

A cisplatin-incorporated liposome that targets the epidermal growth factor receptor enhances radiotherapeutic efficacy without nephrotoxicity

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Abstract. Radiotherapy (RT) is one of the major modalities for non-small cell lung cancer (NSCLC), but its efficacy is often compromised by cellular resistance caused by various mechanisms including the overexpression of epidermal growth factor receptor (EGFR). Although *cis*-diamminedichloroplatinum(II) (cisplatin, CDDP) has been well characterized as an effective radiosensitizer, its clinical application is limited by its severe nephrotoxic effects. In our current study, we developed a CDDP-incorporated liposome (LP) conjugated with EGFR antibodies (EGFR:LP-CDDP) and evaluated its potential to radiosensitize EGFR-overexpressing cells without exerting nephrotoxic effects. EGFR:LP-CDDP showed higher cytotoxicity than non-targeting liposomal CDDP (LP-CDDP) in the cells expressing EGFR *in vitro*. In an A549 cell-derived xenograft tumor mouse model, increased delays in tumor growth were observed in the mice treated with a combination of EGFR:LP-CDDP and radiation. Notably, the

EGFR:LP-CDDP-treated animals showed no differences in body weight loss, survival rates of nephrotoxicity compared with untreated control mice. In contrast, the use of CDDP caused lower body weights and poorer survival outcomes accompanied by a significant level of nephrotoxicity [e.g., decreased kidney weight, increased blood urea nitrogen (BUN) and creatinine, and pathological change]. These findings suggest the feasibility of using EGFR:LP-CDDP to radiosensitize cells in a targeted manner without inducing nephrotoxic effects. This compound may therefore have clinical potential as part of a tailored chemoradiotherapy strategy.

Introduction

Radiotherapy (RT) is commonly used for the treatment of non-small cell lung cancer (NSCLC) but tumor control and survival outcomes remain poor for affected patients due to RT resistance (1,2). Combination therapies involving radiosensitizing drugs and RT are therefore currently recommended for NSCLC cases (3). *Cis*-diamminedichloroplatinum(II) (cisplatin, CDDP) is a well-known radiosensitizing agent and is administered as part of a primary intervention, particularly for advanced NSCLC treatment regimens (4). However, the use of CDDP is often limited as it is severely nephrotoxic (5). CDDP metabolites also induce nephrotoxicity through a biotransformation pathway (6). Hence, the development of alternatives to CDDP is of great interest.

Liposome (LP) is well characterized as a classical carrier for drug delivery system (DDS) (7). LP can alter both the pharmacokinetics and the biodistribution of drugs by affecting the size, surface charge and membrane lipid packing (8). A size-controlled LP can efficiently deliver a drug to the site of a tumor through an enhanced permeability and retention (EPR) effect (passive targeting) and protect the drug from metabolic

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processes that may clear it from the body prematurely (9). LP is insufficient however to actively target a specific site or sustain the long-term circulation in the bloodstream because of its elimination through the reticuloendothelial system (RES).

Epidermal growth factor receptor (EGFR) is frequently targeted as an anticancer therapy strategy as its overexpression has been identified in many types of human cancer including NSCLC (10-12). Furthermore, EGFR overexpression plays a major role in reducing the radiosensitivity of NSCLC cells (13,14). Recently, an active targeting approach has emerged involving the display of a tumor-specific ligand or antibody on an LP (15,16). In our current study, we conjugated an EGFR antibody to an liposomal CDDP (LP-CDDP) and evaluated its ability to enhance the efficacy of targeted RT without the adverse nephrotoxic effects of CDDP.

Materials and methods

Preparation of CDDP-incorporated immunoliposome conjugated with EGFR antibodies (EGFR:LP-CDDP). CDDP was purchased from Sigma (St. Louis, MO, USA). Monoclonal anti-EGFR antibodies were prepared from the hybridoma line HB-8509 (ATCC, Manassas, VA, USA). LP-CDDP was prepared as previously described (17). Briefly, dipalmitoylphosphatidylcholine, cholesterol, ganglioside, diacetyl phosphate and dipalmitoylphosphatidylethanolamine (35:40:15:5:5 as the molar ratio; Katayama Chemical Industries Co., Ltd., Osaka, Japan) were dissolved in methanol/chloroform (1:1 v/v) solution. The lipid film was produced by evaporating and drying under vacuum. It was dissolved in 10 mM N-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid (TAPS) buffer (pH 8.4), followed by sonication to obtain small unilamellar vesicles. The LP encapsulated CDDP (17). The lipid concentration was measured as total cholesterol in the 0.5% Triton X-100 (Sigma-Aldrich Korea, Ltd., Gyeonggi, Korea), using a Determiner TC 555 kit. Total lipid concentration was calculated by multiplying 2.5 by cholesterol concentration. Anti-EGFR antibodies were displayed on the LP surface using 3,3'-dithiobis(sulfosuccinimidylpropionate) (DTSSP) (Pierce Biotechnology, Inc., Rockford, IL, USA). Tris was then added to a final concentration of 132 mg/ml to terminate the reaction (18). To quantify the number of EGFR antibodies on the LP-CDDP, western blotting was used. Briefly, the samples were boiled in sample buffer and separated using 4-15% gradient SDS-PAGE. The resolved proteins were then transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, USA) which was blocked in a 5% skim milk solution (Becton-Dickinson & Co., Sparks, MD, USA) in Tris-buffered saline with Tween-20 (TBST) (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% Tween-20) for 1 h. The filter was then incubated with peroxidase-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) for 2 h. Immunoreactive protein was visualized using ECL Western Blotting Detection Reagents (GE Healthcare, Buckinghamshire, UK).

Physicochemical characterization. The sizes and ζ -potentials of the LP-CDDP and EGFR:LP-CDDP were measured at 25°C by dynamic light scattering (DLS) using a Zetasizer

Nano ZS device (Malvern Instruments Ltd., Worcestershire, UK). The polydispersity index (PDI) is a width parameter for the ζ -average as an intensity mean. The compounds were adsorbed onto a carbon-coated grid, negatively stained using 2% (w/v) phosphotungstic acid (pH 7.0), and subjected to transmission electron microscopy (TEM) (JEM-1011; JEOL, Tokyo, Japan).

Measurement of in vitro anticancer effects. Human epidermoid carcinoma A431 cells (ATCC no. CRL-1555), human lung carcinoma A549 cells (ATCC no. CCL-185) and human colon carcinoma RKO cells (ATCC no. CRL-2577) were obtained from ATCC and maintained in Dulbecco's modified Eagle's medium, Ham's F12K medium and Eagle's minimum essential medium, respectively, supplemented with 10% fetal bovine serum (all from Invitrogen Life Technologies, Grand Island, NY, USA). These cell lines were selected as they have been shown to express EGFR at different levels; A431 and A549 cells show high EGFR expression and RKO cells demonstrate low expression of this receptor (10). Cells were seeded in a 96-well culture plate, grown overnight, and treated with CDDP, LP-CDDP or EGFR:LP-CDDP. After incubation for 24 h, cell viability was assayed using Cell Counting kit-8 (Dojindo, Kumamoto, Japan) in accordance with the manufacturer's instructions. For clonogenic assays, A549 cells were seeded onto 6-well plates at a density of 100-1,000 cells/well depending on the intended doses of CDDP and ionizing radiation (IR) (CL/1800; Varian Medical Systems, Inc., Palo Alto, CA, USA). After CDDP treatment, cells were irradiated at 0-10 Gy and added fresh media in the next day, then incubated for 12 days to allow colony formation. The emerging colonies containing >50 cells were counted. The plating efficiency was defined using the non-irradiated cells as: plating efficiency (PE) = (mean colonies counted)/(cells plated). The survival fraction was calculated as: survival fraction (SF) = (mean colonies counted)/[(cells plated) x PE], as previously described (19).

In vivo tumor growth delay. All animal experiments were performed in accordance with the protocols of the Institutional Animal Care and Use Committee of the Asan Institute for Life Sciences (Seoul, Korea) (2010-12-180). To generate a xenograft tumor model, A549 cells (1×10^6 cells) were subcutaneously injected into the right hind legs of Balb/c nude mice. When the tumors grew to a size of ~ 200 mm³, the mice were randomly divided into eight experimental groups (n=12) and injected intravenously with 10 mg/kg (CDDP dose equivalent) of CDDP, LP-CDDP or EGFR:LP-CDDP. At 2 h after these injections, the tumors were irradiated with 5 Gy using a 6 MV photon beam linear accelerator (CL/1800; Varian Medical Systems, Inc.). The tumor size and body weights of the mice were then measured every week using caliper (Mitutoyo, Kanagawa, Japan). The tumor volume (V) was calculated as: V (mm³) = [(largest length) x (shortest length)²]/2. To evaluate the efficacy of each treatment, change of tumor growth was compared between treated group and control group (T/C). The T/C (%) on the final date of this experiment was calculated as: T/C (%) = [(change in tumor growth for treated group)/(change in tumor growth for control group)] x 100. On the final day of the experiment, the kidneys were collected and weighed.

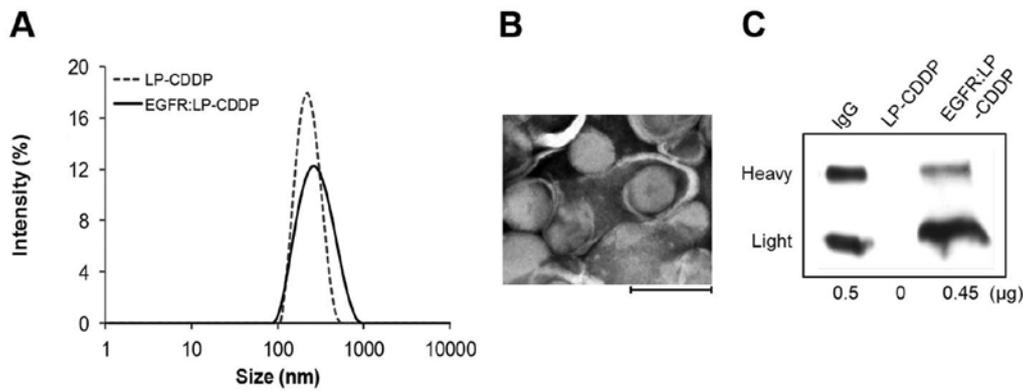


Figure 1. Characterization of EGFR:LP-CDDP. (A) The sizes of liposomal CDDP (LP-CDDP) and EGFR:LP-CDDP were determined using the dynamic light scattering (DLS) method. (B) Transmission electron microscopy (TEM) of EGFR:LP-CDDP. (C) Quantification of mouse monoclonal anti-epidermal growth factor receptor (EGFR) antibody conjugation on EGFR:LP-CDDP.

Table I. Analytical information.

	Size (nm)	ζ potential (mV)
LP-CDDP	212.4	-64
EGFR:LP-CDDP	247.9	-60
LP-CDDP, liposomal CDDP.		

Evaluation of nephrotoxicity. To evaluate kidney function, the blood urea nitrogen (BUN) and creatinine levels were measured in 5-week-old Balb/c mice following a single injection of CDDP, LP-CDDP or EGFR:LP-CDDP (10 mg/kg CDDP dose equivalent) (n=6). At 3 days after this treatment, the BUN levels were determined using the modified Berthelot reaction of Bio-Quant, Inc. (San Diego, CA, USA). Creatinine was measured using Creatinine Colorimetric Detection kit (Enzo Life Sciences, Inc., Farmingdale, NY, USA). To assess nephrotoxicity, Balb/c nude mice bearing an A549-derived tumor were treated with CDDP, LP-CDDP or EGFR:LP-CDDP (10 mg/kg CDDP dose equivalent) (n=6) and sacrificed 30 days later. The kidney, lungs and liver were harvested and fixed in 4% paraformaldehyde and the tissues were embedded in paraffin and sliced at a 5 μ m thickness. The resulting sections were stained with hematoxylin and eosin and observed under a microscope (DP71; Olympus, Tokyo, Japan).

Statistical analysis. Statistical analysis of the group differences in these assays were performed by one-way ANOVA, Tukey's test. The values are the mean \pm standard deviation. The value of $P < 0.05$ was considered to be statistically significant.

Results

Characterization of EGFR:LP-CDDP. The size distribution of LP-CDDP and EGFR:LP-CDDP, measured by the DLS method, is shown in Fig. 1A and was measured at 212.4 nm (PDI, 0.100) and 247.9 nm (PDI, 0.148), respectively (Table I). A TEM image of EGFR:LP-CDDP revealed a size and its spherical shape that was consistent with

these values (Fig. 1B). The level of CDDP incorporated into EGFR:LP was 3.2 mg CDDP/14.2 mg lipid/ml and the loading efficiency was 22.5%. The amount of EGFR Ab displayed on the LP was calculated by measuring the density of the bands detected by western blotting (Fig. 1C). A total of 20 μ l of LP-CDDP (containing 280 μ g of lipid) contained 0.45 μ g EGFR Ab.

In vitro anticancer effects of EGFR:LP-CDDP. The targeting ability of EGFR:LP-CDDP in EGFR-expressing cancer cells was evaluated in A431 and A549 cells that express EGFR at a high level. Both lines showed a lower viability following treatment with EGFR:LP-CDDP compared with LP-CDDP. Specially, cytotoxicity of EGFR:LP-CDDP was higher than that of LP-CDDP at 10 μ g/ml against A431 and A549 cells. In contrast, the viability of RKO cells which express a rare variant of EGFR did not differ between EGFR:LP-CDDP and LP-CDDP treatments (Fig. 2A). These results indicated that EGFR:LP-CDDP can target EGFR-expressing cancer cells leading to enhanced cytotoxicity. To further investigate their chemoradiotherapeutic effects, A549 cells were treated with LP-CDDP or EGFR:LP-CDDP (5 μ g/ml) and irradiated at 0, 2, 5, or 10 Gy after 2 h. The survival fractions were calculated using a colony formation assay as described in Materials and methods as 1, 0.79, 0.20, and 0.01 at the radiation doses of 0, 2, 5, and 10 Gy, respectively (Fig. 2B). The cells treated with a combination of LP-CDDP with IR showed survival fractions of 0.6, 0.57, 0.14, and 0.008 at 0, 2, 5, and 10 Gy. In the cells exposed to a combination of EGFR:LP-CDDP with IR, these values were 0.6, 0.54, 0.13, and 0.006 at 0, 2, 5, and 10 Gy. These data revealed that both LP-CDDP and EGFR:LP-CDDP enhance radiosensitivity but that EGFR:LP-CDDP was slightly more potent.

Enhanced in vivo chemoradiotherapeutic efficacy and reduced toxicity of EGFR:LP-CDDP. The chemoradiotherapeutic efficacy of LP and EGFR:LP containing CDDP was compared with that of free CDDP in mice bearing A549-derived tumors in the right hind leg. The mice were intravenously injected with CDDP, LP-CDDP, or EGFR:LP-CDDP at 10 mg/kg (CDDP concentration equivalent). After 2 h, the tumors were irradiated at 5 Gy. As shown in Fig. 3A, the tumor growth in

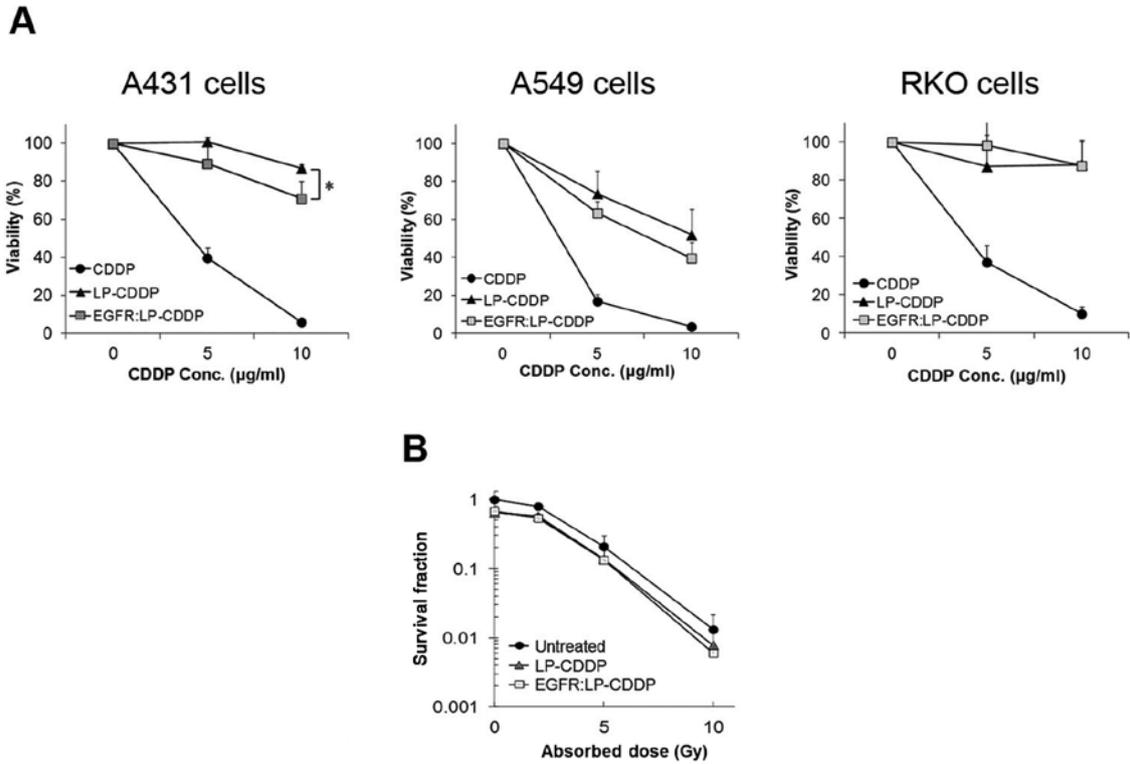


Figure 2. *In vitro* anticancer effects. (A) Assessment of cytotoxicity. A431, A549 [high epidermal growth factor receptor (EGFR)-expressing cell line] and RKO cells (low EGFR-expressing cell line) were treated with *cis*-diamminedichloroplatinum(II) (CDDP), liposomal CDDP (LP-CDDP) or EGFR:LP-CDDP for 24 h. (B) Clonogenic assay. A549 cells were treated with CDDP, LP-CDDP or EGFR:LP-CDDP and irradiated 2 h later.

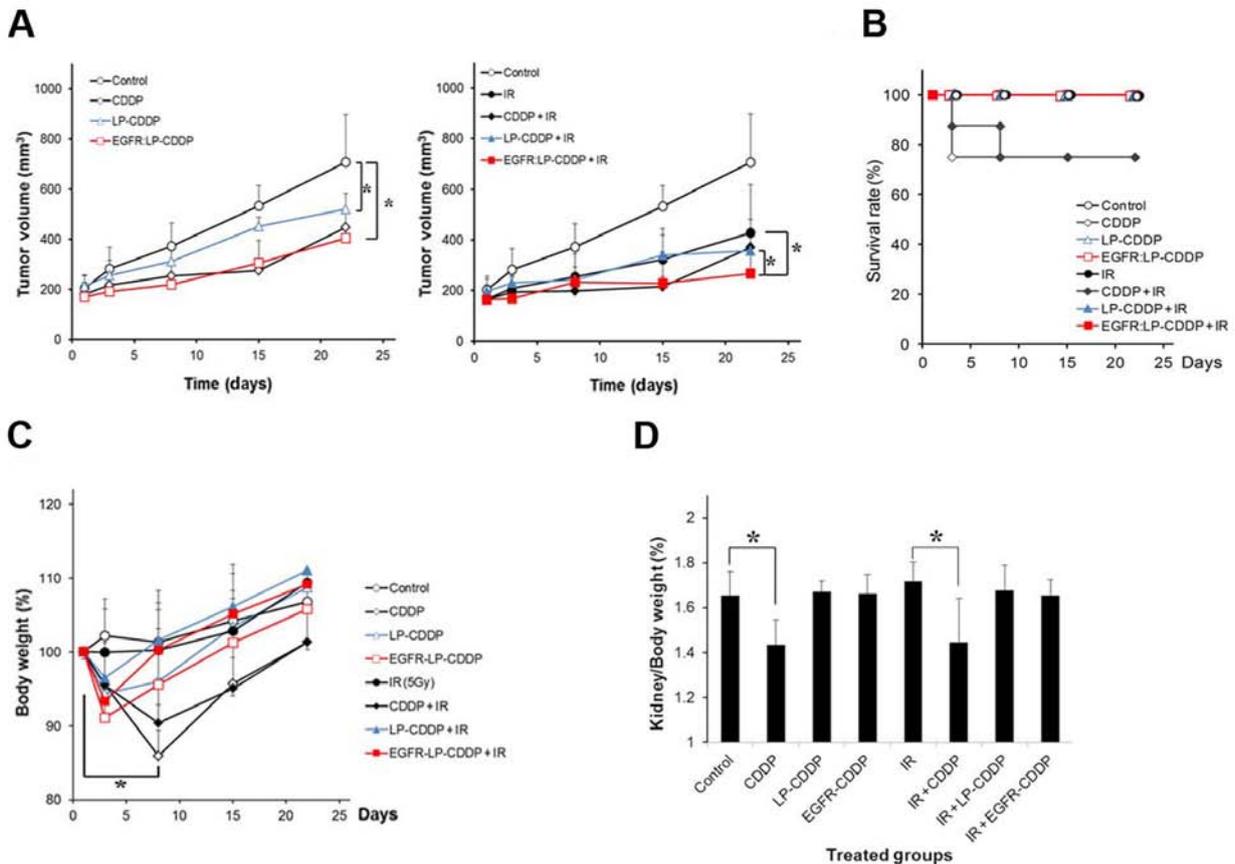


Figure 3. *In vivo* antitumor effects and toxicity levels. *Cis*-diamminedichloroplatinum(II) (CDDP), liposomal CDDP (LP-CDDP) or EGFR:LP-CDDP (as CDDP 10 mg/kg) were intravenously injected into A549 tumor-bearing mice. After 2 h, the tumors were irradiated with 5 Gy. (A) Tumor growth delay (n=12). (B) Survival rate (%) (n=12). (C) Body weight change (%) (n=12). (D) Ratio of kidney weight to body weight (%) (n=6).

Table II. Kaplan-Meier analysis.

Groups	Survival rate (%)				
	(days) 1	3	8	15	22
Control	100	100	100	100	100
CDDP	100	75	75	75	75
LP-CDDP	100	100	100	100	100
EGFR:LP-CDDP	100	100	100	100	100
IR	100	100	100	100	100
CDDP + IR	100	87.5	75	75	75
LP-CDDP + IR	100	100	100	100	100
EGFR:LP-CDDP + IR	100	100	100	100	100

CDDP, *cis*-diamminedichloroplatinum(II); LP-CDDP, liposomal CDDP; IR, ionizing radiation.

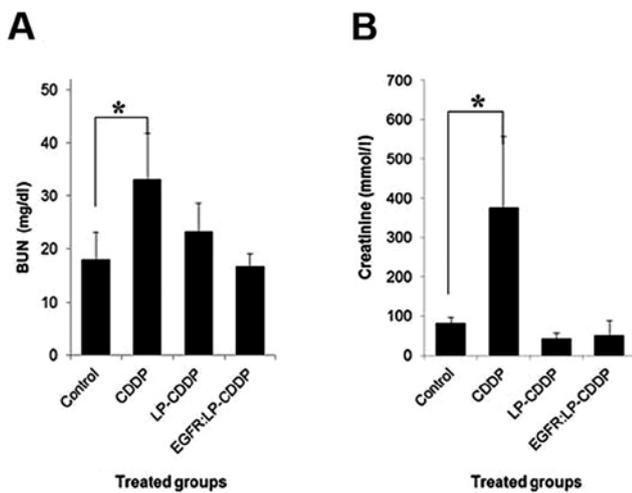


Figure 4. Effects of EGFR:LP-CDDP on blood urea nitrogen (BUN) and creatinine levels. *Cis*-diamminedichloroplatinum(II) (CDDP), liposomal CDDP (LP-CDDP) or EGFR:LP-CDDP (as CDDP 10 mg/kg) were intravenously injected into Balb/c mice. After 3 days, blood was collected from the inferior vena cava (n=6).

the CDDP-, LP-CDDP-, or EGFR:LP-CDDP-treated animals was delayed compared with that of the control. In combination therapies of IR and drugs (Fig. 3A, right panel), CDDP, LP-CDDP, and EGFR:LP-CDDP all enhanced the radiotherapeutic efficacy of the treatment. At day 22, the T/C (%) values of the control-, CDDP-, LP-CDDP-, or EGFR:LP-CDDP-treated mice were 100%, 53.1±4.9%, 61.3±7.3%, and 46.8±4.3%, respectively. Values of 51.8±7.4%, 40.7±4.9%, 32.2±8.4%, and 20.7±4.2% were obtained after IR, CDDP with IR, LP-CDDP with IR, and EGFR:LP-CDDP with IR, respectively. These results reveal a higher chemotherapeutic and chemoradiotherapeutic efficacy of EGFR:LP-CDDP among the compounds tested.

During the experiments, 25% of the mice treated with CDDP or with a combination of CDDP and IR died within 1 week (Fig. 3B and Table II). The body weights of the surviving animals in the groups treated with CDDP or with a

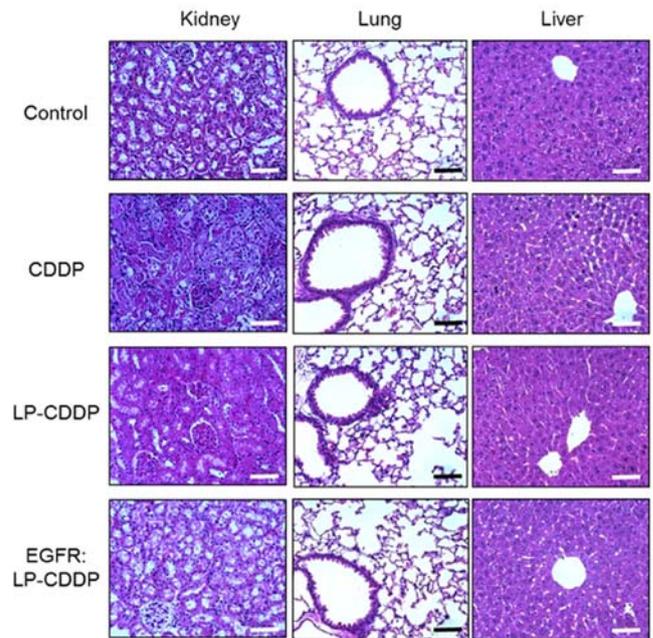


Figure 5. Representative photomicrographs of the kidney, lung and liver of treated mice. *Cis*-diamminedichloroplatinum(II) (CDDP), liposomal CDDP (LP-CDDP) or EGFR:LP-CDDP (equivalent to a CDDP dosage of 10 mg/kg) were intravenously injected into Balb/c nude mice bearing A549-derived tumors. Tissue sections were stained with hematoxylin and eosin (scale bar, 100 μ m).

combination of CDDP and IR fell to 85% of normal levels at the beginning of the therapy and then slowly recovered (Fig. 3C). These results indicate that although free CDDP has anticancer effects, it is severely toxic. The other treatment groups showed equivalent survival rate and body weight profiles. On the final day of the experimental period, the kidney weights of the mice in each treatment group were compared. As shown in Fig. 3D, only two treatments (CDDP and CDDP with IR) caused a significant loss in kidney weight. These results suggest that the LP formulation prevented CDDP-induced damage.

Pathological changes and nephrotoxicity. To examine and compare the effects of LP-CDDP and EGFR:LP-CDDP on renal function, the kidney injury markers BUN and creatinine were assayed in the tumor mice treated with CDDP, LP-CDDP and EGFR:LP-CDDP (Fig. 4). At 3 days after these treatments (10 mg/kg CDDP dose equivalent), the BUN values in the control-, CDDP-, LP-CDDP- and EGFR:LP-CDDP-treated animals were measured at 6.4±1.7, 11.8±3.1, 8.3±1.8, and 6.0±0.8 mmol/l, respectively (Fig. 4A). In the case of creatinine, the values for the control, CDDP, LP-CDDP and EGFR:LP-CDDP animals were 81.6±15.4, 375.9±180, 4.35±14.2, and 50.9±36.9 mmol/l, respectively (Fig. 4B). Only CDDP caused significant nephrotoxicity as expected, whilst LP-CDDP and EGFR:LP-CDDP did not show any evidence of such toxic effects.

The mice treated with CDDP, LP-CDDP or EGFR:LP-CDDP were then histopathologically evaluated. Consistently, acute cortical tubular degeneration and regeneration was observed in the animals treated with CDDP (Fig. 5). On the other hand, the kidneys of the mice treated with LP-CDDP

and EGFR:LP-CDDP did not show any toxic damage (Fig. 5). There were no pathological changes observed in the lungs or liver in any group. These results indicated that encapsulation of CDDP using LP eliminates the nephrotoxic properties of this compound.

Discussion

A combination of chemotherapy and RT is regarded as the standard treatment regimen for various cancers including lung, head and neck, and cervical cancers. For chemoradiotherapy interventions to treat NSCLC, CDDP is frequently used (3). However, although CDDP has remarkable radiosensitization effects, its nephrotoxic properties severely limit its clinical application. To reduce this toxicity of CDDP, encapsulation has been attempted using gelatin nanoparticles, polymeric micelles, carbon nanohorns and LP (20-22). These carriers containing CDDP are a promising new class of radiosensitizers. However, a CDDP-incorporated LP has not been studied previously in this context. In our present study, we have developed the EGFR:LP-CDDP compound and we provide compelling evidence that it enhances theradiotherapeutic efficacy of a combination IR regimen without causing nephrotoxicity *in vivo*. In particular, it is meaningful for further tailored chemoradiotherapy strategies that EGFR:LP-CDDP targets radioresistant cells expressing a high level of EGFR.

The conjugation of LP-CDDP to EGFR antibodies via a crosslinker produced a strong interaction (Fig. 1) which contributed to the effective targeting ability of the resulting EGFR:LP-CDDP both *in vitro* and *in vivo*. The surface charge of EGFR:LP-CDDP is also sufficiently negative to avoid the binding of non-specific blood proteins (Table I). The cytotoxicity of EGFR:LP-CDDP or LP-CDDP was found to be weaker than that of CDDP in an *in vitro* assay, suggesting that these compounds might be slowly taken up by cells (Fig. 2A) (23). However, the cellular selectivity of EGFR:LP-CDDP was observed to be dependent on the EGFR expression levels. The radiosensitizing effects of EGFR:LP-CDDP and LP-CDDP were compared using a clonogenic assay. Although few differences were found, we speculate this was due to an insufficient time for CDDP uptake into the cells.

Whilst the anticancer effects of EGFR:LP-CDDP were weak *in vitro*, these effects were found to be significant *in vivo* (Figs. 3-5). In terms of tumor growth, the EGFR:LP-CDDP-treated mice appeared to show a delay in comparison with the LP-CDDP- or CDDP-injected animals. This suggested that EGFR:LP-CDDP had successfully targeted the tumor. Moreover, the combination of EGFR:LP-CDDP and IR enhanced tumor growth delay in the model mice compared with separate EGFR:LP-CDDP or IR therapies, indicating that EGFR:LP-CDDP effectively radiosensitizes tumor cells. The free CDDP-treated group seemed to show a greater delay in tumor growth than the LP-CDDP-treated animals since several mice in this group died. Additionally, body weight loss was significant in the free CDDP-treated group. Although the EGFR:LP-CDDP- or LP-CDDP-treated groups showed slight body weight loss at 3 days after the injections, these weights quickly recovered. This indicated that the encapsulation of CDDP by LP reduces its toxicity. The kidney weights were also significantly reduced in the mice treated with CDDP or with

a combination of CDDP and IR (Fig. 3D). Nephrotoxicity is a well-known side effect of CDDP and several markers of this complication have been reported including urea, creatinine, and a fractional excretion of sodium (24,25). Creatinine and BUN levels are easily measurable via blood tests. The normal value of creatinine is <1.3 mg/dl and of BUN is <23 mg/dl (24). Both of these values were increased in mice treated with CDDP, but not in the other treatment groups. CDDP-induced nephrotoxicity was also observed by histopathological analysis (Fig. 5). Renal tubular degeneration and regeneration were observed only in the mice treated with CDDP. In the renal cortical tubules of these mice, tubular dilation, cell necrosis, and sloughing of cells were also evident. In addition, nephrotoxicity was similarly observed in the animals treated with a combination of CDDP and IR (data not shown). Overall, our results indicate that EGFR:LP-CDDP in combination with IR enhances the radiotherapeutic efficacy through the active targeting of EGFR-expressing NSCLC cells and that CDDP-induced nephrotoxicity is eliminated by LP encapsulation.

In conclusion, EGFR:LP-CDDP is an effective targeted radiosensitizer in EGFR-overexpressing NSCLC cells. This maximizes the chemoradiotherapeutic efficacy of combination regimens in NSCLC cells by neutralizing both the toxicity of CDDP and the IR resistance of the cells.

Acknowledgements

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