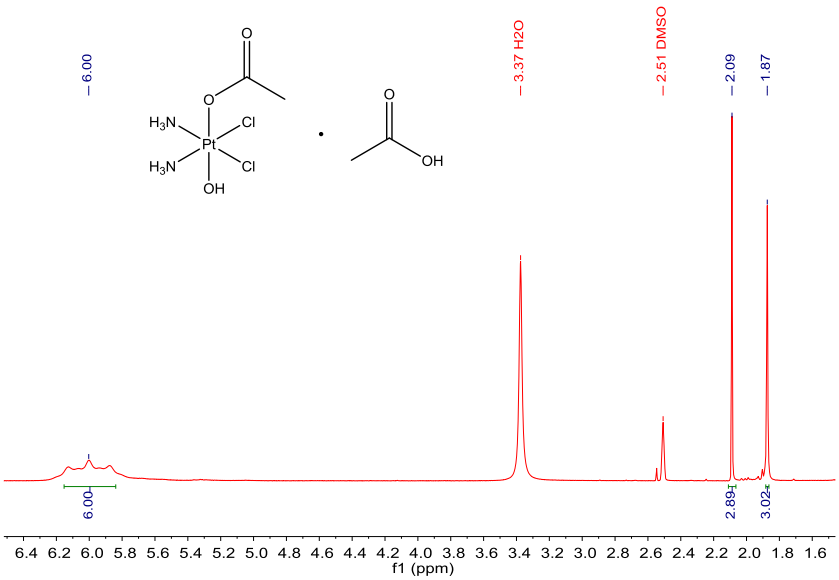

Fig. S2 ^{1}H-NMR spectroscopy of $[Pt(NH_3)_2Cl_2(OH)(OAc)]$ (**1**) in DMSO-d_6.

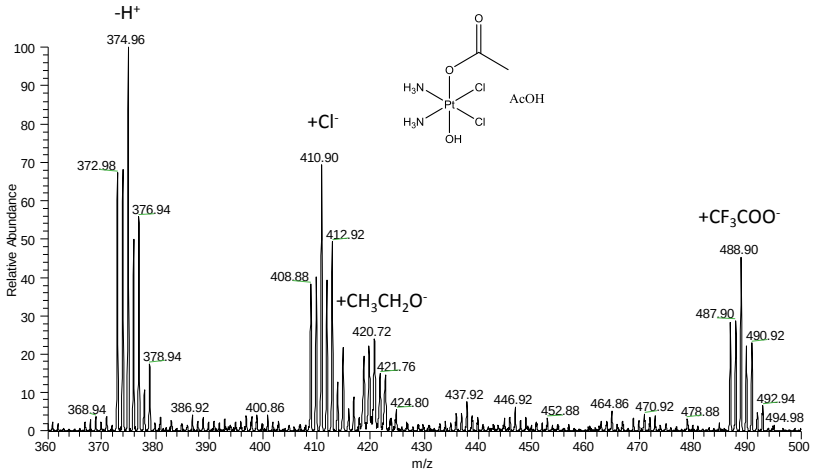

Fig. S3 ESI-MS spectroscopy of $[Pt(NH_3)_2Cl_2(OH)(OAc)]$ (**1**) in the negative mode. m/z of $[C_2H_{10}Cl_2N_2O_3Pt\text{-}H^+]^-$: calculated 374.96, found 374.96.

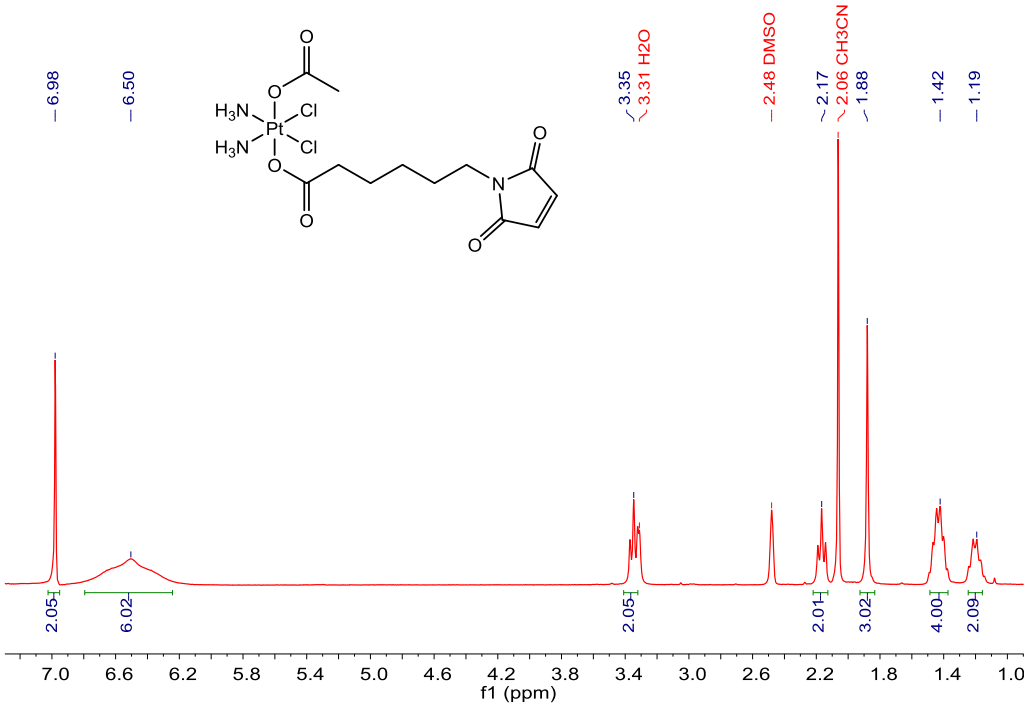

Fig. S4 ^{1}H-NMR spectroscopy of Mal-Pt (**2**) in DMSO-d_6.

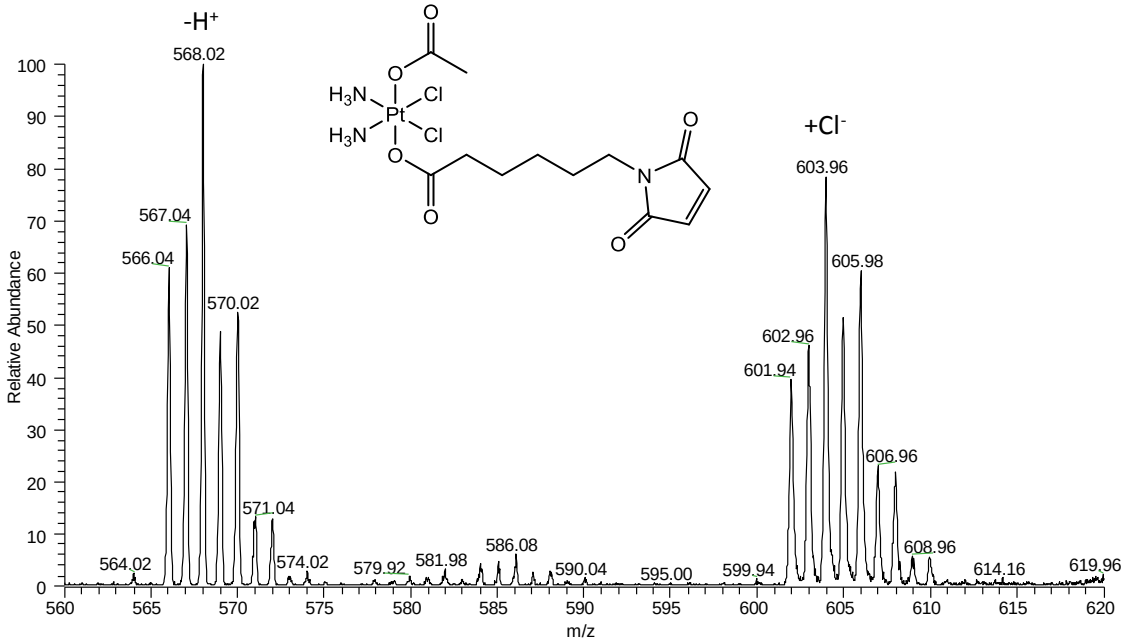

Fig. S5 ESI-MS spectroscopy of Mal-Pt (**2**) in the negative mode: m/z of $[C_{12}H_{21}Cl_2N_3O_6Pt\text{-}H^+]^-$: calculated 568.04, found 568.02.

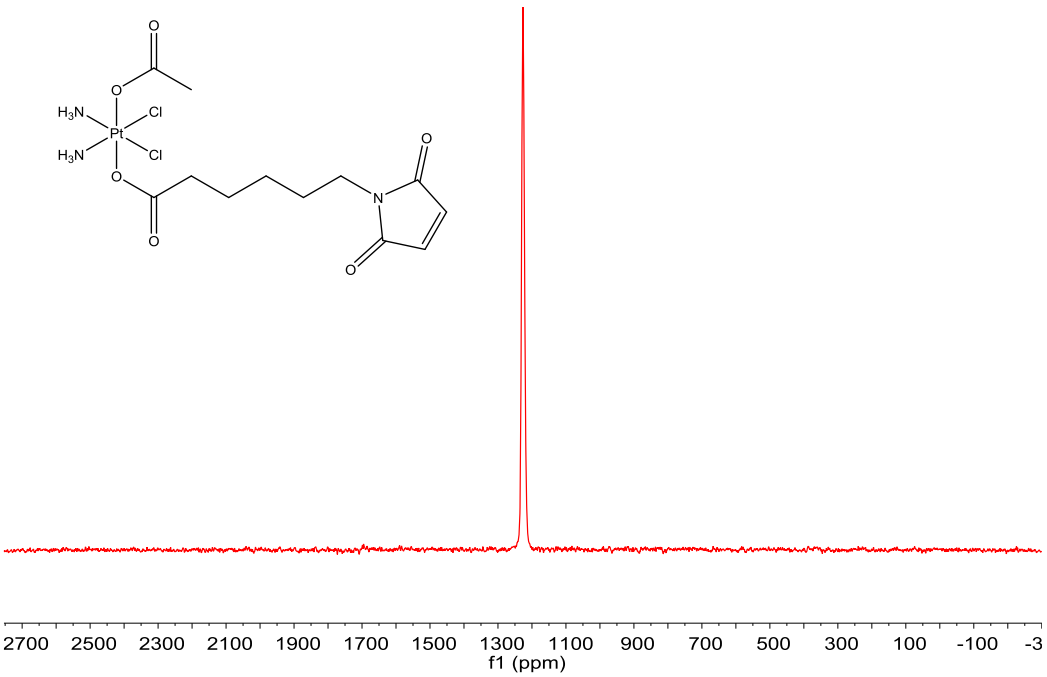

Fig. S6 ^{195}Pt-NMR spectroscopy of Mal-Pt (**2**) in DMSO-d_6.

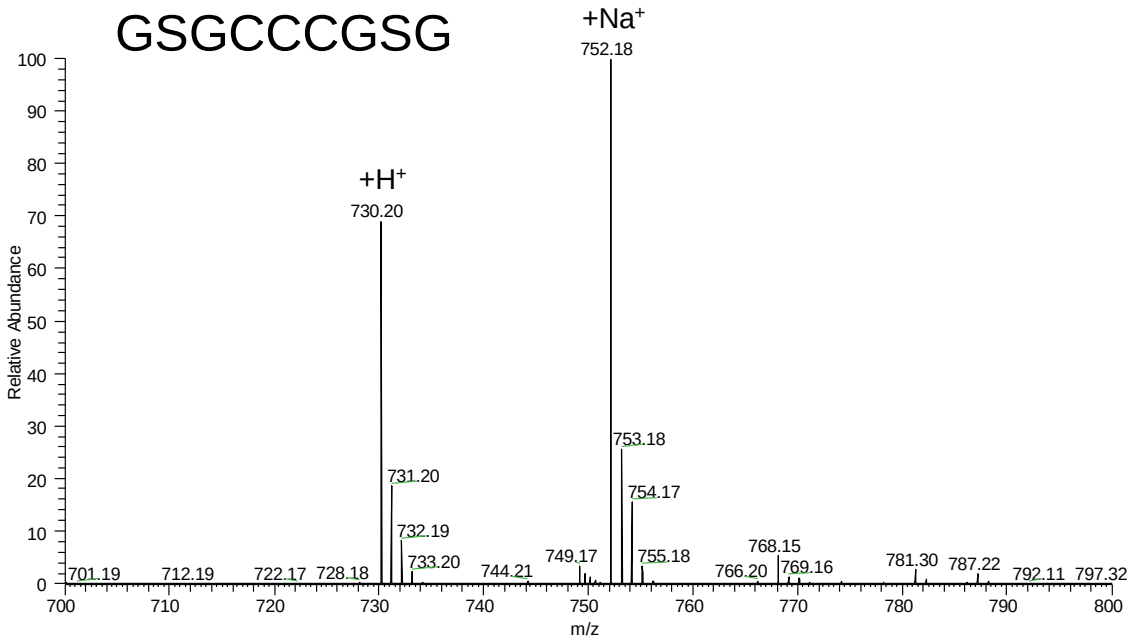

Fig. S7 ESI-MS spectroscopy of the Cys_3-containing model peptide (GSGCCCGSG) in the positive mode. m/z of $[C_{23}H_{39}N_9O_{12}S_3+H]^+$ was measured at 730.20 (calculated 730.19).

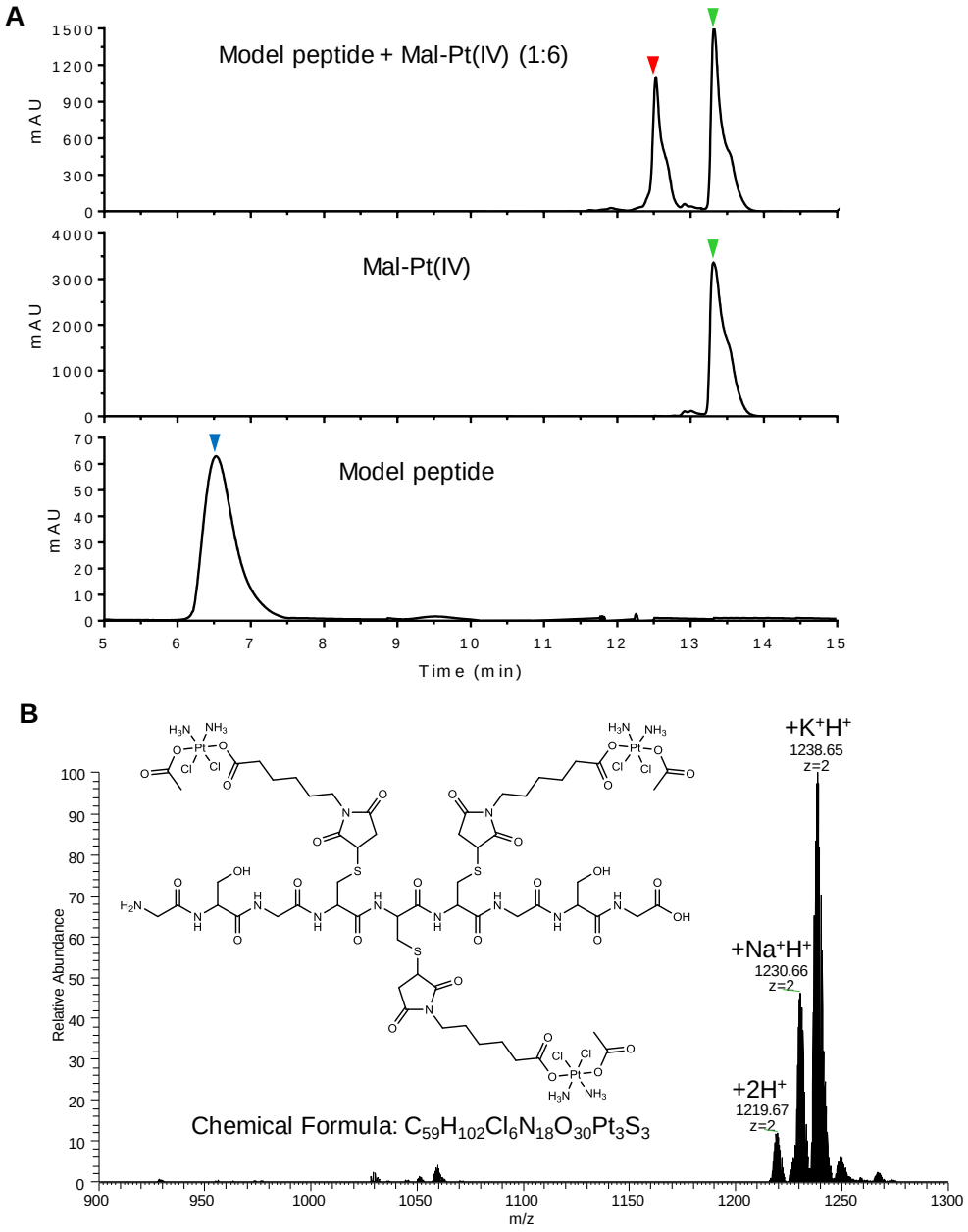

Fig. S8 The conjugation of Mal-Pt to the Cys_3-containting model peptide (GSGCCGSG). (A) Analysis of the reaction by HPLC. 6-molar ratio of Mal-Pt was used in the reaction. Symbols denote free peptide (blue), Mal-Pt (green) and the Mal-Pt/peptide conjugate (red). (B) ESI-MS spectroscopy of the conjugate of Mal-Pt/peptide collected from HPLC (peak at retention time 12.5 min). The peak at m/z 1219.67 (+2) indicates the composition of product $C_{59}H_{102}Cl_6N_{18}O_{30}Pt_3S_3$, confirming the binding of Mal-Pt to peptide through maleimide-thiol conjugation. The peaks at 1230.66 and 1238.65 (+2) are corresponding to the binding of Na^+ and K^+, respectively.

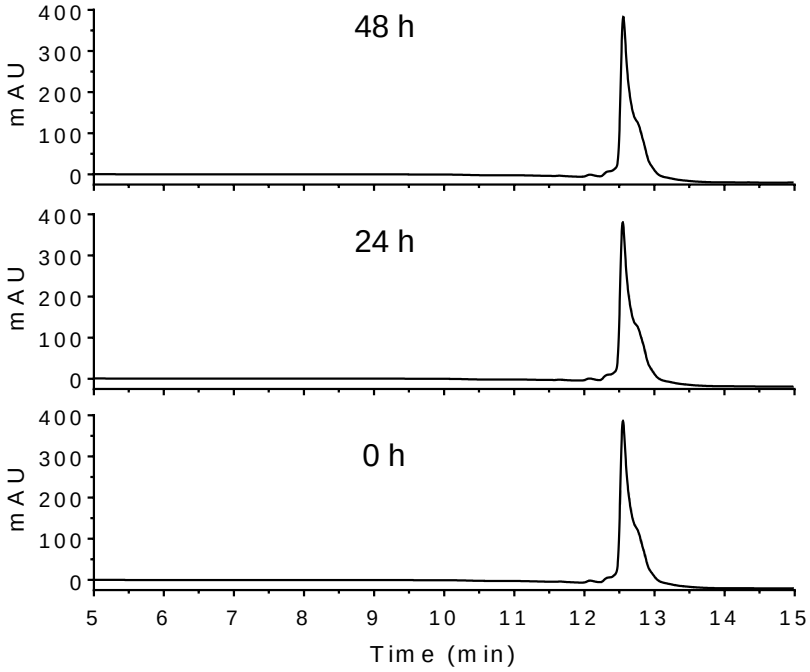

Fig. S9 The stability of the conjugate of Mal-Pt(IV) with Cys_3-containting peptide. The conjugate was collected from HPLC and kept at room for 0, 24 or 48 h before HPLC analysis.

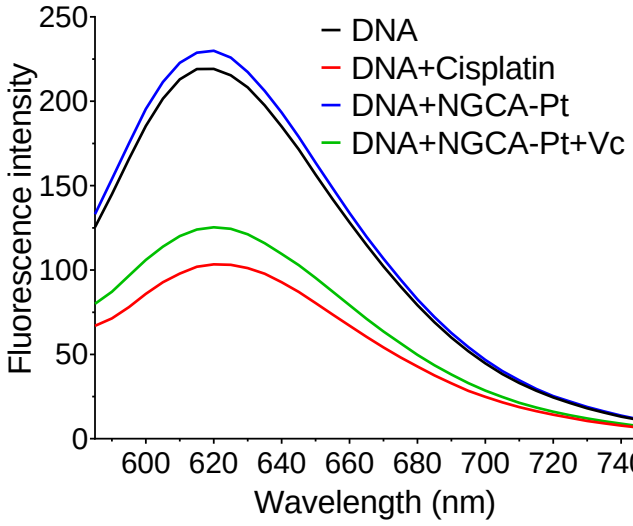

Fig. S10 Fluorescence measurement of the binding of Pt agents to using an EtBr probe. Interactions were performed on Herring sperm DNA (100 ng/μL) with Pt agents a [Pt]/[nucleotide] ratio of 1:2 in 10 mM phosphate buffer (pH 7.4) containing 10 mM $NaClO_4$ at 25°C for 24 h. EtBr (0.04 mg) was added before fluorescence measurements. The result clearly shows that the binding of cisplatin to DNA quenched the fluorescence. NGCA-Pt(IV) can react with DNA only upon the reduction of Pt(IV) by ascorbic acid (V_C), indicating that the platinum was in Pt(IV) form on the conjugate.

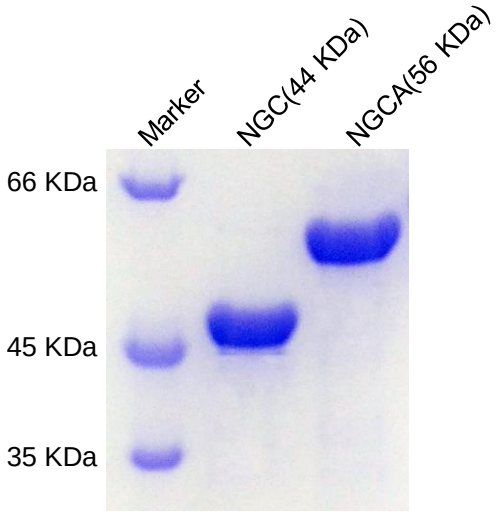

Fig. S11 Electrophoresis analyses of the purified proteins NGC and NGCA.

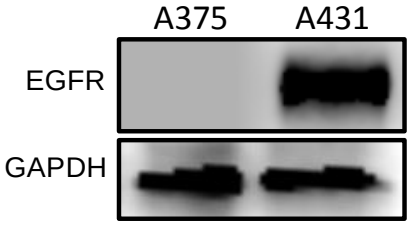

Fig. S12 EGFR expression in human cutaneum carcinoma A375 cells and A431 cells measured by western blotting.

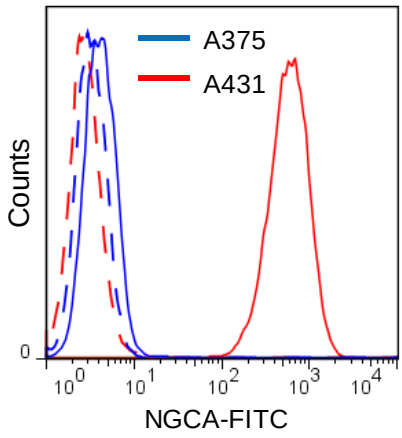

Fig. S13 The binding of FITC labeled NGCA to A375 or A431 cells measured by flow cytometry. Curves show cells before (dash lines) or after (solid lines) incubation with 10 μM NGCA-FITC at 4°C for 1 h.

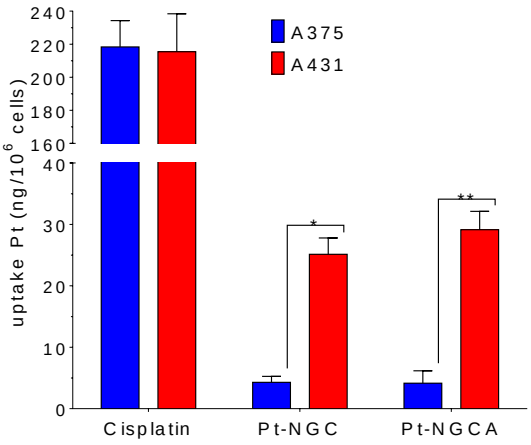

Fig. S14 Cellular uptake of platinum in A375 and A431 cells determined using ICP-MS. Cells were treated with cisplatin or Pt-nanobody conjugates in 100 μM Pt for 4 h. Error bars denote standard deviations of thrice independent experiments and asterisks indicate *p*-values (*: $p < 0.05$; **: $p < 0.01$).

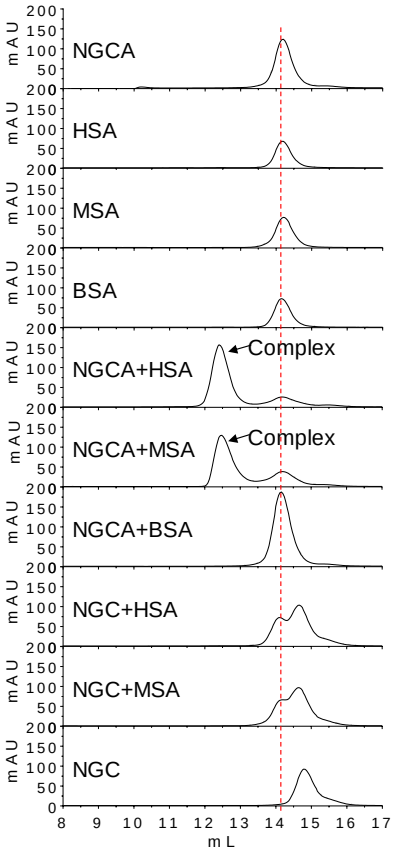

Fig. S15 Gel filtration chromatographic analysis of the binding of NGCA to albumin. 20 μM NGC or NGCA was incubated with 40 μM albumin and analyzed on a Superdex 200 column (GE Healthcare).

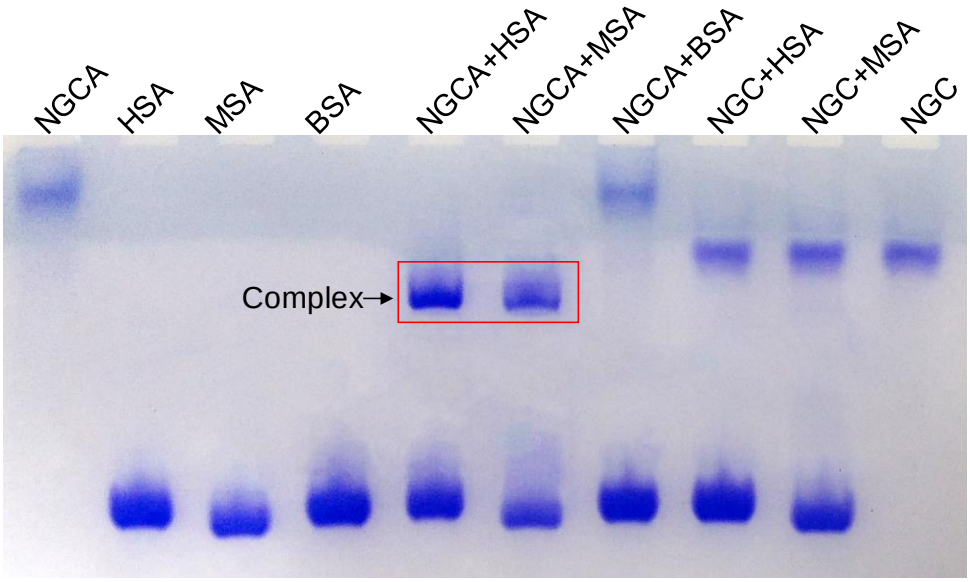

Fig. S16 Native-PAGE analysis of the interaction of NGCA with albumin.

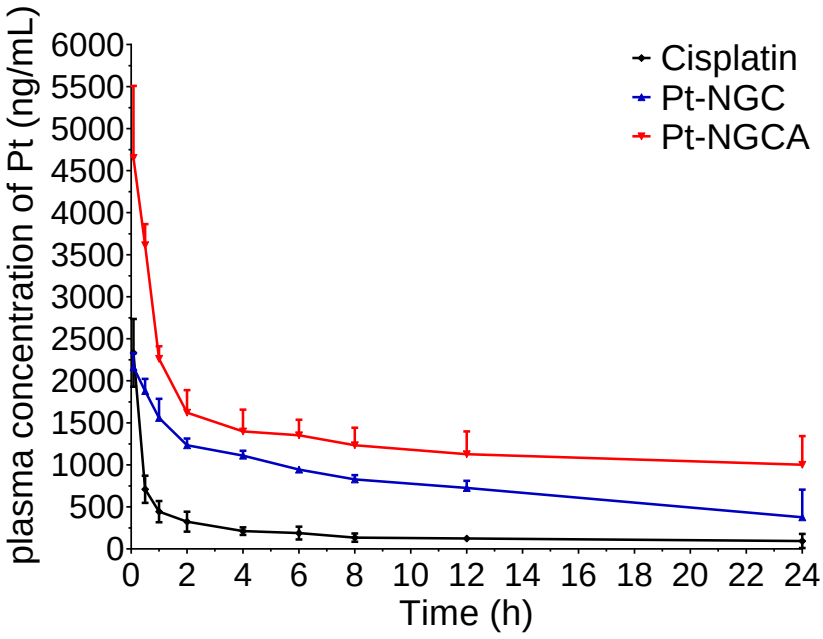

Fig. S17 *In vivo* pharmacokinetics. Balb/c nude mice were treated with cisplatin, Pt-NGC or Pt-NGCA in 2 mg/kg Pt *via* tail vein injection. After 24 h, the Pt concentration in plasma was measured by ICP-MS.

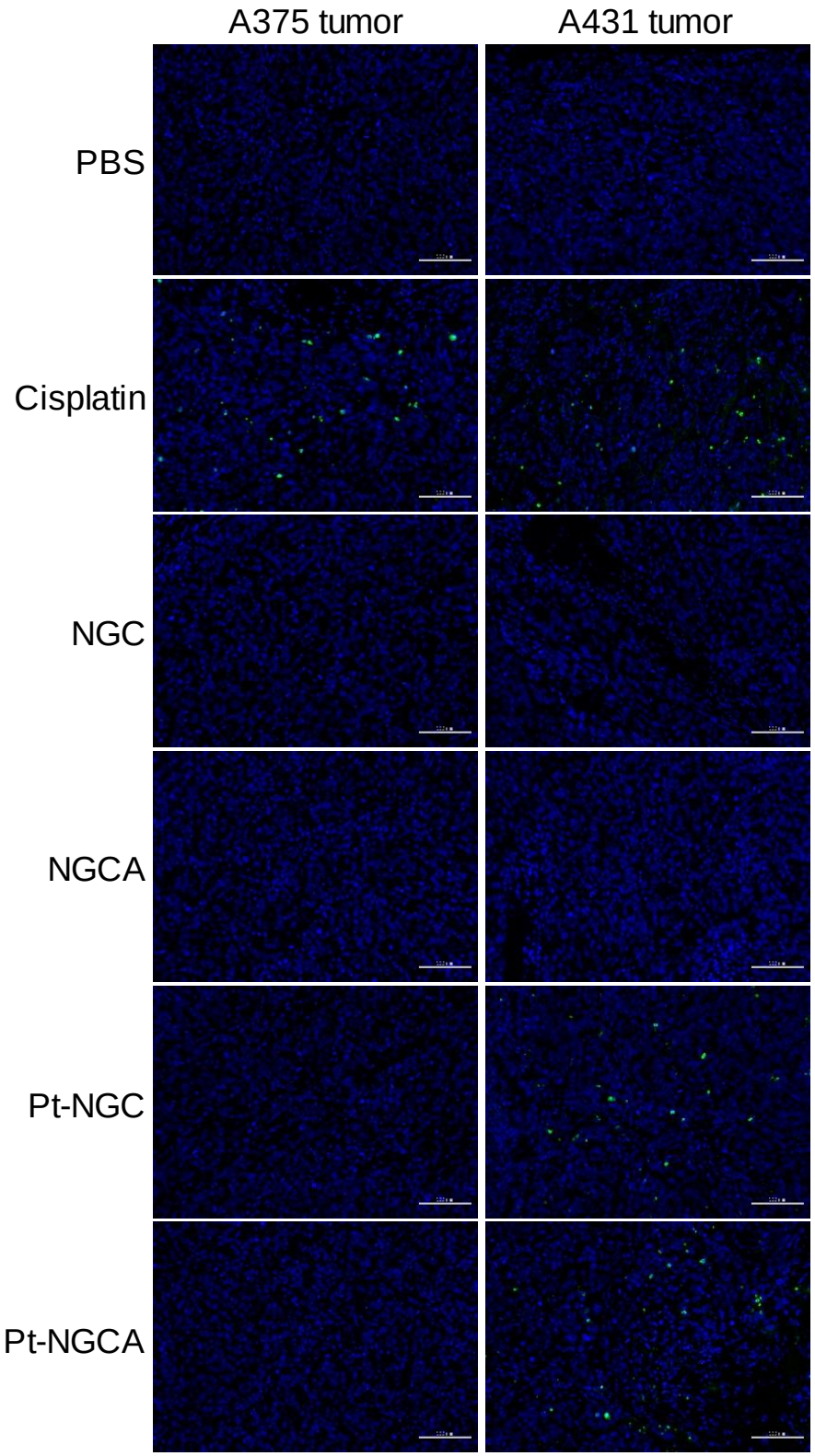

Fig. S18 TUNEL analyses of tumors. The TUNEL-positive apoptotic cells were stain in green, and nuclei were stained by DAPI in blue.

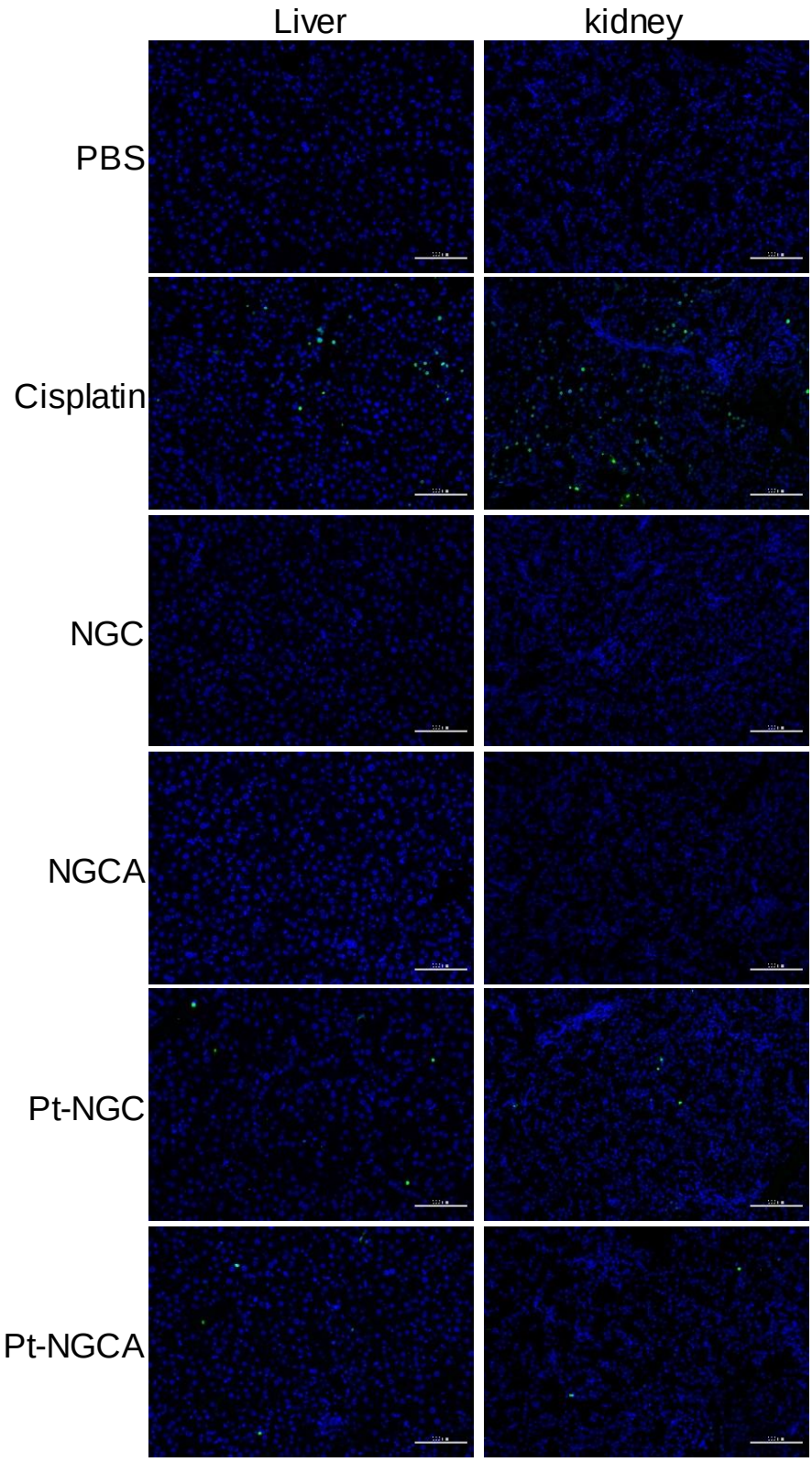

Fig. S19 TUNEL analyses of the liver and kidney after the treatments. The TUNEL-positive apoptotic cells were stain in green, and nuclei were stained by DAPI in blue.

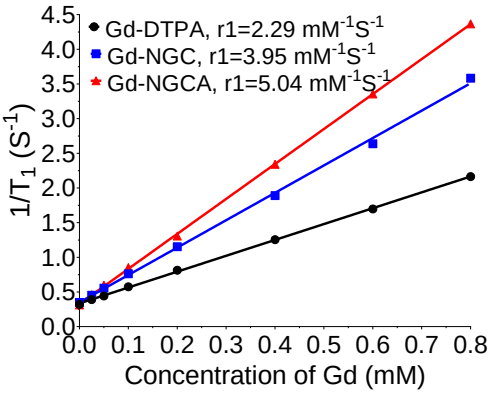

Fig. S20 The T_1 relaxation rate of Gd-DTPA, Gd-NGC and Gd-NGCA, which were obtained from the slopes of linear fits of experimental data.

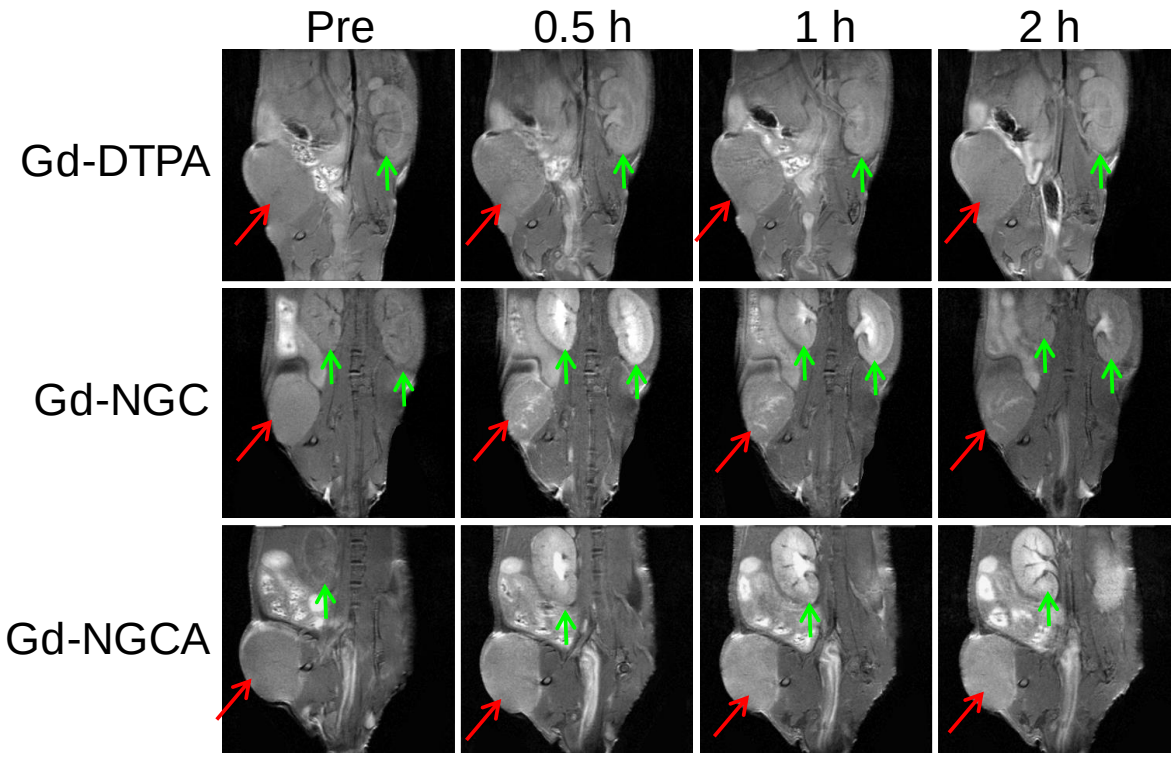

Fig. S21 *In vivo* T_1-weighted MRI on the mice. Tumors are indicated with red arrows and kidneys are indicated with green arrows.

Supporting Tables

Table S1. Quantification of the Pt and Gd bound to NGC or NGCA using ICP-MS.

Protein concentration [a]	NGC [b]		NGCA [b]	
	Pt (ratio)	Gd (ratio)	Pt (ratio)	Gd (ratio)
10	27.4 (2.74)	21.4 (2.14)	25.8 (2.58)	19.3 (1.93)
20	55.2 (2.76)	38.7 (1.94)	57.3 (2.86)	44.3 (2.22)
50	130.9 (2.62)	104.2 (2.08)	140.2 (2.80)	99.6 (1.98)
100	278.5 (2.78)	193.1 (1.93)	266.1 (2.66)	203.4 (2.03)
Average ratio [c]	2.72 [c]	2.02 [c]	2.73 [c]	2.04 [c]

a: Protein concentration in μM.

b: Pt or Gd bound to protein in μM. The ratios of [metal]/[protein] are given in the parentheses.

c: The average ratios of [metal]/[protein] measured from samples in different concentration.

Table S2. IC_{50} values (μM) of different drug agents on the growth of A375 and A431 cells after a 72 h drug exposure.

	A375	A431
Cisplatin	1.3 ± 0.6	1.3±0.3
NGC	>200	>200
NGCA	>200	>200
Pt-NGC	>200	72.6±17.4
Pt-NGCA	>200	42.9±13.8

Table S3. Variations of the liver indexes (ALT and AST) and kidney indexes (BUN and CR) after the treatment.

Group	Liver Function		Kidney Function	
	ALT (U/L)	AST (U/L)	BUN (mg/dL)	CR (μM)
PBS	61.2±6.6	230±19	42.4±1.2	79.6±3.1
Cisplatin	80.0±8.1	271±20	49.2±4.8	93.4±9.4
NGC	58.7±8.1	207±34	30.8±1.0	65.7±3.2
NGCA	53.9±8.8	178±17	35.5±2.7	71.3±4.2
Pt-NGC	62.6±3.5	240±18	37.8±0.8	72.2±6.0
Pt-NGCA	67.9±8.1	260±27	36.4±2.3	71.6±4.2