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Cisplatin-loaded Poly(L-glutamic acid)-*g*-Methoxy Poly(ethylene glycol) Nanoparticles as a Potential Chemotherapeutic Agent against Osteosarcoma*

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Abstract Herein, cisplatin-loaded poly(L-glutamic acid)-*g*-methoxy poly(ethylene glycol) nanoparticles were evaluated as a potential chemotherapeutic agent against osteosarcoma by using alone or with an iRGD (internalizing RGD, CRGDKDPDC). The release rate of platinum from the cisplatin-loaded nanoparticles CDDP/PLG160-*g*-mPEG2K (CDDP-NPs) accelerated with the increase of the acidity of the environment. *In vitro* test demonstrated that CDDP-NPs could inhibit the proliferation of MNNG/Hos osteosarcoma cells with IC_{50} (72 h) of 12.2 μg·mL^{-1}. *In vivo* test for MNNG/Hos osteosarcoma tumor bearing mice exhibited that CDDP-NPs had comparable or slightly higher efficacy but significantly lower side effects in comparison with free CDDP. The coadministration of iRGD could further enhance the anticancer efficacy of CDDP-NPs against MNNG/Hos osteosarcoma without bringing obvious side effects. Therefore, CDDP-NPs using alone or with iRGD have great potential for the treatment of osteosarcoma.

Keywords: Nanoparticles; Biodegradable polymers; Cancer chemotherapy; Polymeric drug carrier.

INTRODUCTION

Osteosarcoma is the most common bone malignancy in children and adolescents[1]. Owing to the high rate of systemic spread, the cure method is rare with surgical treatment alone[2–4]. In fact, the 5-year survival rate of patients is no more than 20% with amputation treatment alone. With the application of adjuvant chemotherapy, the current 5-year survival rate of patients has currently markedly increased to approximately 65% for localized and 20% for metastatic osteosarcoma[5, 6].

Cis-diamminedichloroplatinum (II) (CDDP, cisplatin or cisplatinum) is a frequently used anticancer drug in adjuvant chemotherapy for the treatment of osteosarcoma[7]. It exerts antitumor effects by binding to deoxyribonucleic acid (DNA) and bringing about the formation of intrastrand and interstrand cross-links which

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triggers the apoptosis of cancer cells[8]. The therapeutic activity of cisplatin is proportional to the intensity of dosage, however the dose-dependent nephrotoxicity and neurotoxicity have limited its use as a treatment for osteosarcoma[9, 10]. In addition, *in vitro* studies have shown cisplatin to have a direct effect on growth plate chondrocytes that results in decreased growth and final height in animals[11]. Since osteosarcoma usually occurs in children and teenagers, these side effects would have a negative impact on the quality of life of patients. Therefore, the strategy that can reduce the side effects without loss of anticancer efficacy of CDDP against osteosarcoma is highly desired.

Polymeric nanocarriers can dramatically improve the pharmacokinetics and biodistribution of cisplatin, thus the enhanced anticancer efficacy and reduced side-effects can be obtained[12–15]. Since most solid tumors have elevated vascular permeability and usually lack effective lymphatic drainage, nanomaterials, such as liposomes[16], nanoparticles[17] and macromolecular drugs[18], tend to accumulate in tumor tissue much more than in normal tissues, this is referred to as the enhanced permeability and retention (EPR) effect[19]. As compared with free CDDP, PEGylated CDDP-loaded polymeric nanoparticles usually have remarkably prolonged blood circulation time[12, 13, 20–26]. This leads to that the CDDP-loaded polymeric nanoparticles have more opportunity to target tumor tissue through the EPR effect, suggesting the enhanced anticancer efficacy and reduced side-effects[27]. In particular, recently Chen and coworkers have developed cisplatin loaded poly(L-glutamic acid)-*g*-methoxy poly(ethylene glycol) (CDDP/PLG-*g*-mPEG) nanoparticles that showed comparable *in vivo* antitumor efficacy and significantly lower systemic toxicity as compared with free cisplatin in MCF-7 tumor bearing mice[28]. It is interesting to investigate the possibility of treating osteosarcoma with the CDDP/PLG-*g*-mPEG nanoparticles.

In addition, the penetration of nanoparticles into tumor tissue is usually poor. Allen *et al*. found the tumor penetration depth of methoxy poly(ethylene glycol)-*b*-poly(ε-caprolactone) block copolymer micelles (BCMs) to be dependent on the particle size. BCMs with diameters of 25 nm (BCM-25) diffused further awayfrom the blood vessels (D_{mean} = (42 ± 9) μm) following extravasation, compared to BCMs with diameters of 60 nm (BCM-60) which mainly remained in the perivascular regions (D_{mean} = (23 ± 4) μm). This suggested that reducing the particle size promotes nanoparticles penetration into solid tumors. But the traveling distance of nanoparticles in a solid tumor was extremely limited (42 μm) even if the size of nanoparticles was as small as 25 nm[29]. Furthermore, because of the poor penetration of nanoparticles into tumor tissue, there is a low contact probability for some nanoparticles to reach a majority of target cells within the tumor[30, 31]. As a result, the therapeutic efficacy of many anticancer nanomedicines is limited[32]. But nevertheless Sugahara *et al*. reported an internalizing RGD (iRGD) that could selectively enhance the vascular and tissue permeability of tumors overexpressing α_v integrins and neuropilin-1[33]. The activity of effector drugs, such as albumin-embedded paclitaxel and DOX liposomes, was significantly enhanced when iRGD was chemically conjugated to or co-administrated with the chemotherapeutic agents. Therefore the coadmistration of iRGD may have some possibility to enhance the anticancer efficacy of CDDP/PLG-*g*-mPEG nanoparticles against osteosarcoma.

Herein, we present the treatment of osteosarcoma with the CDDP/PLG-*g*-mPEG nanoparticles alone or with iRGD. MNNG/Hos is used as the osteosarcoma cancer model. The CDDP delivery systemis evaluated *in vitro* and *in vivo* in detail. The coadminstration of iRGD is expected to enhance the permeability of tumor vascular and tissue, consequently enhancing the efficacy of the CDDP-loaded nanoparticles for the treatment of osteosarcoma (Fig. 1).

METHODS

Materials

The preparation of poly(L-glutamic acid)-*graft*-methoxy poly(ethylene glycol) (PLG160-*g*-mPEG2K) with PLG/mPEG (*W*/*W*) ratio of 2 was published elsewhere[28]. *cis*-Diaminodichloroplatinum (II) (Cisplatin, CDDP) was purchased from Kunming Guiyan Pharmaceutical Co., Ltd., China. iRGD (CRGDKGPDC) peptide was custom-synthesized from Apeptide Co. Ltd. (Shanghai, China). 3-(4,5-Dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was purchased from Sigma and used as received. All other reagents and solvents were purchased from Sinopharm Chemical Reagent Co. Ltd., China and used as received.

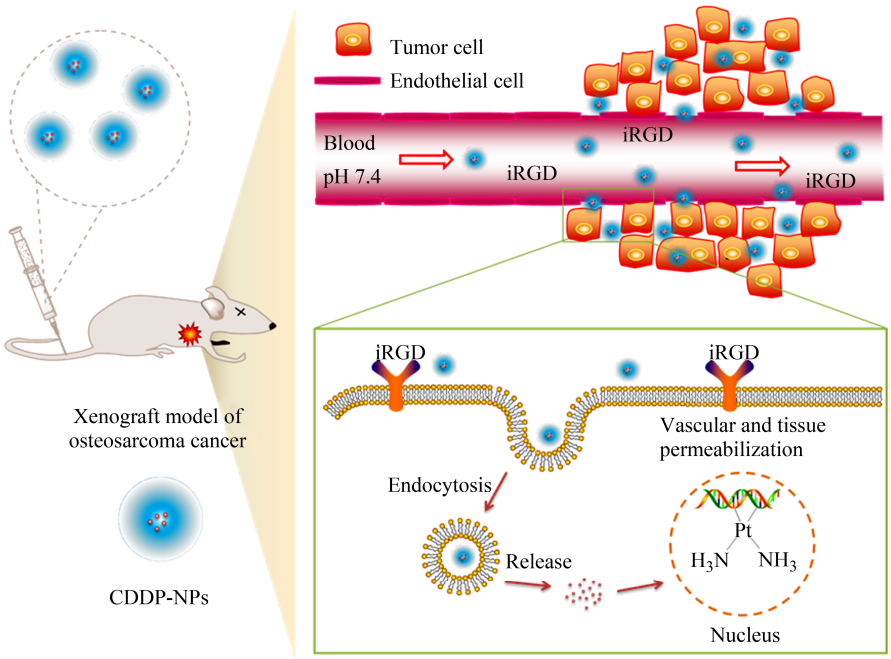


Fig. 1 The schematic illustration of the intravenous injection, blood circulation, tumor accumulation, cellular uptake and intracellular drug release of the CDDP-loaded nanoparticles (CDDP-NPs)

Preparation of CDDP/PLG160-g-mPEG2K Nanoparticles

PLG160-*g*-mPEG2K (90 mg) and CDDP (10 mg) were dissolved in distilled water (50 mL) and reacted at 37 °C for 72 h in the dark. The CDDP/PLG160-*g*-mPEG2K nanoparticleswere then obtained by lyophilization of the reaction mixture in the dark without further purification. The particle size and zeta potential of the CDDP/PLG160-*g*-mPEG2K nanoparticles (CDDP-NPs) were evaluated by a Zeta Potential/BI-90Plus particle sizeanalyzer (Brookhaven, USA) at 25 °C.

In vitro Drug Release

The release of platinum from the CDDP/PLG160-*g*-mPEG2K nanoparticles in phosphate buffered saline (PBS, pH 5.0 or 7.4, 0.01 mol·L^{-1}, [Cl^-] = 20 or 150 mmol·L^{-1}) was evaluated by dialysis. Typically, 5.0 mg of CDDP-NPs in 5 mL of PBS was added into a dialysis tube (MWCO 3500 Da), which was then incubated in 40 mL PBS buffer at 37 °C with a shaking rate of 100 r·min^{-1}. At selected time intervals, 2 mL of incubating solution was taken out and replaced with an equal volume of fresh media. The platinum content was determined by an Inductively Coupled Plasma Optical EmissionSpectrometer (ICP-OES, iCAP 6300, Thermoscientific, USA).

Cell Cultures

MNNG/Hos (Human osteosarcoma cell line) was obtained from the Cancer Institute and Hospital, Chinese Academy of Medical Sciences (Beijing, China). Cells were cultured at 37 °C in a 5% CO_2 atmosphere in a culture medium (CM) containing Roswell Park Memorial Institute 1640 Medium (RPMI-1640) supplemented with 10% fetalbovine serum (FBS), penicillin (50 U·mL^{-1}) and streptomycin (50 U·mL^{-1}). CM renewal was every 2 days. Cells were passaged every two to three days, and trypsinized using Trypsin-EDTA solution (0.05% Trypsin, 0.53 mmol·L^{-1} EDTA) at 37 °C and in a 5% CO_2 atmosphere.

In vitro Evaluation

MNNG/Hos cells were seeded in 96-well culture plates at a density of 5 × 10^3 cells per well in 200 μL RPMI-1640 and allowed to attach for 24 h. Then cells were reseeded with PLG160-*g*-mPEG2K, free CDDP or CDDP-NPs at different concentrations and incubated for another 48 h or 72 h. At each time point, cell viability was analyzed using MTT and measured in a Bio-Rad 680 microplate reader at a wavelength of 490 nm.

In vivo Antitumor Efficiency

Balb/C nude mice (SPF grade) with a body mass from 16 g to 18 g were obtained from Experimental Animal Center of National Institute of Food and Drug Control, China. MNNG/Hos tumor tissues were obtained from Cancer Institute and Hospital, Chinese Academy of Medical Sciences and cut into small pieces of approximately 2 mm^3 in size, and four or five pieces of tissue were transplanted subcutaneously into the right flanks of the Balb/C nude mice. After tumor nodules grew to a volume ~140 mm^3, the mice were randomly divided into five groups (n = 6). The mice were injected intravenously *via* tail vein four times at 3 day intervals with PBS (pH 7.4), free CDDP (4 $mg \cdot kg^{-1}$), free CDDP + iRGD (4 $mg \cdot kg^{-1}$ CDDP, 10 $mg \cdot kg^{-1}$ iRGD), CDDP-NPs (4 $mg \cdot kg^{-1}$ on the basis of CDDP), and CDDP-NPs nanoparticles + iRGD (4 $mg \cdot kg^{-1}$ on the basis of cisplatin, 10 $mg \cdot kg^{-1}$ iRGD). The antitumor activity was evaluated in terms of the tumor size, which was estimated by the following equation: $V = a \times b^2/2$, where a and b are major and minor axes of the tumor measured by a caliper, respectively. Tumor suppression rate = $[(V_c - V_x)/V_c] \times 100\%$, here, V_c represented the tumor volume of the control group, while V_x represented the tumor volume of a treatment group. The body weight was measured as an indicator of systemic toxicity.

Histopathology Evaluation

The histopathology damage evaluation was assessed by hematoxylin and eosin (H&E) method. Briefly, after 3 days of the last administration, MNNG/HOS tumor-bearing mice were sacrificed and the chests were cut open, PBS and paraformaldehyde were perfused from the left atrium. The tumors were collected, embedded in paraffin, and cut into sections of 5 $\mu mol \cdot L^{-1}$ in thickness. The obtained sections were then stained with hematoxylin and eosin to assess histological change in tumors using a microscope (Nikon TE2000U).

Statistical Analysis

All experiments were performed at least three times and expressed as means ± SD. Data were analyzed for statistical significance using Student's test. $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Preparation and Solution Behavior of CDDP-NPs

CDDP-NPs were prepared by the complexation reaction of CDDP with PLG160-*g*-mPEG2K copolymer in aqueous solution. The average hydrodynamic diameter (D_h) of CDDP-NPs was (43.4 ± 1.3) nm. The size was suitable for the "passive targeting" delivery of nanoparticles to solid tumors because CDDP-NPs were large enough to avoid the filtration by kidney (D_h > 20 nm) and small enough to avoid a specific sequestration by sinusoids in spleen and fenestra of liver (D_h < 100 nm). Furthermore, because the solid tumors exhibited pores or fenestrations in vascular endothelium in the range of 100 nm to 1 μm depending on the tumor type[34, 35], CDDP-NPs should have the ability to penetrate through the leaky vasculatures of tumors[36–38]. The zeta potential of CDDP-NPs was (–13.6 ± 0.6) mV, which is good for prolonged circulation time owing to the slightly negative charge being able to effectively reduce protein absorbance in blood circulation[39]. The negative zeta-potential of the CDDP-NPs should be caused by the free carboxylate groups from the PLG160-g-mPEG2K.

In vitro Release

The *in vitro* release of Pt(II) from CDDP-NPs was investigated in PBS buffer (pH 5.0 or 7.4, 0.01 $mol \cdot L^{-1}$, $[Cl^-]$ = 20 or 150 $mmol \cdot L^{-1}$) at 37 °C using dialysis method. As reported by Kataoka *et al*.[12, 21], the release of CDDP from the metal complex nanoparticles was due to the inverse ligand exchange reaction of Pt(II) from the carboxylates of copolymer to the chloride ions in the surroundings. The CDDP-loaded nanoparticles showed a sustained and time dependent release behavior at all pH and Cl^- concentrations, no burst release was observed (Fig. 2). The release rate of CDDP-NPs at pH 5.0 ($[Cl^-]$ = 150 $mmol \cdot L^{-1}$) was faster than that at pH 7.4 ($[Cl^-]$ = 150 $mmol \cdot L^{-1}$), which could be explained by that the protonation of carboxylates of PLG-*g*-mPEG2K copolymer at acidic pH weakened the Pt(II) and PLG160-*g*-mPEG2K coupling bonds[25]. This was good for the tumor specific release of CDDP since pH decreased from 7.4 at blood to 5.0 at lysosome along the endocytic pathway

of nanoparticles[40]. The release rate of the CDDP-NPs at [Cl^-] = 20 mmol·L^{-1} (pH 7.4) was lower than that at [Cl^-] = 150 mmol·L^{-1} (pH 7.4), indicating that the release of platinum was dependant of chloride ion in solution.

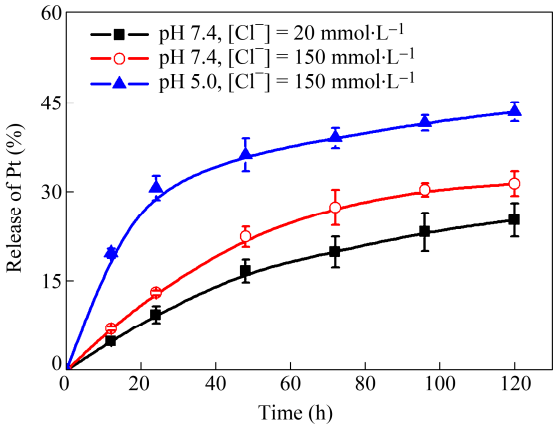


Fig. 2 Accumulative platinum release of CDDP-NPs in PBS of pH 5.0 or 7.4, [Cl^-] = 20 or 150 mmol·L^{-1}

In vitro Cytotoxicity against MNNG/Hos Cells

The *in vitro* cytotoxicity assessment of the PLG160-*g*-mPEG2K copolymer, free CDDP and CDDP/PLG160-*g*-mPEG2K was carried out using MTT method. As shown in Fig. 3(a), the viabilities of MNNG/Hos cells treated with the PLG160-*g*-mPEG2K copolymer were around 80% to 100% at all test concentrations in 48 h, suggesting the excellent biocompatibility of the PLG160-*g*-mPEG2K. Both CDDP and CDDP-NPs showed dose and time dependent cytotoxicity towards MNNG/Hos cells (Fig. 3b). In consistent with the literature reports, the *in vitro* cytotoxicity of CDDP against MNNG/Hos cells was significantly reduced after incorporation into polymeric nanoparticles[12, 20, 24]. CDDP-NPs exhibited slight inhibition to the proliferation of MNNG/Hos cells (cells viability > 80%) at CDDP concentration of 16 μg·mL^{-1} in 48 h incubation. After exposure of MNNG/Hos cells for 72 h to CDDP-NPs, distinct inhibition was observed with IC50 of 12.2 μg·mL^{-1} on the basis of CDDP. This value was 3.0-fold of the IC_{50} (4.0 μg·mL^{-1}) of CDDP at 72 h, which could be explained by the slow drug release rate of CDDP from CDDP-NPs.

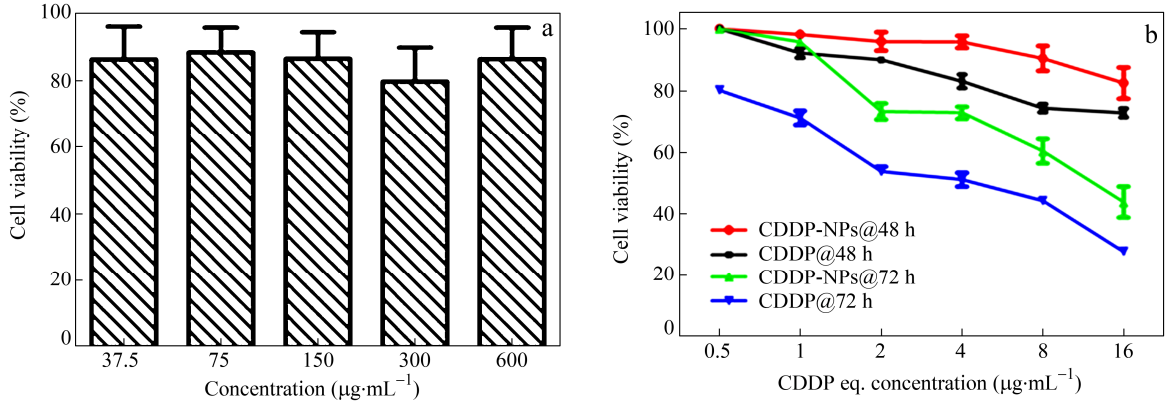


Fig. 3 MTT assay with MNNG/Hos cells: (a) PLG160-*g*-mPEG2K incubated with MNNG/Hos cells for 48 h and (b) CDDP and CDDP-NPs incubated with MNNG/Hos for 48 and 72 h

In vivo Antitumor Efficacy

Balb/C nude mice bearing human osteosarcoma tumors (MNNG/Hos) were used as the tumor model to evaluate the *in vivo* antitumor efficacy of free CDDP, CDDP-NPs and the combinations with iRGD. After 13 days of implantation, the MNNG/Hos tumors were grown to about 140 mm^3 on average. Saline, free CDDP (4 mg·kg^{-1}),

CDDP + iRGD (4 mg·kg^{-1} CDDP, 10 mg·kg^{-1} iRGD), CDDP-NPs (4 mg·kg^{-1} on CDDP basis), and CDDP-NPs + iRGD (4 mg·kg^{-1} on CDDP basis, 10 mg·kg^{-1} iRGD) were administrated by intravenous injection *via* tail vein at 3 day intervals for four times.

After treatment, mice of the free CDDP and CDDP + iRGD groups became emaciated (Fig. 4a) and were manifested with low spirits and hunchbacked posture. One mouse died after 2 times treatments in the free CDDP group. Body weight change of mice could be an indicator of systemic toxicity. The average body weight of the mice of free CDDP group decreased from (18.6 ± 0.6) g at day 13 to (17.4 ± 0.4) g at day 25. For the free CDDP + iRGD group, the average body weight decreased from (19.7 ± 1.2) g at day 13 to (18.4 ± 0.9) g at day 25. These should result from the intense systemic toxicity of free CDDP. In contrast, the average mice body weights of the saline, CDDP-NPs and CDDP-NPs + iRGD groups increased after treatment than before treatment (Table 1). These suggested that CDDP-NPs had lower systemic toxicity than free CDDP.

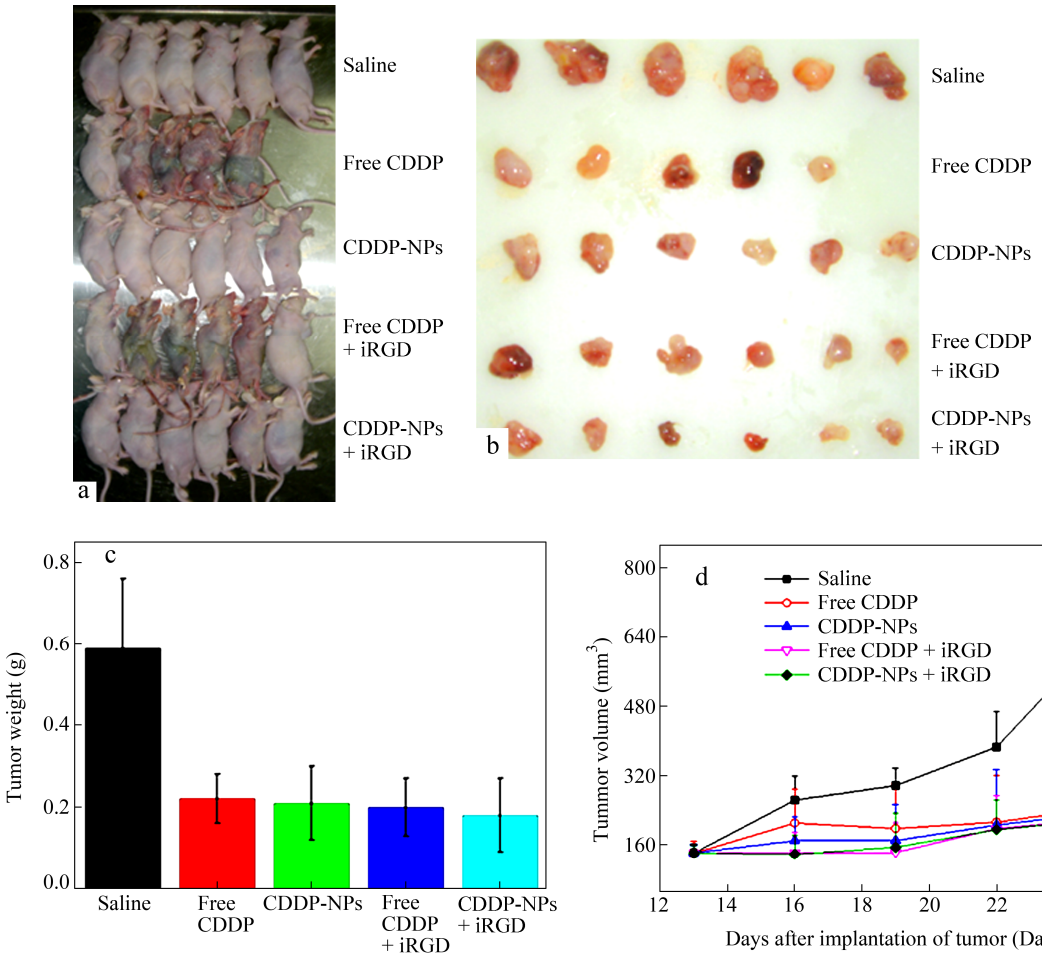


Fig. 4 Treatment of Balb/C nude mice bearing human osteosarcomatumors (MNNG/Hos) by intravenous injection *via* tail vein at 3-day intervals for four times: (a) Pictures of mice bearing MNNG/Hos tumors of each group after 4 times treatments; (b) Pictures of tumor tissues from each group; (c) Average tumor weights from each group and (d) tumor sizes of the Balb/C nude mice as a function of time

Pictures of tumor tissues from each group after 4 times treatments were shown in Fig. 4(b). The average tumor weights of each group were displayed in Fig. 4(c). Figure 4(d) was the tumor sizes of the Balb/C nude mice as a function of time. The tumors from the groups of free CDDP, CDDP-NPs, free CDDP + iRGD and CDDP-NPs + iRGD were much smaller than those from the saline group with significant differences ($P < 0.01$).

At day 25, the tumor weight of the control group was (0.59 ± 0.17) g. After 4 times treatments, the tumor weights of free CDDP, CDDP-NPs, free CDDP + iRGD, and CDDP-NPs + iRGD groups were (0.22 ± 0.06), (0.21 ± 0.09), (0.20 ± 0.07), and 0.(18 ± 0.09) g, respectively. The tumor suppression rates of free CDDP + iRGD (66.85%) and CDDP-NPs + iRGD (68.82%) groups were higher than those of free CDDP (62.25%) and CDDP-NPs (64.33%) groups (Table 1). This should be attributed to the penetration improvement of the CDDP-NPs in the tumor tissue with the use of iRGD. These indicated that both free CDDP and CDDP-NPs could suppress MNNG/Hos tumor growth. CDDP-NPs have comparable or slightly higher efficacy than free CDDP for the treatment of human MNNG/Hos osteosarcoma. The coadminstration of iRGD have potential to enhance anticancer efficacies of free CDDP and CDDP-NPs.

Table 1. Growthinhibition of treatment on Balb/C nude mice bearing human MNNG/Hos osteosarcoma tumors

Group	Number of live mice		Body weight		Tumor weight (g)	TSR [h] (%)	*P* [i]
	Before [f]	After [g]	Before [f]	After [g]			
Control [a]	6	6	19.3 ± 0.8	21.0 ± 0.7	0.59 ± 0.17	–	–
Free CDDP [b]	6	5	18.6 ± 0.6	17.4 ± 0.4	0.22 ± 0.06	62.25	< 0.01
CDDP-NPs [c]	6	6	19.4 ± 0.8	21.9 ± 0.7	0.21 ± 0.09	64.33	< 0.01
Free CDDP + iRGD [d]	6	6	19.7 ± 1.2	18.4 ± 0.9	0.20 ± 0.07	66.85	< 0.01
CDDP-NPs + iRGD [e]	6	6	20.1 ± 0.8	21.9 ± 0.9	0.18 ± 0.09	68.82	< 0.01

[a] Control: Saline; [b] Dosage of free CDDP: 4 mg·kg^{-1}; [c] Dosage of CDDP-NPs: 4 mg·kg^{-1} on CDDP basis; [d] Dosage of free CDDP: 4 mg·kg^{-1}, dosage of iRGD: 10 mg·kg^{-1}; [e] Dosage of CDDP-NPs: 4 mg·kg^{-1} on CDDP basis, dosage of iRGD: 10 mg·kg^{-1}; [f] Before treatment (at day 13); [g] After treatment (at day 25); [h] Tumor suppression rate; [i] *P* value by statistical analysis, tumor weight compared with control group.

Histopathology Evaluation

To further evaluate the therapeutic efficacy of CDDP-NPs against osteosarcoma, MNNG/Hos osteosarcoma tumor bearing mice were sacrificed after 3 days of the last administration. The tumor sections were prepared andstained with H&E for pathology analysis. For H&E staining, haematoxylin stain colored nuclei of cells blue while eosin stain colored cytoplasm and extracellular matrix red. Necrotic cells did not exhibit clear cell morphology, and the chromatin became darker or diffused outside the cell. As shown in Fig. 5, the tumor cells

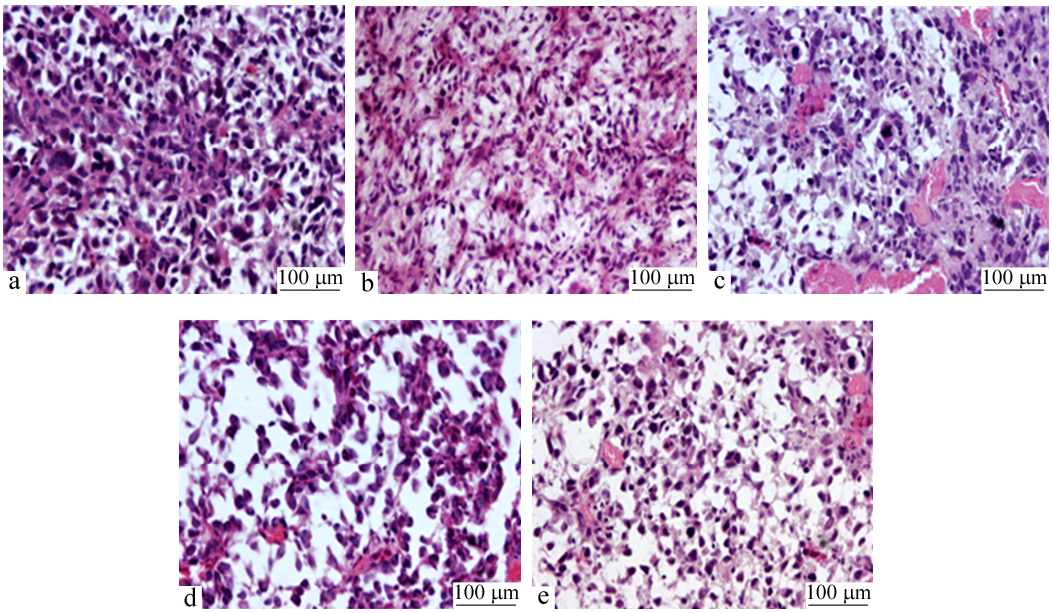


Fig. 5 H&E stainingof tumors (a to e) of nude mice bearing MNNG/Hos tumors after 25 days treatment with saline (a), free CDDP (b), CDDP-NPs (c), CDDP + iRGD (d) and CDDP-NPs + iRGD (e)

with large nucleus and a spherical or spindle shape were observed in the tumor tissue of the saline group (Fig. 5a), in which high nucleus/cytoplasm ratio and some inflammatory cells were also seen, indicating a rapid tumor growth. In contrast, various degree of tissue necrosis, extensive nuclear shrinkage and fragmentation were observed in other groups. Chromatin was concentrated and distributed around the edge, and nuclei became pyknotic, fragmented or absence, especially for the tumor cells of CDDP-NPs + iRGD group (Fig. 5e). The necrosis area in the CDDP-NP + iRGD group was the largest among the tested groups, while the free CDDP, CDDP-NPs and CDDP + iRGD groups displayed a relatively lower necrotic level, which was consistent with *in vivo* tumor growth inhibition study.

CONCLUSIONS

In this study, CDDP-NPs were evaluated for the treatment ofosteosarcoma by using alone or with iRGD. CDDP-NPs showed a dose and time-dependent proliferation inhibition to MNNG/Hos cells. In an *in vivo* test for MNNG/Hos osteosarcoma tumor bearing mice, CDDP-NPs and CDDP-NPs + iRGD showed slightly higher anti-tumor effect and a significantly lower body weight loss in comparison with free CDDP, suggesting the advantages of CDDP-NPs over free CDDP in anticancer efficacy and systemic side effects for the treatment of osteosarcoma. The combination of CDDP-NPs + iRGD (TSR: 66.85%) could further enhance the efficacy of CDDP-NPs (TSR: 64.33%) without bringing obvious side effects. Therefore, CDDP-NPs was a promising new therapy for osteosarcoma.

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