Design and Delivery of Camplatin to Overcome Cisplatin Drug Resistance

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General Materials and Methods

The carrier polymer P1 (MPEG-b-PCL-b-PLL) was used as previously reported [1] and is a tri-block copolymer with two hydrophilic blocks polyethylene glycol (mPEG, molecular weight: 5000 Da, roughly 114 repeating units of ethylene glycol) and polylysine (PLL, 10 repeating units) as well as one hydrophobic poly-caprolactone (PCL, 20 repeating units). All other commercially sourced chemicals and solvents were used without further purification. N-hydroxysuccinimide (NHS), 1-ethyl-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl), succinic anhydride and (±)-Camphoric acid anhydride were purchased from Sigma-Aldrich. Cisplatin (purity 99%) was bought from Shandong Boyuan Chemical Company, China. 1H NMR spectra were measured using a Unity-300MHz NMR spectrometer (Bruker). Fourier Transform Infrared (FT-IR) spectra were recorded on a Bruker Vertex 70 spectrometer. Electrospray mass spectrometry (ESI-MS) measurements were performed on a Quattro Premier XE system (Waters) equipped with an electrospray interface (ESI). An inductively coupled plasma optical emission spectrometer (ICP-OES, iCAP 6300, Thermoscientific, USA) was used to measure platinum loading in the polymer-Pt(IV) conjugates and dialysis samples in drug release experiments. An inductively coupled plasma mass spectrometer (ICP-MS, Xseries II, and Thermoscientific, USA) was used for quantitative determination of trace levels of platinum in the cell lysis liquid. Micelle size and polydispersity was measured using a DAWN EOS DLS (Wyatt Technology, USA) equipped with a vertically polarized He-Ne laser. TEM images were taken using a JEOL JEM-1011 electron microscope. Particle size and zeta potential measurements were conducted on a Malvern Zetasizer Nano ZS.

Synthesis of cisPt(IV)-OH

CisPt(IV)-OH was synthesized as previously described [1].

Synthesis of camplatin

To a solution of cisPt(IV)-OH (0.334 g) in anhydrous DMSO (30 ml) was added camphoric anhydride (0.182 mg) and the reaction

mixture was stirred at room temperature for 24 hours in the dark. Cooled diethyl ether (500 ml) was added to precipitate the product to obtain a bright yellow solid, which was washed several times with diethyl ether, and dried.

Preparation of micellar nanoparticles of camplatin M(camplatin)

Camplatin was dissolved in water in a flask, to which an aqueous solution of EDC and NHS (2 eq. per carboxyl group each) was added under stirring for ten minutes. The polymer P1 (1 amine eq. per camplatin carboxyl group) was then added and the solution was stirred in the dark for 24 h. P1 has 114 ethylene glycol, 20 caprolactone (CL) and 10 (lysine) LL repeat units. The polymer conjugates of camplatin were then purified by dialysis (1,000 MWCO) for 12 h and lyophilized.

Cell lines and culture conditions:

A2780 and A2780DDP cells were purchased from Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, Shanghai, China, and grown in RPMI 1640 (Life Technologies) supplemented with 10% fetal bovine serum, 0.03% L-glutamine and 1% penicillin/streptomycin in 5% CO2 at 37 °C.

MTT (3-(4,5-dmethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) Assay

A2780 and A2780DDP cells harvested in a logarithmic growth phase were seeded in 96-well plates at a density of 105 cells /well and incubated in RPMI-1640 for 12 h. The medium was then replaced by RPMI 1640 containing cisplatin, cisplatin + camphoric acid, camplatin and M(camplatin) at a final equivalent Pt concentration from 0 to 200 μM; Moreover, camphoric acid and camphoric anhydride at a concentration ranging from 0 μM to 200 μM was used to determine the cytotoxicity of camphoric acid and camphoric anhydride. The incubation time for all drugs was 48 h. After incubation, 20 μL of MTT solution in PBS with the concentration of 5 mg/mL was added and the plates were incubated for another 4 h at 37 °C, followed by removal of the culture medium containing MTT and addition of 150 μL of DMSO to each well to dissolve the formazan crystals formed. Finally, the plates were shaken for 10 minutes, and the absorbance of formazan product was measured at 492 nm by a microplate reader.

Intracellular uptake of M(camplatin) and Pt-DNA adducts measurement

A2780 and A2780DDP were seeded in 6-well plates at a density of 106 cells per well. At their logarithmic phase of growth, the cells were treated with cisplatin, camplatin and M(camplatin) with the final platinum concentration in the culture medium regulated to 10 μM and incubated at 37 °C for 1 h and 4 h. As previously described (2), to remove surface-bound drugs, cells were washed three times with ice-cold PBS, incubated with 1.5 mL of 0.15 M sodium chloride (pH was adjusted to 3.0 by acetic acid) for 3 min at 4 °C, then rinsed with 2 mL of cold PBS, harvested by scraping in ice-cold PBS, and centrifuged. Thereafter, the cell pellet were lysed by adding 200 μL cell lysis buffer (Promega lysis buffer) and then the cell lysis solution was frozen at −20°C for 20 min and thawed at room temperature. 100 μL of the cell lysis solution for each sample was used directly to measure the Pt content by ICP-MS. The other 100 μL of the cell lysis solution was used to determine the protein content in each cell sample by using bicinchoninic acid (BCA) protein assay kit (Beyotime Institute of Biotechnology, Shanghai, China) according to previously published data [2]. The platinum content was expressed as nano-grams of Pt per milligram of total proteins.

A2780 and A2780DDP were seeded in 6-well plates at a density of 106 cells per well. At their logarithmic phase of growth, the cells were treated with cisplatin, camplatin and M(camplatin) with the final platinum concentration in the culture medium regulated to 10 μM and

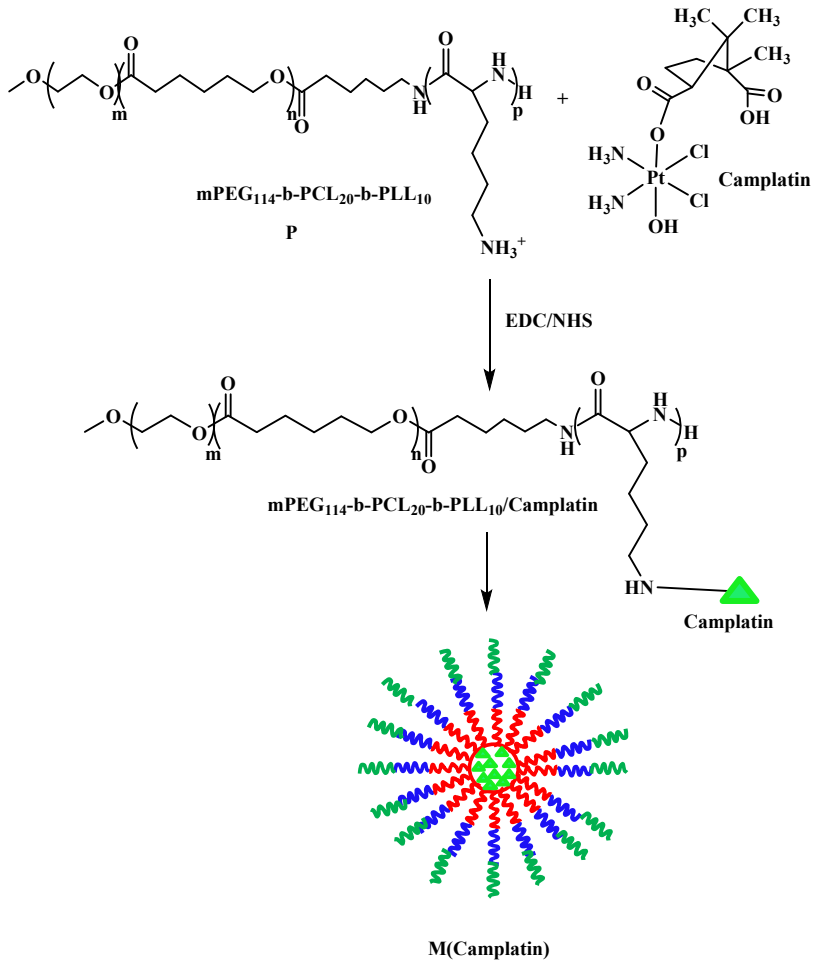
incubated at 37 °C for 24 h. Next we used genomic DNA extraction kit (TIANGEN BIOTECH, Beijing, China) to isolate the genomic DNA of the cells treated with different drugs, and finally 200 μL distilled water was used to dissolve the DNA and the DNA content in the solution was quantified. The remaining 200 μL DNA was diluted into 1 mL volume. The platinum content was measured by ICP-MS and expressed as nano-grams of Pt per milligram of total genomic DNA.

Quantitative real-time PCR

A2780 and A2780DDP in logarithmic phase of growth were seeded in 6-well plates at a density of $3*10^5$ cells per well. The cells were exposure to cisplatin, camphoric acid, camplatin, M(camplatin) and combination of cisplatin and camphoric acid for 24 h, with both platinum and camphoric acid concentration regulated to 10 μM. The total RNA from drug-exposured cells was extracted using TRIzol Reagent (Life Technologies, USA), and cDNA was synthesized by M-MLV Reverse Transcriptase (Promega, USA). The primers used for quantitative real-time PCR (qPCR) were: Bcl-2 GGTCATGTGTGTGGAGAGC(sense) and GATCCAGGTGTGCAGGTG (antisense); Bax: TCTGGAAGAAGATGGGCTGAG (sense) and TTTGTGTCCCGAAGGAGGTT(antisense); â-actin: GTCCACCGCAAATGCTTCTA (sense) and TGCTGTCACCTTCACCGTTC(antisense). The qPCR reactions were conducted on StepOnePlus Real-Time System (Applied Biosystems, USA) using SYBR Premix Ex Taq (Takara, China), according to procedures: 95°C for 30 seconds; then 40 cycles of 5 seconds at 95°C and 30 seconds at 60°C. The amplification products were verified by a melting curve. The relative mRNA levels were calculated using the 2−ÄÄCt method. Each experiment was performed in triplicate.

Drug release from MPEG-b-PCL-b-PLL/Pt(IV) micelles

50 mg of camplatin was dissolved in 20 ml of phosphate buffered saline (0.1 M PBS, pH 7.4). The solution was then placed into a pre-swelled dialysis bag with a molecular weight cutoff of 3.5 kDa and immersed into 140 ml of 0.1 mol/L PBS (pH 7.4). The dialysis was conducted at 37 degree in a shaking culture incubator. 1.5 mL of sample solution was withdrawn from the incubation medium at specified time intervals and measured for Pt concentration by ICP-OES. After sampling, equal volume of fresh PBS was immediately added into the incubation medium. The platinum released from the micelles was expressed as the percentage of cumulative platinum outside the dialysis bag to the total platinum in the micelles. The same drug release procedure was performed in the presence of 5 mM and 0.1 mM sodium ascorbate, respectively.



Scheme S1 Synthesis of polymer-camplatin conjugates ($mPEG_{114}$-PCL_{20}-b-PLL_{10}/Camplatin) and preparation of the micellar nanoparticles with camplatin M(camplatin). $mPEG_{114}$-PCL_{20}-b-PLL_{10} is a tri-block copolymer with two hydrophilic blocks polyethylene glycol (mPEG, molecular weight: 5000 Da, roughly 114 repeating units of ethylene glycol) and polylysine (PLL, 10 repeating units) as well as one hydrophobic poly-caprolactone (PCL, 20 repeating units). The platinum content in M(camplatin) was determined by ICP-OES to be 10.4% in weight, corresponding to cisplatin content of 16% and camphoric acid content of 10.6% in weight.

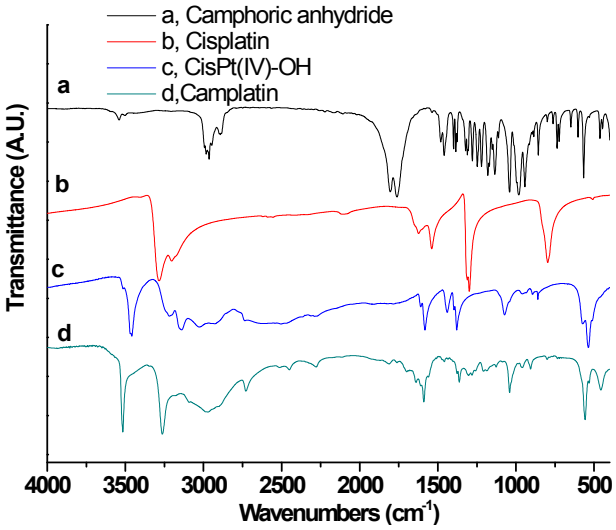


Figure S1 FT-IR spectra of camphoric anhydride (a), cisplatin (b), cisPt(IV)-OH(c), and camplatin (d).

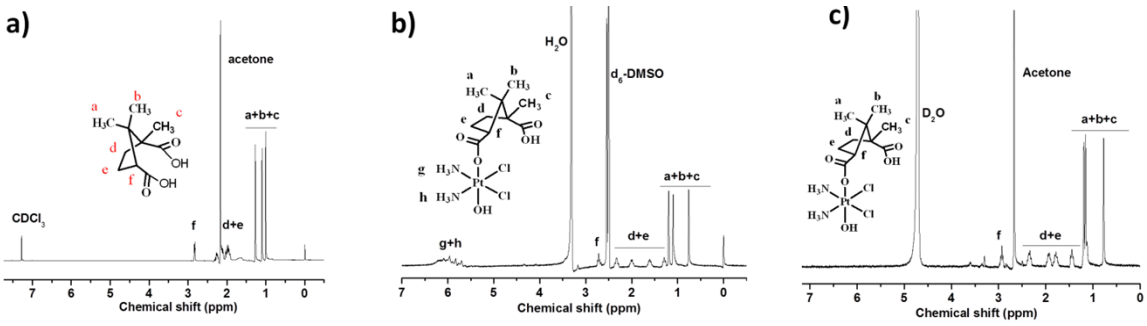


Figure S2 ^{1}H NMR spectra of camphoric acid in $CDCl_3$ (a), camplatin in d_6-DMSO (b), and camplatin in D_2O (c). Camplatin (Figure S2b) shows all the characteristic protons of camphoric acid (Figure S2a) with a new broad peak around 6.0 ppm which could be assigned to the NH_3 of camplatin. Shifting the solvent from d-DMSO to D_2O, the chemical shift of active protons in NH_3 disappeared (Figure S2c).

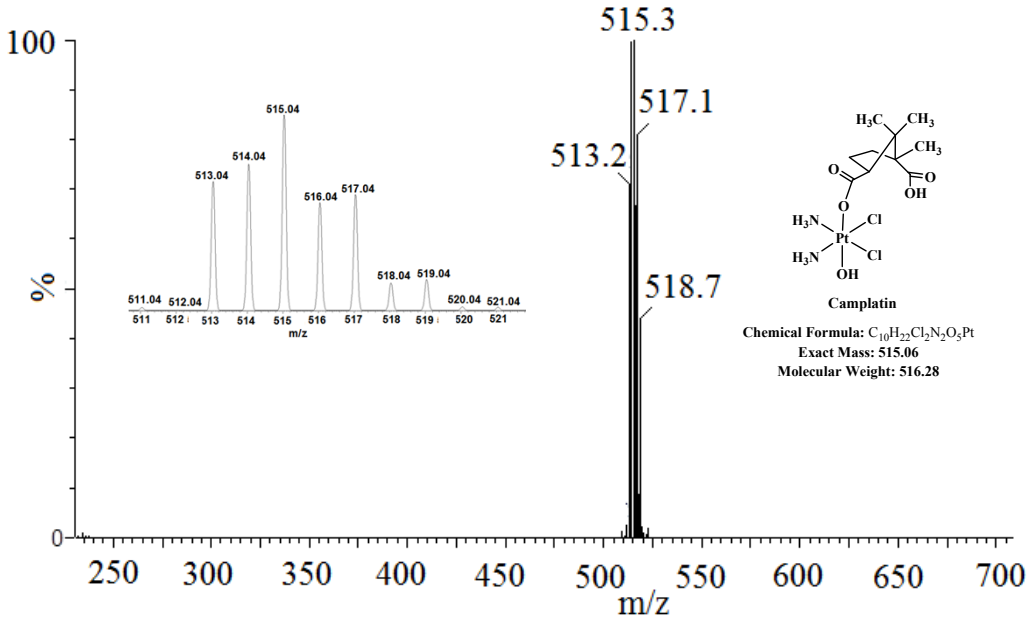


Figure S3 ESI-MS spectra of camplatin (negative mode). The chemical structure of camplatin and its molecular weight calculated by Chemical-Draw are shown in the insets. There is a major peak at m/z=515.3, standing for its molecular ion peak. Moreover, the isotopic pattern is nearly the same as the calculated one (inset).

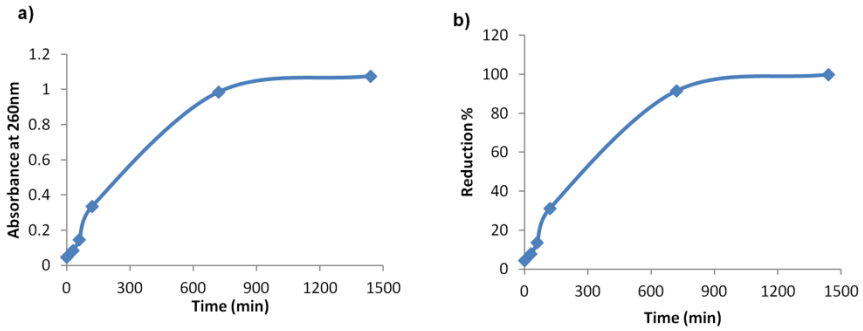


Figure S4 Reduction kinetics of camplatin by GSH over a time period of 12 h. 32 μM Pt drugs were incubated with 16 mM GSH at room temperature and the UV-absorbance at 260 nm were monitored.

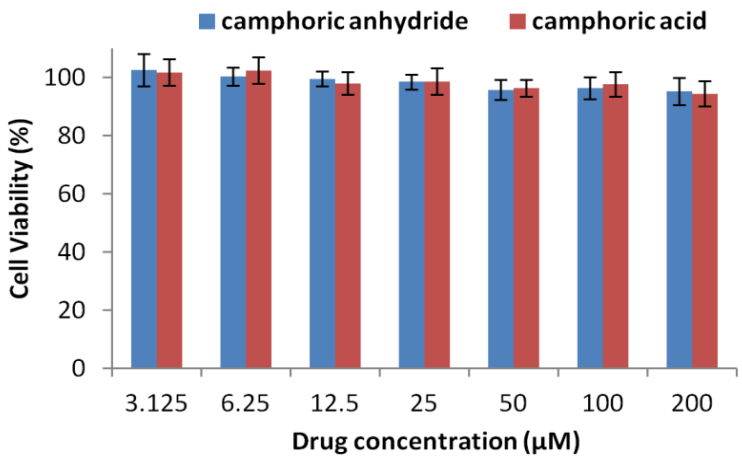


Figure S5. MTT assay of camphoric anhydride and camphoric acid towards A2780 cancer cells. The drug concentrations were regulated from 3.125 μM to 200 μM. Results show that both camphoric anhydride and camphoric acid have minimum cytotoxicity towards the ovarian cancer cells A2780.

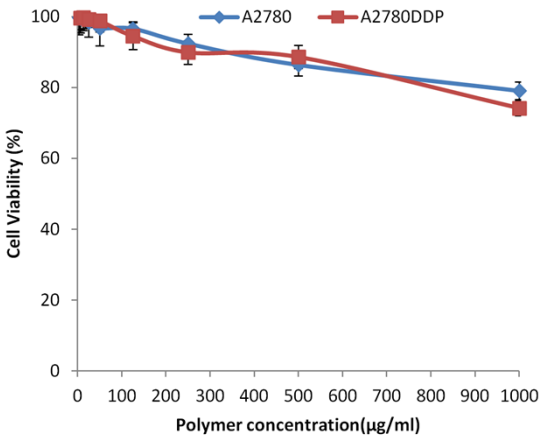


Figure S6. In vitro cytotoxicity study of the polymer MPEG-b-PCL-PLL on A2780 and A2780DDP cells for 48 h determined by MTT assay

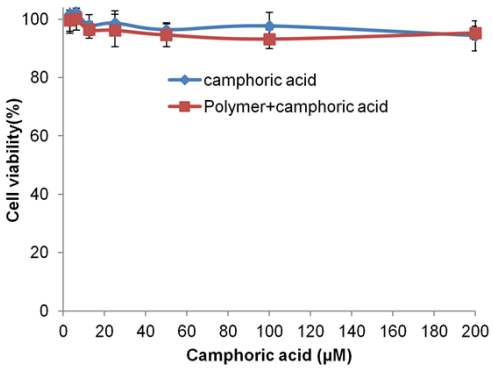


Figure S7. Representative in vitro MTT assay evaluation of polymer plus camphoric acid on A2780 cells at 48 h.

References

[1] Xiao H, Qi R, Liu S, Hu X, Duan T, Zheng Y, Huang Y, Jing X. Biodegradable polymer - cisplatin(IV) conjugate as a pro-drug of cisplatin(II), Biomaterials, 2011, 32, 7732-7739.

[2] Xiao H, Song H, Zhang Y, Qi R, Wang R, Xie Z, Huang Y, Li Y, Wu Y, Jing X. The use of polymeric platinum(IV) prodrugs to deliver multinuclear platinum(II) drugs with reduced systemic toxicity and enhanced antitumor efficacy, Biomaterials, 2012, 33,8657-8669.