Biotin Guided Pt^{IV} Amphiphilic Prodrug Synergized with CDK4/6 Inhibition for Enhanced Tumor Targeted Therapy

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Materials and methods

Hydrogen peroxide (H_2O_2), hexadecyl isocyanate, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and Calcein were purchased from Aladdin in Shanghai, China. PKH26 red fluorescent cell linker mini kits were obtained from Sigma-Aldrich, Missouri, USA. Cisplatin (99% purity) was acquired from Boyuan in Shandong, China. Ribociclib was purchased from Selleck. The Annexin V-FITC apoptosis detection kit was obtained from Beyotime Institute of Biotechnology in Jiangsu, China. 2-(4-Amidino-phenyl)-1H-indole-6-carboxamidine (DAPI) and propidium iodide (PI) were sourced from Sigma-Aldrich, Shanghai, China. RPMI-1640 medium, 0.25% trypsin-EDTA, fetal bovine serum (FBS), and penicillin/streptomycin (P/S) were obtained from Gibco (Grand Island, NY, USA). Cell culture vessels were sourced from Corning (Corning, NY, USA).

General measurements

NMR spectra were recorded using a 400 MHz NMR spectrometer (Bruker) at room temperature. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS, Waters, USA) was employed to determine the mass of the samples. Inductively coupled plasma mass spectrometry (ICP-MS, Xseries II, Thermo Scientific, USA) was utilized for the quantitative analysis of total platinum content in the nanoparticles, in vitro drug release samples, and cells. The morphology and size of the

nanoparticles were analyzed using transmission electron microscopy (TEM) on a JEOL JEM-1011 electron microscope. Size and zeta potential measurements were conducted with a Malvern Zetasizer (Nano ZS, UK). Localization of calcein-labeled APtIV was performed using confocal laser scanning microscopy (Leica, Germany). Micro-CT analysis was conducted using a Bruker-MicroCT SkyScan1172 (Karlsruhe, Germany), while in vivo imaging of mice was carried out with an In Vivo Imaging System (IVIS, PerkinElmer). The MTT assay was performed using a microplate reader (SpectraMax).

Synthesis of BioPt^{IV}

BioPt^{IV} was synthesized, Bio-NHS (82 mg, 0.429 mmol) and Pt(IV)-OH were suspended in 10 mL anhydrous DMSO. The reaction continued for 12 h at 60 °Ct. **BioPt^{IV}** was separated by preparative chromatography to give a yellow solid (yield 75%). **BioPt^{IV}** was characterized by 1H NMR and HRMS (ESI) m/z calcd for $C27H55Cl2N5NaO5PtS^+$ $[M+H]^+$, 849.2481; found, 849.2484. HPLC: Ammonium dihydrogen phosphate buffer solution (pH=3) and acetonitrile was used as the mobile phase with a volume ratio 1:3 for purity detection by HPLC. The results showed that the purity of **BioPt^{IV}** exceed 95%.

Nanoparticles formulation of BioPt^{IV}

The DMSO (1 mL) solution of Lipo-CisPtIV-Bio (5 mg) was added dropwise to deionized water (10 mL). After 15 minutes, the solution was

transferred to an ultracentrifuge tube and subjected to ultrafiltration for 20 minutes at 4000 rpm, repeated three times to obtain $BioPt^{IV}$. For $BioPt^{IV}$@Rib, the DMSO (1 mL) solution of Lipo-CisPtIV-Bio (5 mg) and 1 mg of Rib were similarly added dropwise to deionized water (10 mL), followed by the same ultrafiltration process.

Critical Micelle Concentration Determination

The critical micelle concentration (CMC) of $BioPt^{IV}$ was measured using a dye assay. Stock solutions of $BioPt^{IV}$ were prepared at concentrations of 5.0 × 10^-3, 1.0 × 10^-3, 5.0 × 10^-4, 1.0 × 10^-4, 5.0 × 10^-5, 1.0 × 10^-5, 5.0 × 10^-6, and 1.0 × 10^-6 mg/mL. Eighty μL of a pyrene/acetone solution at a concentration of 1.0 × 10^-4 mg/mL was added to ten brown flasks, which were then protected from light to allow the acetone to evaporate. The stock solutions were mixed with the pyrene/acetone solution and shaken for 24 hours in the dark. Fluorescence excitation spectra were measured using a Fluoromax-4 spectrophotometer (Horiba Ltd., Kyoto, Japan) with an emission wavelength (λ_{em}) of 390 nm. The excitation spectra were recorded between 280 and 360 nm, and the intensity ratios (I397/I339) of the pyrene excitation spectra were plotted against the logarithm of the polymer concentration. The CMC was determined from the intersection of two straight lines.

Stability experiments for $BioPt^{IV}$

The stability of $BioPt^{IV}$ in PBS (pH = 7.4) was assessed by examining size

and distribution changes of the freshly prepared $BioPt^{IV}$ solution at 0, 1, 3, 5, 7, and 14 days. Additionally, the stability of $BioPt^{IV}$ in PBS containing 10% serum was evaluated, with size and distribution changes measured at the same time points.

Cell lines and cell incubation conditions

MB49 and MBT2-3 cells were obtained from the Chinese Academy of Sciences. The cells were cultured in DMEM medium containing 10.0% FBS, supplemented with 1.0% Penicillin-Streptomycin, and incubated at 37 °C in a 5.0% CO2 and 95.0% air atmosphere (approximately 20.0% O2). Mouse breast cancer 4T1 cells were purchased from BeNa Culture Collection Co., Ltd. (Beijing, P.R. China) and were cultured in RPMI 1640 medium containing 10.0% FBS, supplemented with 1% penicillin and 1% streptomycin, and incubated under the same conditions.

Platinum uptake in the cell

MB49 cells (5×10^5) were seeded in 6-well plates and cultured for at least 24 hours. The cells were then treated with Cisplatin, Cis+Rib, BioPtIV, and $BioPt^{IV}$@Rib at a concentration of 20 μM in the culture medium at 37 °C for 1, 4, and 7 hours. After treatment, the cells were washed three times with PBS. The cells were then collected, nitrified, and diluted with 1.0 wt% nitric acid for inductively coupled plasma mass spectrometry (ICP-MS) analysis.

Cell viability studies

The cells (MB49, MBT2-3) were seeded in 96-well plates at a density of 5×10^3 cells/well and incubated for at least 24 hours. The medium was then replaced with culture medium containing platinum-based drugs: Cisplatin, Cis+Rib, BioPtIV, and BioPt^{IV}@Rib, with concentrations ranging from 0.05 to 40 μM based on Pt. After incubating for 48 hours, 5 mg/mL MTT solution in medium was added, and the plates were further incubated at 37 °C. Following the removal of the culture medium containing MTT, 150 μL of DMSO was added to each well to dissolve the formazan crystals formed. Finally, the plates were shaken for 10 minutes, and the absorbance of the formazan product was measured at 492 nm using a microplate reader (Infinite M1000 Pro, Tecan, Switzerland).

Apoptosis analysis

Apoptosis of Mb49 cells induced by Cisplatin, Cis+Rib, BioPt^{IV}, and BioPt^{IV}@Rib at 20 μM for 24 hours was assessed using Annexin V/PI staining. The extent of apoptosis was measured with the Annexin V-FITC apoptosis detection kit, following the manufacturer's instructions. [2]

Animal welfare and protocols

Six-week-old female BALB/c mice were purchased from Beijing Vital Lihua Company and housed in SPF animal rooms. All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Qingdao University and approved by the Animal Ethics Committee of Qingdao University.

***In vivo* biodistribution imaging.**

In the subcutaneous model, BALB/c mice bearing primary bladder cancer were obtained by injecting Mb49 cells (1.0×10^7 cells/mL, 100.0 μL per mouse) at the leg site. When the tumors reached an approximate volume of 150 mm^3, the mice were intravenously injected with 200.0 μL of normal saline containing 3.5 mg/kg Pt BioPtIV@RIB-Cy7. At specific time points of 0.5, 24-, 48-, and 72-hours post-injection, the mice were anesthetized, and fluorescence signals from BioPtIV@RIB-Cy7 were recorded using the Master in vivo imaging system, equipped with an external 710 nm laser as the excitation source. Additionally, extra mice were sacrificed after 72 hours of treatment, and tumor tissues along with major organs were collected for ex vivo fluorescence imaging.

Antitumor Efficacy toward Subcutaneous Osteosarcoma *in vivo*

Mb49 cells (1.0×10^7 cells/mL, 100.0 μL per mouse) were injected at the right leg area of female BALB/c mice. When the tumors reached an approximate volume of 100 mm^3, treatment groups were administered: PBS (200 μL), Cisplatin (3.5 mg Pt/kg), Cisplatin + ALN (3.5 mg Pt/kg), and APtIV (3.5 mg Pt/kg). After five treatments, the mice were sacrificed. Tumor volume was calculated by measuring the tumor diameter using Vernier calipers, and the volume (mm^3) was estimated using the following equation.

$$V = \frac{4\pi}{3} \times \frac{a}{2} \times (\frac{b}{2})^2$$

In the equation, a represents the length of the major axis measured with the Vernier caliper, while b denotes the length of the minor axis, also measured with the Vernier caliper.

Statistical analysis

Data were presented as mean ± standard deviation. Student's t-test was used to determine the statistical difference between various groups. Differences were considered statistically significant at a level of *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

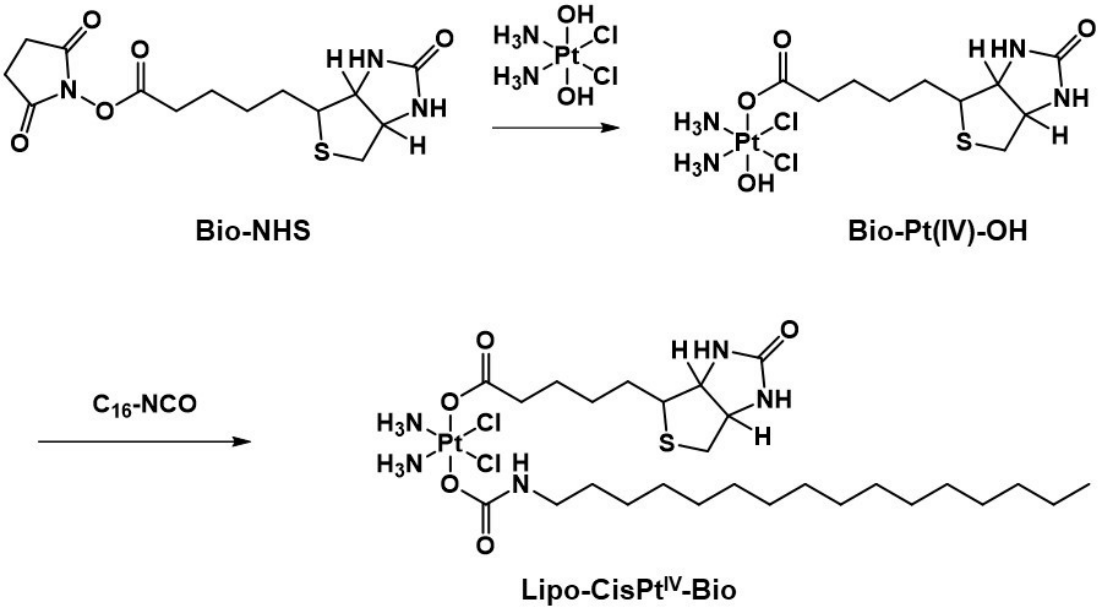

Figure S1. Synthesis procedure and chemical structures of Pt^{IV} prodrug Lipo-CisPt^{IV}-Bio.

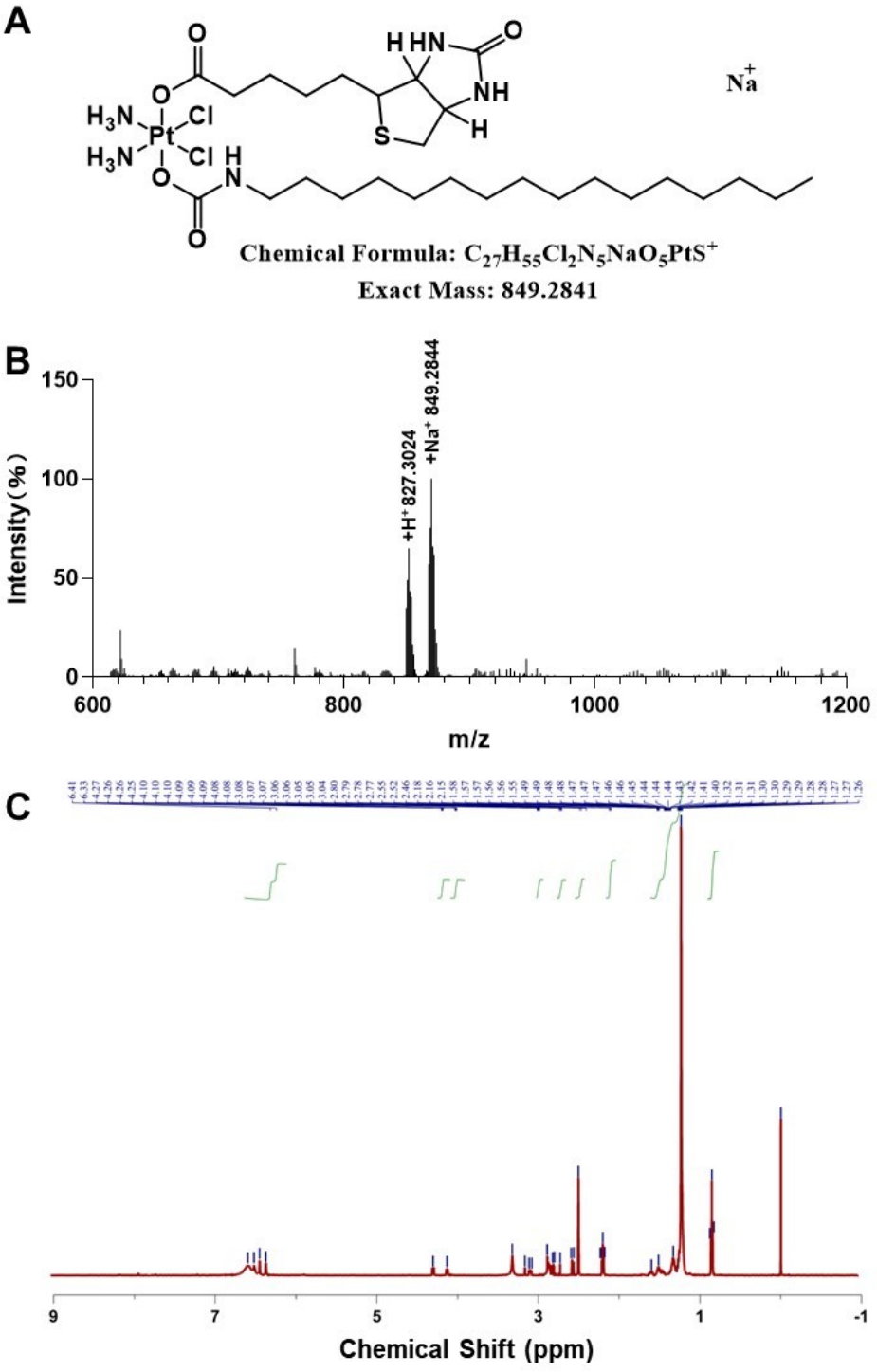

Figure S2. (a) Basic information of Lipo-CisPt^{IV}-Bio. (b) ESI-MS spectrum of Lipo-CisPt^{IV}-Bio. (c) ^{1}H NMR spectrum of Lipo-CisPt^{IV}-Bio in DMSO-d_6. The peaks labeled letters in the spectrum correspond to the protons labeled letters in the structure.

Mass ratio of carrier to drug (Prodrug: Rib)	2:1	3:1	4:1	5:1
Encapsulation percentage	Rib 61%	Rib 72%	Rib 89%	Rib 87%
Loading efficiency	Rib19% Cis23%	Rib17% Cis26%	Rib17% Cis29%	Rib14% Cis30%

Figure S3. Formula optimization of BPt^{IV}@Rib was conducted using various mass ratios of the Lipo-$CisPt^{IV}$-Bio and Rib.