Supplementary Information

Tumor-targeting delivery of hyaluronic acid-platinum(IV) nanoconjugate to reduce toxicity and improve survival

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Experimental section

Materials

Hyaluronic acid (MW, 11 kDa) was obtained from Freda Biochem as sodium hyaluronate. Cisplatin (purity ≥ 99.9%) was supplied by Boyuan Pharmaceutical Chemical Company. Cy7 acid, mono-NHS ester (Cy7, SE) and R isomer [Tetramethylrhodamine-6-isothiocyanate] (TRITC) were provided by Fanbo Biochemicals. Ethylenediamine dihydrochloride, succinic anhydride and 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) were purchased from Sigma-Aldrich. N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC·HCl) and N-Hydroxysuccinimide (NHS) were offered by Aladdin. Sodium ascorbate (NaAsc) and reduced L-Glutathione (GSH) were presented by Sinopharm Chemical Reagent. Dulbecco's Modified Eagle's Medium (DMEM), Minimum Essential Medium (MEM), trypsin, fetal bovine serum (FBS), penicillin-streptomycin solution, phosphate buffered saline (PBS) were bought from Gibco®.

Cell culture and animal use

B16-F10, Hep G2 and HEK-293 were purchased from Cell Bank of Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. B16-F10 was grown in DMEM, while Hep G2 and HEK-293 were cultivated in MEM, supplemented with 10 v/v% FBS, 100 U/mL penicillin and streptomycin in cell chamber. Exponentially growing cultures were maintained at 37 ℃ under an atmosphere of 5% CO_2 and 90% relative humidity.

ICR mice and SD rats were offered by College of Veterinary Medicine Yangzhou University. C57BL/6J mice were obtained from SLRC Laboratory Animal Center. Those animals were acclimatized at 22 ± 2 ℃ and 70 ± 5% relative humidity under natural light-dark conditions with access to food and water ad libitum. The animal use protocol was conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and approved by the Animal Ethics Committee

of China Pharmaceutical University.

Preparation of HA-EDA-Pt(IV) nanoconjugate

Synthesis of *cis,cis,trans*-[$Pt(NH_3)_2Cl_2(OH)_2$]

Cis,cis,trans-[$Pt(NH_3)_2Cl_2(OH)_2$] was synthesized from cisplatin [1]. Briefly, H_2O_2 was dropwise added into a 10% cisplatin suspension, and the mixture was kept stirring at room temperature (R.T.) for 24 h. The product was recrystallized in situ at 4 ℃ overnight, collected via vacuum filtration and washed with ice cold water, ethanol and diethyl ether. Then the solvent was removed to give the expected product as bright yellow powder (yield: 90.3%). δ_H (500 MHz; d^6 DMSO) 5.50 (3 H, q, NH_3).

Synthesis of *cis,cis,trans*-[$Pt(NH_3)_2Cl_2(OOCCH_2CH_2COOH)(OH)$]

Cis,cis,trans-[$Pt(NH_3)_2Cl_2(OOCCH_2CH_2COOH)(OH)$] was prepared as previously described [2]. A mixture of equimolar amounts of *cis,cis,trans*-[$Pt(NH_3)_2Cl_2(OH)_2$] and succinic anhydride in dry dimethyl sulfoxide (DMSO) was kept stirring in the dark at R.T. for 12 h. The solvent was removed by lyophilization and dichloromethane was added to remove residual succinic anhydride. Finally, Recrystallization from acetone (-20 ℃) afforded Pt(IV) as yellow needle crystals which were washed with cold acetone and diethyl ether, and dried in a vacuum oven for two days (yield: 65.2%). δ_H (500 MHz; d^6 DMSO) 5.96 (3 H, q, NH_3), 2.35-2.41 (4H, t, CH_2).

Synthesis of ethylenediamine modified hyaluronic acid

Amine-functionalized hyaluronic acid (HA-EDA) was obtained by condensing amino groups to carboxyl groups [3]. EDC·HCl was added to 2% HA solution in PBS (pH 7.4, 10 mM). After 15 After 15 min standing for activation, ethylenediamine dihydrochloride, also dissolved in PBS (pH 7.4, 10 mM), was added dropwise in 30 min. Whereafter, the reaction solution was adjusted to neutral pH, proceeded for 2 h at R.T. and underwent exhaustive dialysis (Molecular Weight Cut Off, MWCO, 3.5 kDa) against 100 mM NaCl and distilled water before filtration through a 0.45 μm retention size filter. The polymer obtained as a white sponge-like powder after lyophilization was stored at 4 ℃ for further use. δ_H (500 MHz; D_2O) 2.92 (2H, s, N-CH_2), 3.35 (2H, s, N-CH_2).

Conjugate *cis,cis,trans*-[$Pt(NH_3)_2Cl_2(OOCCH_2CH_2COOH)(OH)$] to HA-EDA

EDC (0.19 g), NHS (0.12 g) and Pt(IV) (0.35 g) were mixed in 6.0 mL of PBS (pH 6.5, 10 mM), and the mixture was kept stirring in an ice bath in the dark. After the suspension became clear, 0.10 g HA-EDA in 20.0 mL of PBS (pH 6.5, 10 mM) was added and the mixture was stirred at R.T. for 24 h, then dialyzed (MWCO 3.5 kDa) against distilled water for 12 h and lyophilized to obtain HA-EDA-Pt(IV) nanoconjugate. The drug loading efficiency of platinum was determined using an iCE 3300 Graphite Furnace Atomic Absorption Spectrometry (GFAAS, Thermo Scientific, USA) with Quadline® deuterium background correction and equipped with an adapted autosampler, a platinum lamp (Well Group Scientific, USA) and Extended Lifetime Cuvettes (ELCs, Thermo Scientific, USA). δ_H (500 MHz; D_2O) 2.36-2.68 (4H, m, $COCH_2$).

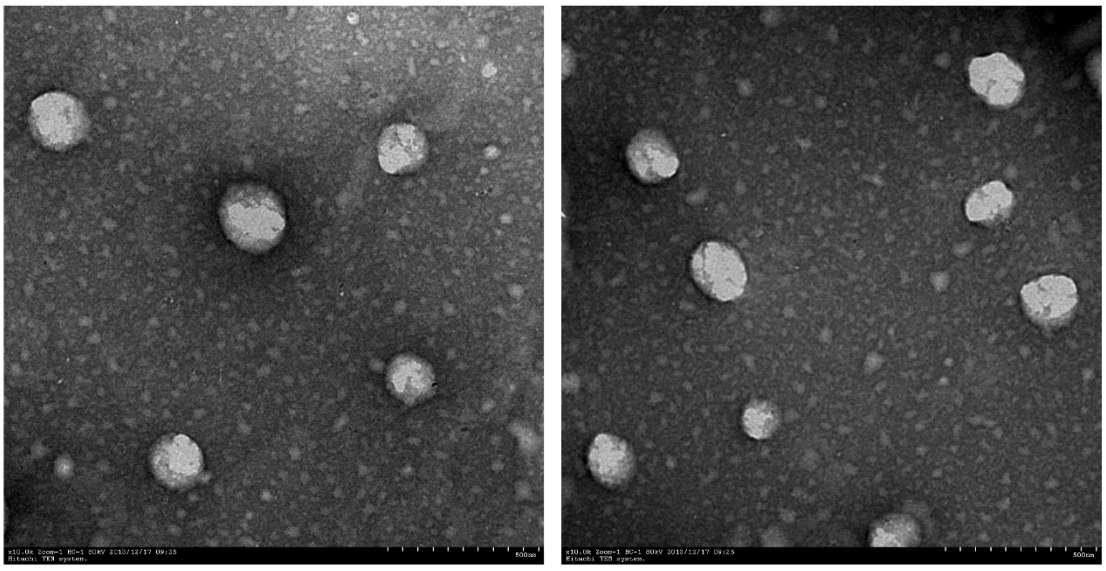
Measurement of particle size, zeta potential and morphology

The particle size and zeta potential of HA-EDA-Pt(IV) nanoconjugate were recorded using a Particle Size & Zeta Potential Analyzer (90Plus Zeta, Brookhaven Instruments Corporation, USA). All the measurements were conducted thrice at 25 ℃ and a scattering angle of 90°. The morphology of HA-EDA-

Pt(IV) nanoconjugate were investigated using Transmission Electron Microscope (TEM, H-7650, Hitachi High-Technologies Corporation, Japan). The nanoconjugate was negatively stained with 1% phosphotungstic acid (pH 8.0) prior to measurement.

Table S1 Hydrodynamic diameter distribution and zeta potential of HA-EDA and HA-EDA-Pt(IV) nanoconjugate.

Sample name	Zeta potential (mV)	Particle size (nm)	Polydispersity index
HA-EDA	-25.84 ± 0.73	177.0 ± 2.1	0.252 ± 0.042
HA-EDA-Pt(IV) nanoconjugate	-28.65 ± 2.01	186.4 ± 1.3	0.267 ± 0.014



(A) (B)

Fig. S1 Typical TEM morphology of (A) HA-EDA and (B) HA-EDA-Pt(IV) nanoconjugate.

Release profile of platinum from HA-EDA-Pt(IV) nanoconjugate in vitro

HA-EDA-Pt(IV) nanoconjugate was dissolved in PBS (pH 7.4 and 5.0, respectively, 100 mM), then transferred into a pre-swelled dialysis bag with 3.5 kDa MWCO. The drug release experiment was conducted at 37 ℃ with stirring at 100 rpm. At designed time points, 1.0 mL of sample solution was withdrawn and its platinum concentration was quantified by GFAAS. Equal volume of fresh PBS was immediately replenished. The same drug release procedure was carried out in triplicates in the presence of 5 mM and 0.1 mM NaAsc, respectively.

Cytotoxicity evaluation

In vitro anti-proliferation of HA, HA-EDA, HA-EDA-Pt(IV) nanoconjugate and cisplatin were evaluated by means of Cell Counting Kit-8 (CCK-8, Beyotime Institute of Biotechnology, China) assay [4]. 3×10^3 cells/well of B16-F10, Hep G2 and HEK-293 were incubated in 96-well plates for 24 h. The medium was replaced with 200 μL fresh medium containing different formulations at various concentrations. The amounts of HA and HA-EDA were equal to those in the corresponding HA-EDA-Pt(IV) nanoconjugate samples. CCK-8 were added to every well 24 and 48 h later, then the plates were incubated for another 1 h, followed by reading at 450 nm in an Absorbance Microplate Reader (ELx800, BioTek Instruments, USA). The relative cell viability was calculated and data were presented as means ± SD (n = 5).

Confocal laser scanning microscopy analysis for cellular uptake

HA-EDA-Pt(IV) with remanent amino groups dissolved in carbonate buffer solution was further used to obtain the fluorescence labeled nanomedicine, after that, TRITC dissolved in DMSO was added. The reaction was maintained at R.T. in the dark for 24 h, dialyzed (MWCO 3.5 kDa) for 72 h, and finally lyophilized to obtain HA-EDA-Pt(IV)-TRITC. The loading efficiency was determined using a Fluorescence Spectrophotometer (Lumina, Thermo Fisher Scientific, USA).

Cells were seeded in the special confocal microscopy dish (MatTek Corporation, USA) with a density of 5×10^5 cells/well and then exposed to the TRITC-labeled nanoconjugate (final TRITC concentration: 5 and 50 mg/L). At prearranged time points, cells were washed by 4 ℃ PBS thrice, fixed with 4% paraformaldehyde for 20 min, followed by nuclei staining with DAPI for 30 s. The fluorescent images were analyzed using a Confocal Laser Scanning Microscope (CLSM 710, Zeiss, Germany). In receptor competition study, prior to addition of HA-EDA-Pt(IV)-TRITC, excess of HA (200 mg/mL) was pre-incubated with cells for 2 h.

Determination of platinum contents in the cells

Cellular uptake was also evaluated according to a protocol with minor modification [5]. B16-F10 and Hep G2 were seeded in 6-well plates at the density of 5×10^5 cells per well. After overnight incubation, cells were treated with cisplatin and HA-EDA-Pt(IV) nanoconjugate (at the equivalent cisplatin concentration of 0, 20, 40, 80 mg/L). At specified time points, cells were washed with PBS thrice, incubated with 1.5 mL of 0.15 M sodium chloride (pH 3.0) for 3 min at 4 ℃, rinsed with 2.0 mL cold PBS, harvested by scraping in ice-cold PBS, centrifuged, and finally lysed by adding 100 μL Cell Lysis Buffer (Beyotime Institute of Biotechnology, China). Thereafter, the lysis solution was froze at -20 ℃ for 20 min and thawed at R.T. The protein contents were immediately measured using a Bicinchoninic Acid protein assay kit (BCA, Beyotime Institute of Biotechnology, China), and Pt contents were directly determined by GFAAS. Besides, cells were cultured with free HA and uptake study was performed as pre-described for the sake of mechanism study.

Cell apoptosis

B16-F10 and Hep G2 were seeded in a 6-well plate and grown for 24 h. The medium was replaced with culture medium containing HA, HA-EDA, cisplatin or HA-EDA-Pt(IV) nanoconjugate. After treatment for 24 and 48 h. cells were collected and suspended in 100 μL binding buffer and incubated in the dark for 10 min with 5 μL Annexin V-FITC (20 μg/mL) and 5 μL PI (50 μg/mL) (Annexin V-FITC/PI apoptosis detection kit, BD Biosciences, USA). Flow cytometric analysis was performed in a Flow Cytometer (FACSCalibur, BD Biosciences, USA). In order to determine receptor competition on cell apoptosis, B16-F10 and Hep G2 were incubated with excess of HA prior to incubation with HA-EDA-Pt(IV) nanoconjugate. Next, apoptosis study was conducted as described above.

XPS study on the reduction of Pt(IV)

X-ray photoelectron spectroscopic (XPS) determination (Pt_{4f}) for various samples was conducted using a Thermo Scientific K-Alpha X-ray Photoelectron Spectrometer System [6]. Samples included:

(1) Cisplatin, *cis,cis,trans*-$[Pt(NH_3)_2Cl_2(OH)_2]$, Pt(IV) and HA-EDA-Pt(IV) nanoconjugate.

(2) HA-EDA-Pt(IV) nanoconjugate + GSH or NaAsc. HA-EDA-Pt(IV) nanoconjugate was incubated with 6 mM of GSH or 3 mM of NaAsc for 24 h at 25 ℃, followed by lyophilization.

(3) HA-EDA-Pt(IV) nanoconjugate + B16-F10 or HepG2. As described in Section 7, the cell lysis solutions were collected and lyophilized for XPS determination.

(4) HA-EDA-Pt(IV) nanoconjugate + rat plasma. HA-EDA-Pt(IV) nanoconjugate was incubated with 2.0 mL rat plasma for 24 h at 25 ℃, followed by lyophilization.

Maximum tolerated dose studies in normal mice

ICR mice (22-25 g) were weighed and divided into 21 groups (three males and three females in each group) at random. Single dose of 3.375, 4.500, 6.000, 8.000, 10.670 or 14.220 mg cisplatin /kg were injected intravenously. Another 14 groups of mice receiving HA-EDA-Pt(IV) nanoconjugate at doses of 25.816, 34.421, 45.894, 61.192, 81.590, 108.787, 145.049, 193.398, 257.865, 343.819, 458.426, 611.234, 814.979, 1086.639 mg/kg. Physiological saline was injected as a negative control. Then body weights and physical states of all mice were monitored. The maximum tolerated dose (MTD) was defined as the allowance of an average body weight loss of 20% and neither death due to toxic effects nor remarkable change in the general signs within 14 days after administration [7].

Pharmacokinetics studies

36 SD rats (240-250 g) composed of male and female in equal were randomly divided into 3 groups: a) cisplatin (8 mg/kg); 2) HA-EDA-Pt(IV) nanoconjugate (an equivalent dose of 8 mg cisplatin/kg); 3) physiological saline. Blood samples were collected from orbital cavity at 5 min, as well as 0.25, 0.5, 1, 2, 4, 8, 12, 24 h after intravenous administration, heparinized and centrifuged at 4000 rpm for 20 min at 4 ℃. The supernatant plasma was collected and stored at -80 ℃ until GFAAS analysis. The pharmacokinetic parameters were calculated using a Phoenix WinNonlin 6.3 Program (Pharsight Cooperation, St. Louis Missouri, USA). Hence, the following parameters were obtained: the area under the plasma concentration-time curve from time zero to time infinity ($AUC_{0\rightarrow inf}$), total body clearance (CL), volume of distribution at steady state (V_{ss}), mean residence time from time zero to time infinity ($MRT_{0\rightarrow inf}$), hybrid constants (A, α, B, β), apparent plasma half-life of the distribution and elimination phases ($t_{1/2\alpha}$, $t_{1/2\beta}$), first-order rate constants (k_{10}, k_{12}, k_{21}).

Anticancer efficacy evaluation in tumor-bearing mice

The mouse melanoma model was generated by subcutaneously injecting B16-F10 suspension (2×10^5 cells in 0.1 mL PBS) into the right medial lower extremity of C57BL/6J mouse (4-5 weeks old). When the tumors reached approximately 50 mm^3 in volume, mice were randomly assigned to 4 groups and intravenously injected with physiological saline, HA-EDA, HA-EDA-Pt(IV) nanoconjugate (6 mg/kg on cisplatin basis) or cisplatin (6 mg/kg) every other day, with the first drug injection day as day 0.

Five female mice from each group were sacrificed at the day after the fourth treatment. Blood was collected from the retro-orbital plexus, then centrifuged to obtain plasma for measuring clinic parameters including alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), blood urea nitrogen (BUN), serum creatinine (Scr) using an Automatic Biochemical Analyzer (UniCel DxC 800 Synchron Clinical System, Beckman Coulter, USA). Major organs (heart, liver, spleen, lung, kidney, brain) and tumors were deprived, weighted and pictured. The tumor inhibition rate (IR) was calculated according to the equation: $IR\,(\%) = (m - m_t)/m \times 100$, where m was the average weight of tumors in control group; m_t was the average weight of tumors in other groups.

For histological analysis, organs and tumors were fixed in 10% formalin overnight, then the paraffin-embedded tissues were sectioned and stained with hematoxylin and eosin (HE) to appraise histological

alteration by an Upright Metallurgical Microscope (BX45-DP72, Olympus Corporation, Japan).

The frozen sections of tumors were used to evaluate the expression of p53 protein by immunohistochemistry [8]. Sections were incubation with the primary antibodies (Mouse Monoclonal Antibody, Clone DO7, Isotype IgG2b, BioGenex Laboratories, USA) to p53, Elivision™ plus Polyer HRP IHC kit (Maixin Biotech, China) and DAB Horseradish Peroxidase color development kit (Beyotime Institute of Biotechnology, China) according to the manufacturers' instructions.

Apoptotic cells of tumors were detected with an in situ cell death detection kit (Maixin Biotech, China) after digestion with proteinase K. Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labelling (TUNEL) stain was performed using a FragELTM DNA fragment detection kit (colorimetric-TdT enzyme method) in accordance with the instructions provided by manufacturer. A microscope was employed to observe substrate labeled with DAB and nuclei stained with hematoxylin.

The rest of mice (n = 20, half male and half female) continued to receive treatments till death. The tumor volumes were monitored every two days with vernier calipers and calculated using the following equation [9]: $V = a \cdot b^2/2$, where a and b were the major and minor axis of tumors. The survival state and body weights were simultaneously recorded as indicators of systemic toxicity.

Biodistribution of cisplatin and HA-EDA-Pt(IV) nanoconjugate

Cisplatin (5 mg/kg) and HA-EDA-Pt(IV) nanoconjugate (5 mg cisplatin /kg) were injected into healthy ICR mice (20-22 g, 6 in each group, half male and half female). At 5 min as well as 0.25, 0.5, 1, 2, 4, 8, 12, 24 h, blood and organs were collected. Blood was centrifuged to obtain plasma. Organs were rinsed with ice-cold physiological saline and treated with 65 v/v% nitric acid under heating. The amount of Pt were quantitatively measured by GFAAS.

***In vivo* tumor-targeting observed by Near Infrared Fluorescence (NIRF) imaging**

Cy7, SE dissolved in DMSO and HA-EDA-Pt(IV) nanoconjugate dissolved in carbonate buffer solution were mixed together ($^{Cy7,\,SE}/_{-NH_2} = 10$) under stirring. After overnight incubation, the mixture was purified by dialysis (MWCO 3.5 kDa) for 72 h, then lyophilized to yield HA-EDA-Pt(IV)-Cy7, SE, and fluorescence spectrum was measured in a Fluorescence Spectrophotometer.

The C57BL/6J mice melanoma xenograft tumor model was established as in Section 12. When the tumor nodules grew to volumes of *ca.* 50-80 mm^3, mice were randomly allocated into 3 groups. HA-EDA-Pt(IV)-Cy7, SE, free Cy7, SE and physiological saline were administrated into the blood stream through lateral tail vein injection. A series of fluorescent images were collected from mice under chloral hydrate anesthesia by the Kodak *In Vivo* Imaging System (Carestream Health, USA). For each NIRF imaging, a corresponding X-ray image was recorded to locate the tumor. Finally, mice were sacrificed, and plasma, tumors and organs were excised for *ex vivo* imaging. All the images were analyzed by Carestream Molecular Imaging Software.

Tissues with thicknesses of 20 μm were mounted on glass slides which were pre-washed with polylysine, stained with DAPI and assessed fluorescent intensity by CLSM.

Statistical analysis

All experiments were repeated at least three times and data were expressed as mean ± standard deviation (SD). Unpaired student's t-test was used for between two-group comparison and one-way analysis of variance (ANOVA) with LSD and S-N-K tests were for multiple-group analysis. Statistical significance

was set at a level of $p < 0.05$ while $p < 0.01$ was considered as extreme significance. Data were analyzed using SPSS 20.0 software.

Notes and References

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