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The effect of lipocisplatin on cisplatin efficacy and nephrotoxicity in malignant breast cancer treatment

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ABSTRACT

A lipid–cisplatin conjugate was synthesized for super-molecular assembly with lipids to form a new generation of liposomal cisplatin formulation, lipocisplatin. *In vitro*, lipocisplatin has higher efficacy in human ovarian cancer A2780 and human breast cancer MCF-7 with the murine breast cancer cell line 4T1 which is currently an established model for stage IV breast cancer as the most sensitive strain. Moreover, lipocisplatin demonstrated a greater MTD value and relatively longer blood circulation as compared to cisplatin. Lipocisplatin preferentially accumulate drugs to the tumor site, resulting in a better tumor inhibition efficacy. Moreover, lipocisplatin exceeds the size cutoff for kidney clearance, hence it bypasses the nephrotoxicity of cisplatin which is a major curse of one of the most efficient anticancer drugs nowadays in clinic. The results here indicated lipocisplatin may be translated into a new generation of liposomal based cisplatin drug in clinic.

1. Introduction

Since its discovery in 1965 by Rosenberg, cisplatin has been in worldwide use to successfully treat several solid tumors, including testicular, ovarian, cervical, and head-and-neck cancer [1,2]. The major biological target of cisplatin is cellular DNA to form stable Pt-DNA adducts which can thereafter interfere with transcription and replication, and trigger apoptosis, resulting in the death of the cancer cell [3,4]. Despite of its great efficacy and widespread use, cisplatin is limited by serious toxicities such as nausea, vomiting, ototoxicity, and peripheral neuropathy with nephrotoxicity as the most notorious and to be the major side effects [5,6]. This is due to the fact that excessive binding of cisplatin to plasma and tissue proteins and fast blood clearance of the drugs results in a rapid loss of its bioavailability with more than 95% of the administered drugs inactivated within 24 h [7] hence continuous higher doses of cisplatin should be administered to ensure its effect.

The most fashionable strategy to prolong the blood circulation, enhance anti-tumor efficacy and reduce systemic toxicity nowadays for a certain drug is to load the drugs to a so-called drug delivery carrier [8–10]. Among the developed drug delivery systems, liposomes are the most successful ones till far. Liposomes are vesicles, which are composed of one or more phospholipids bilayers surrounding an aqueous lumen [11]. Due to the nature of the leaky vasculature and scarce lymphatic vessels in tumors, treatment with liposomes with drugs leads to extravasation and accumulation of the drugs within tumors [12,13]. Moreover, the liposomal anthracycline agents (doxorubicin and daunorubicin) are approved by FDA with liposomal formulations of other agents (vincristine and camptothecins) are in different clinic phase [14,15]. Considering the drawbacks of cisplatin and the benefits of liposomes, people have also developed a lot of liposome based delivery systems for cisplatin to prevent pre-inactivation of platinum drugs, reduce the nephrotoxicity and increase the drug accumulation in the tumor. For example, SPI-077, a sterically stabilized liposome with cisplatin

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which was shown to be effective in preclinical studies exhibited no efficacy on patients [16]. Lipoplatin, which may be the most advanced liposomal formulation of cisplatin did exhibit anti-tumor efficacy in phase I and II studies with reduced side effects compared to the free drug, and is currently being tested in phase III studies [17].

More recently, Sengupta et al. synthesized a cholesterol-tethered cisplatinum (II) amphiphile [18]. Self-assembling cholesterol cisplatin drug with lipid into liposomal nanoparticles exhibited increased potency and efficacy *in vitro* and *in vivo*, respectively. However, the synthesis of the cholesterol cisplatin amphiphile includes several steps and will result in difficulty in engineering a large scale of drugs. Here we describe using a commercialized lipid with a tailed short carboxyl acid (Fig. 1a, 1-palmitoyl-2-glutaryl-sn-glycero-3-phosphocholine, PGPC) rather than the cholesterol to chelate cisplatin to prepare a lipid–cisplatin conjugate (PGPC–cisPt, Fig. 1a). By super-molecular assembly of this lipid–cisplatin conjugate PGPC–cisPt with other lipids such DSPE (1,2-distearoyl-sn-glycero-3-phosphoethanolamine), $MPEG_{2k}$–DSPE (1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[Methoxyl(polyethylene glycol)-2000]) and cholesterol, an alternative liposomal cisplatin formulation which we called thereafter lipocisplatin was prepared (Fig. 1b). Different from the existing lipoplatin where free cisplatin is encapsulated [17], the cisplatin prodrug PGPC–cisPt was assembled into the bilayer of the lipocisplatin. Due to the drug conjugation, cisplatin would be more stable in the liposomes and can be released in a sustained way. Further *in vitro* study revealed that lipocisplatin exhibited increased efficacy as compared to cisplatin and carboplatin on human ovarian cancer cell line A2780 and human breast cancer cell line MCF-7 with the murine breast cancer 4T1 cells as the most sensitive cell line to lipocisplatin treatment. Due to the fact that breast cancer accounts for 22.9% of all cancers worldwide (excluding non-melanoma skin cancers) in women and breast cancer caused 458,503 deaths worldwide In 2008 (13.7% of cancer deaths in women) [19], we choose 4T1 breast cancer model to evaluate lipocisplatin because 4T1 cells are highly malignant and 4T1-induced tumor model is now used as an animal model for stage IV human breast cancer [20].

2. Materials and methods

2.1. Chemicals

Silver nitrate ($AgNO_3$) and cisplatin (CDDP) was purchased from Aladdin (Shanghai, China). DSPE (1,2-distearoyl-sn-glycero-3-phosphoethanolamine), $MPEG_{2k}$–DSPE (1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[Methoxyl(polyethylene glycol)-2000]), 1-palmitoyl-2-glutaryl-sn-glycero-3-phosphocholine (PGPC) and cholesterol were bought from Avanti Lipids (USA). All other chemicals were of analytical grade and used as received.

2.2. Synthesis of PGPC–cisPt

Cisplatin (30 mg, 0.1 mmol) was suspended in 10 mL of de-ionized water in a flask, to which $AgNO_3$ (16.9 mg, 0.1 mmol) was added. The reaction mixture was kept stirring in the dark for 12 h. Thereafter, it was subjected to filtration to get a clear yellow solution of mono-aquated cisplatin. Then, PGPC (60.9 mg, 0.1 mmol) was dissolved in 10 mL DMF in a flask kept in dark, to which the mono-aquated cisplatin solution was added. The mixed solution was kept stirring in the dark for 24 h and then it was lyophilized to get a powder of crude PGPC–cisPt. Then it was purified by dialysis in a 500-Da molecular cutoff dialysis membrane for 24 h against de-ionized water followed by lyophilization.

2.3. Preparation of lipocisplatin

Cisplatin loaded liposome was prepared by film evaporation followed by hydration and extrusion method. Briefly, a total of 1 mmol of lipid, to be specific, cholesterol (0.154 mg, 386.6), DSPE (0.22 mg), $MPEG_{2k}$–DSPE (0.14 mg), and PGPC–cisPt (0.29 mg) (molar ratio: cholesterol:DSPE:$MPEG_{2k}$–DSPE:PGPC–cisPt = 0.4:0.295:0.05:0.3) were dissolved in 2 mL dichloromethane. Solvent was then evaporated into a thin and uniform lipid–drug film using a rotary evaporator. The lipid–drug film was then hydrated with de-ionized water for 1 h at 65 °C. The hydrated sample was then subjected to an extruder with 100 nm polycarbonate membrane. It was extruded by 15 passages and then collected for using.

2.4. Dynamic light scattering and zeta potential

Dynamic light scattering study of lipocisplatin to gain the size and size distribution information is completed on a spectrophotometer equipped with a ZETASIZER Nano Series Nano-ZS ZEN3600 (Malvern Instruments Ltd., UK) at 25 °C. 0.2 mL of liposomal suspension sample was diluted with 2.5 mL of double distilled water immediately after preparation. Each measurement was repeated three times. The zeta potential was also measured by using the Nano-ZS ZEN3600 (Malvern Instruments Ltd., UK) at 25 °C. Liposomal suspensions were diluted 10-fold with double distilled water before the measurement. Each test was repeated by three times.

2.5. Physical stability study of lipocisplatin

Lipocisplatin freshly prepared was kept in a 4 °C refrigerator for a period of 15 days. Changes in average hydrodynamic diameter at desirable time intervals were monitored. The particle size and size distribution were measured via DLS.

2.6. Drug release of lipocisplatin

Lipocisplatin (500 μL) was suspended in buffered solution at pH 5.0 and pH 7.4 respectively and sealed in a dialysis membrane with a molecular weight cutoff equal to 500 Da. The dialysis bag was then immersed in 100 mL PBS buffer at room temperature with gentle shaking. An aliquot was collected from the incubation medium at predetermined time intervals, and the cumulative released drug was quantified via inductively coupled plasma optical emission spectrometry (ICP–OES).

2.7. Chelation with 5′-GMP by the released Pt species

The buffered solution out of the dialysis membrane in Section 2.6 was collected (pH 5.0, 5 mL) in a glass vial, to which 5′-GMP (guanosine 5′-monophosphate sodium salt) was added to a final concentration of 100 μM. The vial was kept in the dark and shaken for 24 h at room temperature. After that, an aliquot of the buffered solution was collected and subjected to matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI–TOF–MS, Bruker, Autoflex series) study.

2.8. Cell lines and culture conditions

Ovarian cancer cell A2780, breast cancer cell line 4T1 and MCF-7 were purchased from ATCC. MCF-7 was cultured in DMEM and A2780 and 4T1 were cultured in RPMI medium 1640 supplemented with 10% FBS, 50 unit/mL penicillin, and 50 unit/mL streptomycin. The cells were incubated in 5% CO_2 atmosphere at 37 °C.

2.9. Cell viability study

Cells (A2780, MCF-7 and 4T1) were seeded separately into 96-well plates at a density of (1×10^4 cells per well). Cisplatin, carboplatin and lipocisplatin at a concentration ranging from 0 μM to 100 μM were added at equivalent Pt concentration and incubated for 48 h. Thereafter, 20 μL MTT solutions at 5 mg/mL were added to each well and incubated for another 4 h. Then the medium was removed and 150 μL DMSO was added. The optical density (OD) was measured at 570 nm using a microplate reader (Bio-Rad, Model 550, USA). The relative cell viability was calculated according to the following equation: Relative cell viability = OD570 (samples)/OD570 (control), where OD570 (samples) refers to the OD values at 570 nm of the drug treated wells, OD570 (control) refers to the OD values at 570 nm of the blank wells (without drug treatment).

2.10. Intracellular uptake and Pt-DNA adducts study via inductively coupled plasma mass spectrometry (ICP–MS)

Cells were seeded in six well plates at a density of 8×10^5 cells/well overnight. Then the cells were treated with either 10 μM of free cisplatin or lipocisplatin at 37 °C for 12 h. For inhibition of the energy-dependent internalization of lipocisplatin, prior to adding lipocisplatin, the cells were treated with 20 mM NaN_3 for 1 h. After that, the culture media with azide was removed and cells were washed three times by PBS. Then lipocisplatin was added to the cells for 12 h incubation. After 12 h of drug incubation, the cells were thoroughly washed 5 times by 1 mL of cold PBS and then the cells were detached and collected by using trypsin and calculated. After that, the cells were lysed by 70% nitric acid over night. The cell lysis liquid was diluted in 5% nitric acid for ICP–MS measurement [21].

For Pt-DNA adducts study in the cells, cells were seeded in six well plates at a density of 8×10^5 cells/well overnight. Then the cells were treated with either 10 μM of free cisplatin or lipocisplatin at 37 °C for 12 h. The cells were thoroughly washed 5 times by 1 mL of cold PBS. Genomic DNA was collected and purified from the using DNAZOL (life TECHNOLOGIES, Inc., Grand Island, NY) according to the manufacturer's instruction. The DNA concentration and purity was determined by measuring absorbance at 260/280 nm. The Pt content in the DNA was tested via ICP–MS and expressed as "pmol Pt/50 μg DNA".

2.11. Mice use and establishment of in vivo 4T1 breast cancer model

Balb/c female mice (4–5 week old, 20–25 g) were obtained from SLRC Laboratory animal center (Shanghai, China). All animal studies were conducted in

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