Department of Pharmacy[1], Zhejiang University City College, Hangzhou, China; Second Affiliated Hospital[2], College of Medicine, Zhejiang University, Zhejiang Provincial People's Hospital[3]; Institute of Pharmaceutics[4], Zhejiang University, Hangzhou, China

Efficacy and toxicity of cisplatin liposomes modified with polyethylenimine

Xiaoyi Sun[1], Jinliang Chen[2], Xiaohui Gu[3], Wenquan Liang[4], Junbo Wang[1]

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Prof. Junbo Wang, Department of Pharmacy, Zhejiang University City College, 51 Huzhou Street, Hangzhou, 310015, P.R.China
wangjb@zucc.edu.cn

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The polycation transfection agent, polyethylenimine (PEI) was introduced into cisplatin (CDDP)-encapsulated liposomes by modification with amphiphilic PEI-cholesterol (PEI-Chol) to evaluate its potential application in chemotherapeutic drug delivery. Compared with unmodified neutral liposomes (CDDP-NL), the remarkable features of PEI-modified cationic liposomes (CDDP-CL) increased cytotoxicity attributed to enhanced cellular uptake and extended cellular retention resulting from endosome escape *in vitro*. In a H22 hepatoma-bearing mouse model, CDDP-CL reduced the nephrotoxicity associated with CDDP and had an antitumor activity similar to free drug, without inducing obvious system toxicity. These results confirm that the cationic modification of liposomes with PEI is efficient and safe for antitumor drug delivery.

1. Introduction

Polycation polyethylenimine (PEI) has been successfully used for gene therapy (Patnaik and Gupta 2013; Won et al. 2011) as an efficient transfection agent. The advantage of strong DNA compaction capacity, endosomolytic activity and nuclear translocation make it outstanding over other cationic polymers. The two latter merits of PEI are also essential for antitumor drugs whose targets exist in the nucleus, which must overcome plasma membrane and nuclear envelope barriers as well as the degradative endolysosomal environment to achieve their efficacy. Although PEI has been extensively studied in gene delivery, its application for small molecular anticancer drugs has not received much attention. The development of PEI-integrated drug delivery systems have focused on the anticancer drugs, paclitaxel, hydroxycamptothecin (Li et al. 2011), curcumin (Lin et al. 2012), doxorubicin (Amjad et al. 2012; Abbasi et al. 2012), and 6-(hydroxymethyl)-1,4-anthracenedione (Ganta et al. 2008). Increased solubility of hydrophobic drugs, sustained drug release and enhanced uptake are important factors for the success of PEI modification. However, the response associated with the intrinsic properties of PEI and their activities *in vivo* are not well understood.

Cisplatin (CDDP) is an effective chemotherapeutic drug that induces the formation of platinum-DNA-adducts. It is widely used to treat a broad spectrum of malignancies including lung, head and neck, testis, and ovarian cancers (Loehrer and Einhorn 1984). However, its clinical application is limited by significant undesirable side effects, such as nephrotoxicity, nausea/vomiting, gastrointestinal toxicity, ototoxicity and neurotoxicity (Nagy et al. 2012). Several attempts have been investigated for improving antitumor activity and reducing CDDP-induced systemic toxicities (Zalba and Garrido 2013), including the use of stealth liposomes (Stathopoulos and Boulikas 2012; Zamboni et al. 2004), which presents a versatile system for drug delivery; however, these methods have not yet demonstrated significant success.

In our previous work, CDDP-encapsulated liposomes were successfully modified with PEI, which dramatically enhanced the antitumor activity against A549 cells *in vitro* (Sun et al. 2012). It was confirmed that their capacity for endosomal escape, alteration of the endocytic pathway and extended cellular retention were the main reasons for improved activity. In this study, we intended to evaluate the efficacy and safety of PEI-modified CDDP-encapsulated cationic liposomes (CDDP-CL) *in vivo*.

2. Investigations and results

2.1. Platinum accumulation and exocytosis

As shown in Fig. 1A, CDDP-CL significantly enhanced intracellular Pt accumulation. The Pt levels were 12.92, 8.44 and 5.19 ng/10^7 cells for CDDP-CL, CDDP and unmodified neutral liposomes (CDDP-NL), respectively. During 6-h exocytosis studies, cellular Pt levels decreased after withdrawal of CDDP (Fig. 1B). Approximately 25% Pt was exported from cells in CDDP-NL group after 1 h, following which the level remained the same until 6 h. In contrast, almost no Pt was expelled from cells treated with CDDP-CL and CDDP solution. Thus, cationic liposomes improved intracellular retention of cisplatin compared with neutral ones.

2.2. Cytotoxicity assay

The *in vitro* antitumor activities of CDDP-NL and CDDP-CL towards H22 cells are shown in Fig. 2A. The cytotoxicity of liposomal CDDP was greatly enhanced by PEI modification, which was even higher than CDDP solution. The IC50 s of CDDP-CL, CDDP-NL and CDDP were 0.45, 1.52 and 0.58 μg/mL,

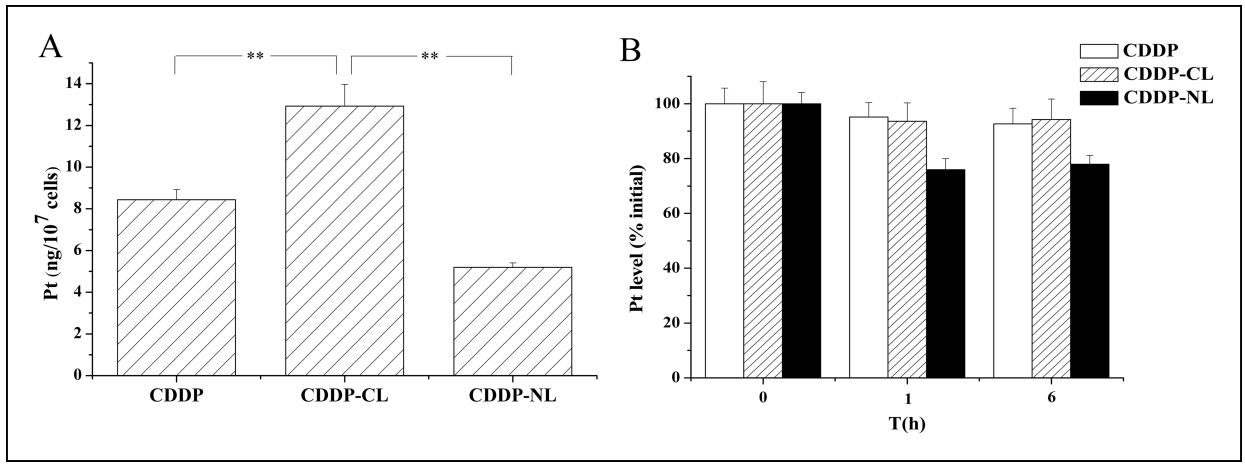

Fig. 1: Cellular platinum accumulation and exocytosis, (A) Intracellular platinum levels in cells after exposure to 5 μg/mL CDDP (** $P < 0.01$). (B) Fractions of Pt remained within the cell after a period of exocytosis.

respectively (Fig. 2B). No cytotoxicity of blank liposomes was observed in the administered concentration range.

2.3. In vivo antitumor efficacy

The antitumor activities of CDDP liposomes *in vivo* were evaluated in H22 tumor-bearing ICR mice. As shown in Fig. 3A, the tumor volumes in groups receiving saline and blank liposomes increased rapidly from day 6, with their mean volumes reaching more than 1000 mm^3 on day 12. In comparison, groups administered CDDP liposomes and free CDDP showed retarded tumor growth or even complete growth suppression with their mean volumes less than 400 mm^3 on day 12. CDDP-CL exhibited more efficient antitumor activity than CDDP-NL. Tumor nodules in the group receiving CDDP-CL remained almost undetectable with mean volumes of not more than 200 mm^3 at the end of therapy, which was comparable with the antitumor effect of CDDP solution. Tumor nodules for the CDDP-NL grew slightly faster and were triple the mean volume of the original at the termination of the experiment. Statistical analysis revealed that the group receiving CDDP-CL had significantly smaller tumors when compared with the group injected with CDDP-NL ($P < 0.01$) from day 6 to day 12, and were not significantly different when compared with the CDDP solution group.

The results of tumor weight and tumor inhibition rate (TIR) (Fig. 3B) were in accordance with the tumor volume results reported above. Tumor weights of both blank liposomes and saline groups reached 700 mg on day 12. Compared with CDDP-NL, cationic liposomes significantly increased the TIR from 83.6% to 93% ($P < 0.01$). There was no significant difference in the TIR between CDDP-CL and free cisplatin.

2.4. Toxicity evaluation

Monitoring of body weight is shown in Fig. 4A. Neither Blank-NL nor Blank-CL demonstrated obvious toxicity. The weight/growth curves of blank liposome groups overlapped with that of the saline group. The mice continued growing during the period of the experiment and showed a nearly 40% body weight increase at day 12. Favorable results were obtained in the groups injected with CDDP-CL and CDDP-NL. The mice lost less than 10% body weight in the following 2 days after therapy and they started to gain weight on day 4. On day 12, they were comparable with the mice receiving blank liposomes. In contrast, free CDDP induced severe weight loss, which caused a remarkable loss greater than 20% of the original weight on day 6 and did not recover their original weight by the end of the experiment. The mice administered CDDP solution had weak movement,

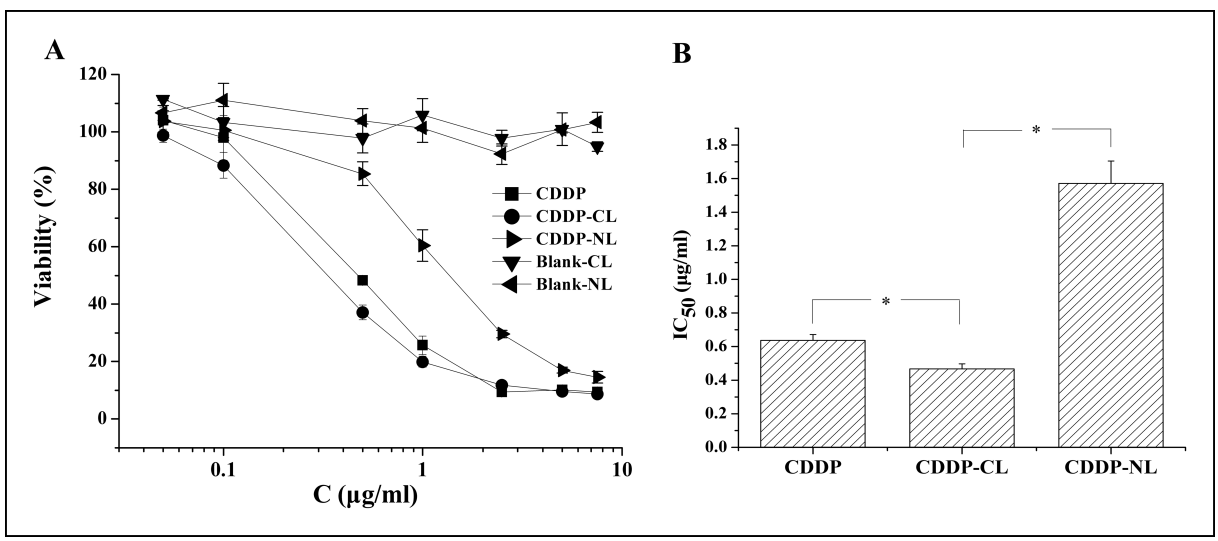

Fig. 2: Cytotoxicity towards H22 cells, (A) Cells were exposed to CDDP for 72 h. (B) IC50 of CDDP-CL, CDDP-NL and CDDP solution. Data are expressed as mean ± S.D of four independent experiments.

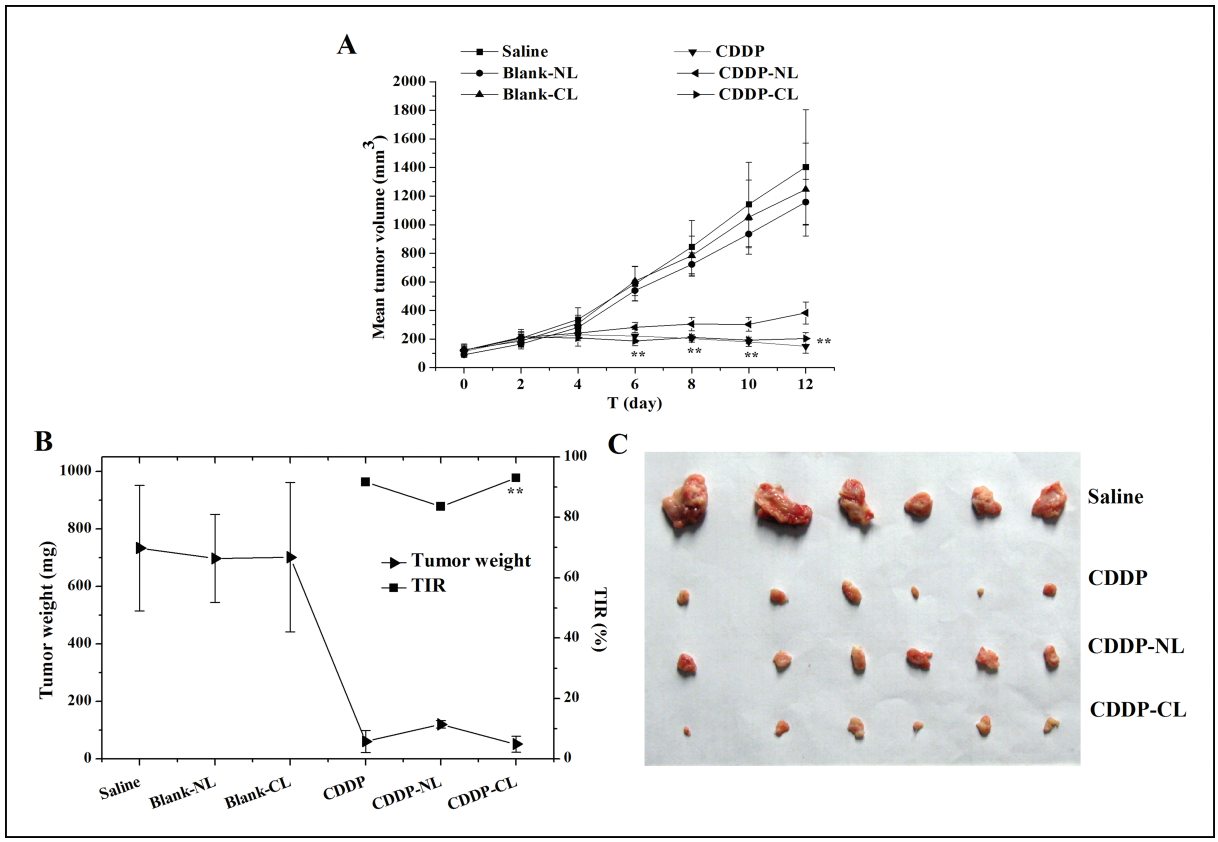

Fig. 3: *In vivo* antitumor efficacy, (A) Mean tumor volume of mice (**$P < 0.01$ CDDP-CL compared with CDDP-NL). (B) Tumor weight and TIR of mice on day 12 (**$P < 0.01$). (C) Tumors on day 12 of therapy.

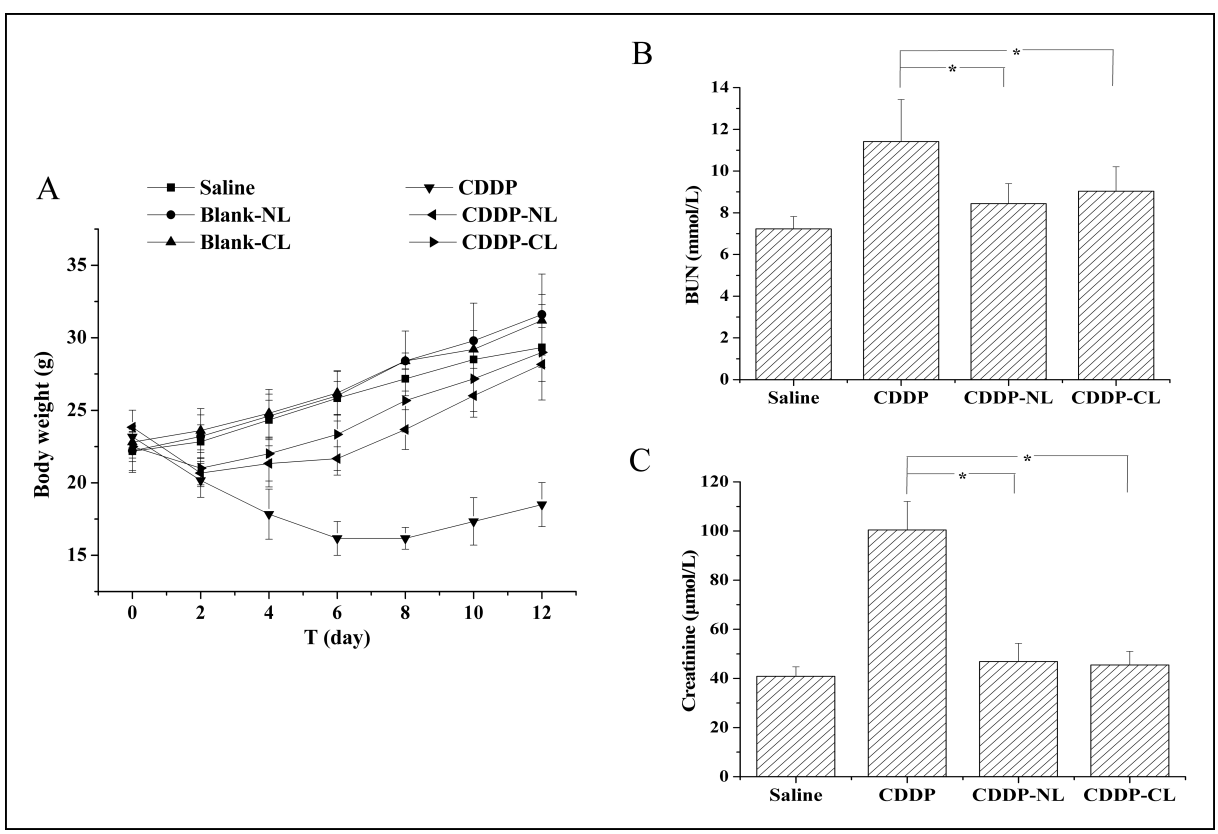

Fig. 4: Toxicity of CDDP liposomes, (A) The body weight of mice treated with CDDP. (B). BUN (C). Serum creatinine after 3 days of treatment (*$P < 0.05$ liposome group compared with CDDP solution). Data are expressed as mean ± S.D of six independent experiments.

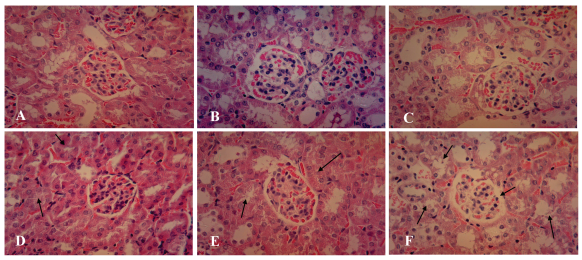

Fig. 5: Representative renal histopathology in the different experiment groups 1 day after cisplatin treatment (× 400), (A) Saline. (B). Blank-NL. (C). Blank-CL. (D). CDDP-NL. (E). CDDP-CL. (F). CDDP. Saline control and blank liposome groups showed normal renal histology. Tubules in mice treated with CDDP-CL and CDDP-NL are partially dilated. In the CDDP solution group, the structure of proximal tubules shows prominent alteration and a dilated Bowman's capsule in the glomeruli is visible (arrow).

spirit and skin luster, while no obvious change was observed in CDDP-NL or CDDP-CL treated animals.

To evaluate nephrotoxicity, blood urea nitrogen (BUN) and serum creatinine levels were analyzed. As shown in Fig. 4B-C, the CDDP solution group exhibited higher BUN and creatinine levels compared with CDDP-CL and CDDP-NL. Particularly, BUN was slightly elevated, but there was no significant difference in creatinine levels between the CDDP-CL and saline groups, which suggest that the nephrotoxicity was dramatically reduced by liposomal encapsulation.

H&E staining showed almost no histological changes in the blank vehicle groups compared with the control group, and the renal morphology and structure were similar (Fig. 5A-C). Cisplatin induced some alteration in liposomal and solution groups to different degrees. CDDP-NL and CDDP-CL caused some tubular dilation, mainly in the mid cortex area (Fig. 5D-E). However, more severe and prominent alterations were observed in the kidney parenchyma treated with free cisplatin. In these samples, not only the proximal tubules displayed disruption of the brush border, but also the glomeruli showed dilated Bowman's capsule (Fig. 5F).

3. Discussion

Amphiphilic PEI derivative for liposomal surface modification can decrease the cytotoxicity of standard polymer PEI with a molecular weight of 25 kDa and maintain the transfection efficiency in gene delivery (Chen et al. 2011, 2012). The enhanced cytotoxicity of cisplatin in A549 cells (Sun et al. 2012) inspired us to do further investigations *in vivo*. The cationic polymer reversed the zeta potential of unmodified liposomes from negative to positive. Other physicochemical characterizations, such as particle size, size distribution and drug loading, showed no significant difference. Sustained released of CDDP-CL and CDDP-NL over long periods indicated structural and functional integrity of the liposomes both *in vitro* and *in vivo* (Table).

Cisplatin is reported to be transported into cells mainly by copper transporter (CTR) proteins and organic cation transporters (OCTs) belonging to the SLC22 subfamily, which have higher efficiency than the internalization of liposomes by endocytosis (Burger et al. 2011). Thus, compared with free drug, the encapsulation of CDDP into neutral liposomes substantially impairs the formation of platinum-DNA adducts after 3 h exposure. In contrast, CDDP-CL promoted Pt accumulation to a similar level as CDDP solution because of the well-known interaction between cations on the surface of CDDP-CL and the negatively charged plasma membrane (Fröhlich 2012). Thus, the IC_{50} towards H22 cells was greatly enhanced by PEI modification. Besides the improvement of uptake, PEI had a significant reduction effect on cellular exocytosis. The expelled rate of intracellular Pt was smaller than those delivered by neutral ones (Fig. 2B). The results were consistent with the slower exocytosis of mesoporous silica nanoparticles modified with PEI (Yanes et al. 2013). Exogenous particles are well known to be internalized and finally delivered to lysosomes. Following this, they are excreted through fusion of lysosomes with the plasma membrane, depending on the various types of particle (Song et al. 2012; Ferrati et al. 2010). Because of osmotic swelling and physical rupture of endosomes caused by the "proton sponge" nature of PEI (Akinc et al. 2005), which was also confirmed in our previous report (Sun et al. 2012), the lysosomal exocytosis pathway was blocked. The prolonged resident time of CDDP-CL within H22 cells, together with its nuclear targeting ability, could facilitate DNA exposure to cisplatin.

To evaluate the antitumor activity of the novel constructed liposomes *in vivo*, H22 tumor volume and TIR were monitored in mice. The results were in accordance with the cytotoxicity *in vitro*. The formulation of CDDP-NL was similar to SPI-077 (Hoving et al. 2005) except for the absence of the long circulation component mPEG-DSPE. Zamboni et al. (2004) described a deficiency of CDDP being released from CDDP-NL into tumor extracellular fluid as the cause of the reduced antitumor activity of unmodified liposomes. However, attempts of PEI modification suppressed the tumor growth more efficiently than CDDP-NL, and the TIR of CDDP-CL was comparable with free CDDP, with some cases leading to the complete remission of the tumor. The mechanisms discussed herein, including uptake enhancement, lysosome escape capacity and prolonged cellular retention time, are different from pH-sensitive (Leite et al. 2012) or fusogenic liposomes (Stathopoulos et al. 2012) whose antitumor activity *in vivo* was similar to treatment with free CDDP.

Although CDDP-CL did not demonstrate a significant advantage over CDDP solution, the cisplatin-induced acute toxicity was dramatically reduced (Fig. 4A) based on the change in body weight. Because nephrotoxicity was a major side effect of CDDP, we chose BUN and serum creatinine as indictors to investigate the effect of CDDP-CL on kidney function (Gandara et al. 1989). Histological examination was also conducted. Because renal tissues and serum were collected at different times after cisplatin therapy, histological results showed some morphological alterations for early nephrotoxicity, while BUN/creatinine levels demonstrated significant change in the cisplatin solution group 3 days after therapy. Rapid clearance of free cisplatin from tumors would cause serious systemic toxicity after intratumoral administration (Ning et al. 1999). However, liposomal entrapment could lower toxicity by reduction of cisplatin distribution in untargeted tissues and sustained release as drug reservoirs (de Carvalho Maroni et al. 2012; Boulikas 2004). In the present study, nephrotoxicity attenuation was achieved by CDDP-CL. Liposomes modified with PEI showed decreased

Table: Characteristics of CDDP-liposomes

	Particle size (nm)	Zeta potential (mV)	Drug loading (μg/mg)	*In vitro* release in 72 h (%)
CDDP-CL	156.0 ± 9.6	51.2 ± 3.5	19.94 ± 0.60	9.55
CDDP-NL	181.3 ± 5.2	-4.9 ± 0.9	20.84 ± 0.32	20.81

toxicity and maintained the antitumor activity as efficient as free CDDP. PEGylation of cationic liposomes may further enhance its therapeutic effects by prolonged circulation time (Ho et al. 2013). It is possible to highlight the beneficial effects of these polymer-modified liposomes as carriers for antitumor drugs.

4. Experimental

4.1. Materials

Cisplatin (CDDP) encapsulated liposomes were prepared by the ethanol injection method followed by extrusion through a 0.22 μm filter. The lipid membrane of cationic liposome was comprised of hydrogenated soy phosphatidylcholine (HSPC), cholesterol and polyethylenimine-cholesterol (PEI-Chol) at an approximate 250:50:1 weight ratio as previously reported (Sun et al. 2012). The PEI-Chol was synthesized from branched PEI with 800 Da with cholesteryl chloroformate (Chen et al. 2007). The neutral liposomes were made of HSPC and cholesterol. Liposome characteristics are summarized in the Table. Particle size and zeta potential of CDDP liposomes were determined by laser light scattering (Malvern Zetasizer 3000HS, Malvern, UK). *In vitro* release was monitored by dialysis method with cell culture medium as the receiving medium.

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) were purchased from Sigma (St. Louis, MO, USA). Platinum (Pt) and Indium (In) standard solution were purchased from Aladdin Reagent (Shanghai, China). Urea Test Kit and Creatinine Test Kit were obtained from Jiancheng Biological Engineering Institute (Nanjing, China).

Murine hepatoma cell line H22 was obtained from Shanghai Institute for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPMI 1640 (Gibco BRL, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Sijiqing Biologic Co., Ltd., Hangzhou, China), 100 U/mL penicillin and 100 μg/mL streptomycin in a 5% CO_2 air incubator at 37 °C.

Male ICR mice with 18–22 g were purchased from Zhejiang Academy of Medical Science (Hangzhou, China). The mice were raised under specific pathogen-free (SPF) circumstances and all the animal experiments were performed in full compliance with guidelines approved by the Animal Care Committee at Zhejiang University.

4.2. Platinum accumulation and exocytosis studies

The level of platinum in H22 cells were determined by inductively coupled plasma mass spectrometry (ICP-MS, Thermo, USA). Briefly, 1×10^7 H22 cells were exposed to 5 μg/mL CDDP solution or CDDP liposomes for 3 h and then cells were washed with PBS containing 0.1% trypsin to remove membrane bound liposomes. Cell pellets were resuspended in 65% HNO_3 and minernalized at 120 °C. Indium was added as the internal standard.

For exocytosis study, cells were incubated with CDDP first, and then the cells were washed. After cultured for the defined time periods, cells were harvested using the protocol descried above for intracellular Pt determination.

4.3. Cytotoxicity assay

The MTT assay was used to assess cytotoxicity of CDDP liposomes. H22 cells (1×10^4) were seeded in 96-well plate and incubated overnight. Then CDDP liposomes were added. Cells were centrifuged, washed with PBS and treated with MTT solution (5 mg/mL) after 72 h exposure. The crystals formed were dissolved by DMSO. Absorbance at 570 nm was measured using microplate reader (ELx 800, Bio-Tek, USA). The cytotoxicity of blank liposomes and CDDP solution were also measured.

4.4. In vivo antitumor efficacy

ICR mice implanted with H22 cells were used to investigate the antitumor efficacy of CDDP liposomes via intratumoral injection. ICR mice were subcutaneously injected at the left axillary space with 0.1 mL of cell suspension containing 5×10^6 H22 cells. Treatments were started after 4–6 days of implantation. The mice with tumor reached at volume of 100 mm^3 were selected, and this day was designated as "Day 0".

Thirty six mice were randomly divided into 6 groups. These mice received a single injection of saline, blank neutral liposomes (Blank-NL), blank cationic liposomes (Blank-CL), CDDP solution, CDDP-NL or CDDP-CL. The CDDP dose was 6 mg/kg.

All mice were weighed and the tumor volumes were measured every other day throughout the whole experiment. The tumor volume was calculated by the formula $(W^2 \times L)/2$, where W is the tumor measurement at the widest point, and L is the tumor dimension at the longest point. Mice were sacrificed on day 12 and the tumors were excised, weighed, and TIR was calculated as follows: TIR = (tumor weight of saline group – tumor weight of tested group)/tumor weight of saline group × 100%.

4.5. Toxicity evaluation

The systemic toxicity was evaluated by monitoring body weight changes and nephrotoxicity. To evaluate the nephrotoxicity, blood samples were collected and centrifuged at 3000 rpm for 5 min for serum separation on the third day of cisplatin therapy (n = 6). The concentration of blood urea nitrogen (BUN) and creatinine levels were measured by a standard enzymatic method using commercial kits.

Renal injury was assessed using a histological examination. Renal tissues were collected the day after cisplatin therapy. Then samples were fixed by immersion in buffered formalin (pH 7.4), dehydrated and embedded in paraffin. Sections (4 μm) were stained with hematoxylin and eosin (H&E) to evaluate renal morphology.

4.6. Statistical analysis

The data are presented as mean ± standard deviation for all treatments. Statistical analysis was done with one-way ANOVA followed by SNK or Dunnett-t test (SPSS 18.0 software) with the significance set at $p < 0.01$ or $p < 0.05$.

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