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Preparation and evaluation of a novel liposomal formulation of cisplatin

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ABSTRACT

A novel liposomal formulation of cisplatin (L-CDDP) was synthesized and characterized. The L-CDDP was formed by conjugating CDDP to the carboxyl of oleic acid incorporated into empty liposomes. Particle size (155.4 ± 16.1 nm) and zeta potential (−50.92 ± 1.19 mV) of the L-CDDP were determined. In addition, pharmacokinetic properties and antitumor activity in vitro and in vivo were evaluated. Pharmacokinetic study demonstrated that L-CDDP had markedly prolonged circulation time relative to the free drug. Furthermore, L-CDDP showed significantly enhanced in vitro cytotoxicity in comparison to free CDDP. A549-engrafted mice treated with L-CDDP had a higher survival rate compared to those treated with free CDDP. Finally, A549-engrafted mice treated with L-CDDP showed no significant loss of body weight, whereas free CDDP treatment at the same dose caused significant loss of body weight. These results suggest further evaluation of the in vivo antitumor efficacy of the novel L-CDDP formulation is warranted.

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1. Introduction

Cisplatin (*cis*-diamminedichloroplatinum(II), CDDP) has been widely used to treat various malignancies such as lung, ovarian, head and neck, and gastric carcinomas (Comis, 1994; Boulikas and Vougiouka, 2003, 2004; Florea and Büsselberg, 2011). However, its significant side effects, especially nephrotoxicity, have limited its use in clinic (Pinzani et al., 1994; Gandara et al., 1995; Hartmann et al., 1999; Arany and Safirstein, 2003; Bosch et al., 2008). In order to decrease the side effects of CDDP, several new platinum compounds, such as carboplatin and oxaliplatin, have been developed (Cleare et al., 1978; Calvert et al., 1989; Raymond et al., 1998; Amin and Buratovich, 2009). Compared with CDDP, these new agents succeed in avoiding nephrotoxicity but bring along new toxicities such as myelotoxicity. Carboplatin is less toxic than CDDP, but also has less potent anti-cancer activity and is affected by the same resistance mechanism. Oxaliplatin circumvents CDDP resistance, but its side effects include dose-limiting neurotoxicity (Tognoni et al., 2002; Ardizzoni et al., 2007; Stathopoulos et al., 2012). In order to overcome these disadvantages, drug delivery systems such as liposomes have been employed for CDDP delivery. Liposomes have proven to suitable carriers for a variety of anticancer drugs. In recent years, many efforts have focused on the development of liposomal formulations of CDDP. Among the many CDDP formulations, only Lipoplatin has produced promising data on anticancer activity in clinical phases I–III trials. The study results showed that Lipoplatin has significantly lower toxicity compared to CDDP, but with comparable efficacy (Boulikas et al., 2007; Lazarioti and Boulikas, 2008). SPI-077 is another liposomal formulation of CDDP. Although SPI-077 demonstrated excellent preclinical data (Newman et al., 1999; Vaage et al., 1999), it produced poor response when evaluated clinically (Harrington et al., 2001; Kim et al., 2001; Veal et al., 2001). Further study results demonstrated that this was due to low CDDP release from SPI-077 at the tumor site (Zamboni et al., 2004). Recently, several studies on carboxylate-CDDP complexes were reported and have shown promising results (Li et al., 2010; Manjari and Bellevue, 2006; Xiong et al., 2012). Here in this study, in order to develop a CDDP formulation with both enhanced efficacy and reduced adverse effects, a novel liposomal CDDP (L-CDDP) was constructed by conjugating CDDP to the carboxyl group of oleic acid on empty liposomes. The cytotoxicity of the liposomal formulation was evaluated using MTT assay. The pharmacokinetics in

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mice was studied, and the antitumor activity was evaluated in nude mice bearing A549 xenograft tumors.

2. Materials and methods

2.1. Materials

Egg phosphatidylcholine (egg PC) was purchased from Avanti Polar Lipids (Alabaster, AL, USA). Monomethoxy polyethylene glycol 2000-distearoyl phosphatidylethanolamine (mPEG–DSPE) was purchased from Genzyme Pharmaceuticals (Cambridge, MA, USA). CDDP (M.W. 300.04 Da) was purchased from Fisher Scientific (Pittsburgh, PA 15275). Cholesterol (Chol), oleic acid (OA), sucrose, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT), and chloroform were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). Platinum Standard Solution 1 mg/ml Pt in 10% HCl was purchased from Acros Organics (Fair Lawn, NJ, USA). All other reagents were of analytical grade.

2.2. Cell culture

A549 human lung cancer cells were purchased from American Type Culture Collection (Rockville, MD, USA). The cells were cultured in RPMI-1640 media (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Invitrogen), 100 units/ml penicillin, and 100 mg/ml streptomycin. Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2.

2.3. Preparation of liposomal-CDDP (L-CDDP)

2.3.1. Synthesis of oleic acid-CDDP conjugate (OA-CDDP)

Cisplatin nitrate was synthesized as reported previously with a slight modification (Ye et al., 2006). Simply, 50 mg CDDP was suspended in 10 ml distilled water and mixed with 27.8 mg silver nitrate ($AgNO_3$/CDDP = 1, mol/mol) with rapid stirring. The solution was kept in dark at room temperature. The mixture was then centrifuged at 12,000 rpm for 10 min. The supernatant was filtrated through a 0.22 μm filter and collected.

Next, 40 mg sodium oleate was added to the above aqueous solution and reacted for 48 h at room temperature with gentle stirring in the dark. Then a dark green precipitate was produced and washed with distilled water. OA-CDDP was obtained by drying the precipitation in a vacuum. The chemical structure of OA-CDDP was confirmed by using FT-IR spectrophotometer (Thermo Nicolet 380; Waltham, MA, USA). The FTIR spectra were collected in the range of 4000–400 cm^{-1} at room temperature with the KBr method. Spectra are recorded at a spectra resolution of 4 cm^{-1}, with 32 co-added scans.

2.3.2. Preparation of empty liposomes

Egg PC, Chol, OA and mPEG–DSPE (50:15:30:5, mol/mol) were dissolved in chloroform and dried into a thin film by rotary evaporation; with further drying under vacuum. Distilled water, adjusted to pH 8.0 with NaOH, was added and the lipid film was hydrated at ambient temperature with three cycles of vortexing, followed by bath sonication for 5 min, and then extrusion for three times at room temperature through a 100-nm polycarbonate membrane (Avestin, Inc., Ottawa, Canada).

2.3.3. Preparation of L-CDDP

First the cisplatin nitrate solution was prepared as described above. Then the empty liposomes were added into the above cisplatin nitrate solution (OA/CDDP = 1:1.2, mol/mol) with stirring to prepare liposomal cisplatin conjugate (L-CDDP). The mixture was stirred continuously for 48 h protected from light. Unreacted CDDP nitrate was separated from liposomes by dialyzing against deionized water with a dialysis bag of MCWO 3500. Ten percent sucrose was added to the liposomal solution as a cryoprotectant. The L-CDDP were sterilized by passing through a 0.22 μm filter, and then freeze-dried in a Labconco FreeZone Triad Freeze Dry System (Kansas City, MO, USA).

Pt concentrations were determined by inductively coupled plasma-optical emission spectroscopy (ICP–OES) (Varian 720-ES, Palo Alto, CA, USA). The particle size was determined by dynamic laser light scattering (Submicron Particle Sizer 370, Nicomp, CA, USA). Zeta potential was measured by ZetaPALS (Zeta Potential Analyzer, Brookhaven Instruments Corporation, NY, USA). Encapsulation efficiency (EE) and drug loading capacity (DL) were calculated according to the following formula:

$$\mathrm{EE}(\%) = \frac{\text{Amount of Pt in L-CDDP}}{\text{Amount of Pt in CDDP initially}} \times 100$$

$$DL(\%) = \frac{\text{Amount of CDDP in L-CDDP}}{\text{Amount of CDDP} + \text{amount of excipients in L-CDDP}} \times 100$$

2.4. Release profile of CDDP from L-CDDP

The release of the CDDP from L-CDDP was evaluated in phosphate-buffered saline (10 mM phosphate buffer, pH 7.4 + 150 mM NaCl) or water at 37 °C by a dialysis-based method (MCWO:3500) Roonasi et al., 2010. The released CDDP outside of the dialysis bag was sampled at defined time periods and measured by ICP–OES.

2.5. In vitro cytotoxicity assay

The in vitro cytotoxicity of L-CDDP was evaluated by MTT assay. Briefly, A549 cells (5.0×10^3 cells/100 μl/well) were seeded into 96-well plates in RPMI 1640 medium supplemented with 10% v/v FBS and 1% penicillin/streptomycin 24 h before treatment. Cells were incubated with 100 μl 1:4 serially diluted free CDDP and L-CDDP for 48 h at 37 °C. After incubation, 20 μl of 5 mg/ml MTT was added to each well, followed by incubation for 4 h at 37 °C. The formazan crystals were dissolved in 200 μl DMSO. The absorbance was determined at 570 nm on a Dynatech MR-600 microplate reader (Biorad, Hercules, CA, USA).

2.6. Animal experiments

2.6.1. Animals

Female imprinting control region (ICR) mice (5–6 weeks, 18–20 g) and athymic nude-Foxn1nu mice (5–6 weeks, 18–20 g) were purchased from Harlan Laboratories (Indianapolis, IN, USA). All experimental procedures were done according to protocols approved by The OSU Institutional Laboratory Animal Care and Use Committee (IACUC).

2.6.2. Pharmacokinetics of L-CDDP in mice

CDDP or L-CDDP was administered to ICR mice by intravenous injection at a dose of 6 mg/kg. At the time points of 0.25, 0.5, 1, 2, 4, 8, 16, 24 h, blood samples were collected in heparinized tubes via heart puncture and centrifuged at 4000 rpm for 10 min. The samples were decomposed in *aqua regia*. After evaporation to dryness, the residues were dissolved in 0.5 M hydrochloric acid. The Pt content was determined using ICP–OES.

2.6.3. In vivo antitumor activity

To prepare A549 tumor-bearing mice, 1×10^7 cells were subcutaneously injected into female athymic nude mice. At 8, 12, 16, and 20 days after injection, L-CDDP or CDDP was injected by tail at a dose of 6 mg CDDP/kg and 25 mg CDDP/kg body weight ($n = 10$).

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