Paclitaxel-functionalized aspragalan-calcium phosphate nanoparticles

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ABSTRACT

Paclitaxel (PTX) is an important anticancer agent widely applied in the treatment of breast, ovarian and lung cancer. Its water solubility is very low; hence the available formulation of PTX for clinical use consists of Cremophor EL, which has been associated with several hypersensitivity reactions. In order to reduce the systemic toxicity of PTX as well as to avoid the use of Cremophor EL, we developed a Cremophor EL-free aspragalan-calcium phosphate nanoparticle delivery system containing PTX and tested its efficacy. Calcium phosphate nanoparticles have attracted attention as a drug delivery system in recent years, and aspragalan polysaccharides extracted from Chinese herbs have been reported to provide protection against tumors. Cremophor EL-free aspragalan-calcium phosphate PTX-containing nanoparticles were prepared by an adsorption and an incorporation technique. The physicochemical characteristics of the nanoparticles, including particle size, zeta potential, and surface morphology, as well as quantification of drug, were measured. The results were: 1. The loading efficiency of PTX was higher with the incorporation technique than with the adsorption technique. 2. The release behavior of PTX exhibited a biphasic pattern characterized by an initial burst release followed by a slower and continuous release. 3. The size of nanoparticle was in 100-500nm range. 4. In an *in vivo* study, the PTX-loaded Cremophor EL-free aspragalan-calcium phosphate nanoparticles shown tumor growth inhibition effect on hepatocarcinoma and few side effects. Therefore, PTX -loaded aspragalan-calcium phosphate nanoparticles may be considered as an effective anticancer drug delivery system for cancer chemotherapy.

Keyword: aspragalan: calcium phosphate nanoparticles; paclitaxel; antitumor

1 INTRODUCTION

Paclitaxel (PTX) is an important chemotherapeutic drug that is given as a treatment for some types of cancer, including ovarian, breast and non-small cell lung cancer [1, 2]. .PTX is commonly known as Taxol®. Clinically, Cremophor EL is added in the formulation to improve its very low aqueous solubility; and this has been associated with side effects e.g. hypersentivity, nephrotoxicity and neurotoxicity, as well as effects on endothelial and vascular muscles causing vasodilation, labored breathing, lethargy and hypotension[3]. Many pharmaceutical attempts have been made to reduce the systemic toxicity of PTX, as well as to avoid the use of Cremophor EL, including using liposomes, nanoparticles (NP), microemulsions and vesicles for delivery [3]. In this study we have developed a Cremophor EL-free aspragalan-calcium phosphate nanoparticles ACPNPs) as a delivery system for PTX. Physicochemical characterization, antitumor activity, and acute toxicity of the PTX-ACPNPs were evaluated.

Previous studies demonstrated that calcium phosphate is the most important inorganic constituents of biological hard tissues in living organisms, and its biology is well understood through extensive research on biomineralization. More recently, calcium phosphate has attracted much attention in inorganic nanoparticle delivery systems because of its easy production, biodegradability properties, good biocompatibility and controlled release of carried drugs [4]. Aspragalan polysaccharides were reported to increase the secretion of mostly Th1 cytokines (IL-1a, IL-9, IL-12, and GM-CSF, but not IL-4 and IL-10) by dendritic cells and result in the rapid maturation of dendritic cells, which is required for tumor treatment. We combine those two components to formulate a nanoparticle delivery system in order to increase efficiency of PTX and host immune response against tumors.

2 MATERIALS AND METHODS

2.1 Preparation of PTX nanoparticles

PTX-ACPNP was prepared using two methods. The first was the adsorption technique. Nanoparticles were prepared by a

simple interfacial deposition method (nanoprecipitation). Briefly, 160 mg of $CaCl_2$, 160 mg NaH_2PO_4, 160 mg sodium citrate and 40 mg aspragalan polysaccharides were added to 180 ml distilled water under magnetic stirring at room temperature for 48h, sonicated for 1h, centrifuged (10,000 rpm, 15 min, 4 °C), and the precipitate separated. Then, 10 mg PTX, dissolved in ethanol, was added and stirred with the above precipitate to absorb the PTX. The resulting suspension was centrifuged and the precipitate was resuspended in pure water.

The second preparation method for PTX- ACPNP was named the incorporation technique. Briefly, 160mg $CaCl_2$, 160mg NaH_2PO_4, 160mg sodium citrate and 40mg aspragalan polysaccharides were added to 180 ml distilled water while stirring, and then 10 mg PTX in ethanol was incorporated into the above solution, prior to the formation of the nanoparticles. The suspension was stirred for 48h at room temperature and sonicated for 1h, followed by centrifugation (10,000 rpm, 15 min, 4°C). The supernatant was discarded, and the precipitate resuspended in pure water.

2.2 Determination of PTX content in nanoparticles

For the determination of the loading efficiency of the preparation process, the nanoparticles were first separated from the aqueous suspension medium by centrifugation (10,000rpm, 15min, 4°C) and dissolved by adding 0.1N HCL-methanol. The amount of PTX in the nanoparticles was measured by HPLC. The loading capacity and the association efficiency of the process were calculated as indicated below:

Loading capacity (%) = the amount of PTX in nanoparticles/Weight of nanoparticles×100

Association efficiency (%) = (the amount of PTX in nanoparticles)/Total amount PTX×100.

2.3. Physicochemical characterization of PTX-ACPNPs

The as-prepared nanoparticles were morphologically characterized by scanning electron microscopy (SEM). The particle size and the zeta-potential of PTX- ACPNP in water were measured using a Zetasizer Nano ZS90 (Malvern Instruments Ltd., UK).

2.4. In vitro drug release studies

The in vitro release test of PTX- ACPNP was performed in PBS at 37 °C. Aliquots of the suspension of nanoparticles containing 1mg of PTX were diluted in 25 ml of PBS (pH 6.8) and incubated, under agitation, at 37 °C. During the release studies, aliquots (1.0 ml) of the medium were withdrawn at preset times, and centrifuged (10,000 rpm, 30 min, 4 °C). The content of PTX in the supernatant was quantitated using HPLC. An equal volume of fresh medium was added to resuspend the sediment by vortexing, and poured into the dissolution beaker. Experiments were performed in triplicate.

2.5. In vivo tumor growth inhibition study

Using adult mice, a laparotomy was performed under general anesthesia (d0) and murine hepatocarcinoma H22 cells (5 x 10^6 /ml) were inoculated subcutaneously under the right armpit (0.2 ml/ mouse). The animals were then randomly divided into 5 groups (10 mice/group), two experimental groups, PTX- ACPNP prepared by the adsorption and by the incorporation technique, two positive controls, Taxol® and commercial PTX liposome, and one negative control group injected with 5% glucose

On d1, d3 and d5 after transplantation, each sample was administered by tail vein at a single dose of 4mg/kg, or an equal volume of glucose solution. The animals were killed on the d9 after tumor inoculation. The tumor was excised and weighed. The rate of tumor inhibition was calculated by the formula: (the tumor weight of negative control group-the tumor weight of experimental group)/ the tumor weight of negative control group×100%.

2.6. Evaluation of toxicity and determination of the LD50

The in vivo toxicity of PTX-ACPNP was investigated in healthy female Balb/C mice. Six groups of mice (*n*=10/group) received a single dose of PTX-ACPNP at 235, 200, 170, 145, 123 or 105 mg/kg respectively. The formulations were administered via the tail vein (20ul/10g). Mouse survival was monitored daily for 14 days in all groups. LD50 was calculated by using SPSS 16 software. Allergy test and hemolytic assay were also preformed according to FDA requirement.

3 RESULTS

3.1. Characterization of PTX-loaded nanoparticles

The physicochemical properties and loading efficiency of PTX- ACPNP are shown in Table 1.

Table 1: Physicochemical and loading properties of PTX-ACAP-NP prepared by two methods

Preparation method	Loading capacity (%)	Association efficiency (%)	Particle Size (nm)	Zeta-potential (mV)
Adsorption	5.3	20.5±3.4	139±15	-22±3.1
Incorporation	6.1	53.2±4.1	267±23	-14±2.0

The loading capacity of the two nanoparticle preparations was similar, but nanoparticles prepared by the incorporation technique had a higher association efficiency (53.2%) than those prepared by the adsorption technique (20.5%). The similar results were reported by Ueno et al (2005) [5, 6]. The likely reason is that the drug was entrapped in particles and also adsorbed on the surface of the solid nanoparticles by the incorporation technique. Therefore, the preparation method influenced the loading efficiency of the ACPNPs

With the absorption technique, the diameter and zeta-potential were similar to the free-drug nanoparticles (data not shown). This is due to PTX only being adsorbed on the surface of nanoparticles. The adsorption sites of the aspragalan calcium phosphate nanoparticles were limited, as indicated by the low association efficiency (20.5%), so the presence of a limited amount of PTX did not affect the nanoparticle size and zeta-potential. On the other hand, with the incorporation technique, the diameter was larger and the zeta-potential was smaller due to PTX participating in the formation of the nanoparticles, resulting in changes in their physicochemical properties. In addition, PTX- ACPNP was made using the incorporation technique and lyophilized overnight. The size, Zeta, and loading percentage of PTX- ACPNP were measured to evaluate the stability of PTX-ACPNP. The result shown that the suspension of PTX- ACPNP was stable for at least 24 hours (Table 2).

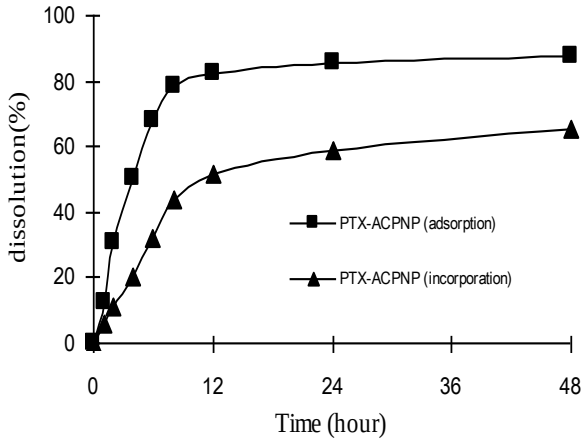
Table 2: Stability of PTX- ACPNP after lyophilized

HOUR	SIZE(nm)	ZETA(mV)	PTX (%)
0	268	-15	100.0
1	297	-12	99.75
2	342	-14	98.21
4	357	-16	98.56
6	370	-15	97.64
8	387	-14	98.31
24	392	-15	97.66

3.2. In vitro drug release

The amount of PTX released over time from the calcium phosphate nanoparticles was cumulatively plotted in Figure 2. The samples prepared by the adsorption technique showed a pronounced bursting behavior for the initial time period of 8 h, while the sample prepared by the incorporation method exhibited a biphasic pattern, characterized by an initial burst release followed by a slower and continuous release. Similar burst release has been previously reported [7, 8]. The burst release of PTX may be due to the desorption of PTX that was only adsorbed on the surface of the nanoparticles, while the slower and continuous release may be attributed to the diffusion of PTX localized inside the nanoparticles, or result from the degradation of the particles.

Figure 2: PTX releasing assay



3.3 In vivo antitumor effect of PTX nanoparticles

Table 3. Percentage of antitumor efficiency of PTX-ACPNP (*,P<0.05)

Group	Weight of tumor (g)	Anti-Tumor rate (%)	t-test *vs* glucose	t-test *vs* Taxol
PTX-ACPNP (adsorption)	0.91±0.34	43.1	0.000*	0.875
PTX-ACPNP (incorporation)	0.74±0.22	53.8	0.000*	0.153
Taxol	0.94±0.36	41.3	0.001*	——
Glucose	1.60±0.39	——	——	——

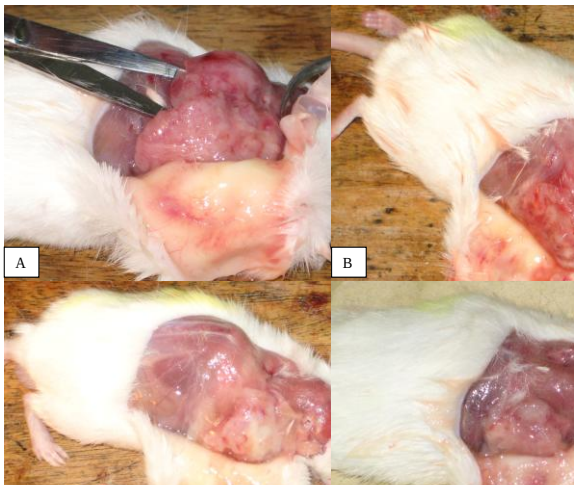
Mouse tumor weight effects of treatment are shown in Table 2. Significant suppression of tumor growth was observed in PTX- ACPNP and Taxol compared with the negative control (P<0.05), among which, the PTX-ACPNP (incorporation) showed the highest antitumor efficacy, and the antitumor

effect of Taxol was lowest. Six mice from Taxol-treated group revealed diffuse proliferation of tumor; only two mice from PTX-ACPNP (incorporation) group had diffusion (Figure 3), Table 4 represented the number of mice with tumor diffusion

Table 4: diffuse proliferation of tumor

Group	% of tumor diffusion
5% Glucose	100%
Taxol	60%
PTX-ACPNP (adsorption)	40%
PTX-ACPNP (incorporation)	20%

Figure 3: The pictures of diffuse proliferation of tumor



A: glucose-treated mouse; B: Taxol group; C: PTX-ACPNP (adsorption) treated mouse; D: PTX-ACPNP (incorporation) treated mouse.

3.4 Preclinical toxicity evaluation of PTX- ACPNP AND determination of LD50

Mice were injected with different dose of PTX-ACPNP and monitored for percentage of death (table 5). The result of statistics shown the LD50 of PTX-ACPNP (incorporation) was 151.976mg/kg. There were no allergy side effect and haemolysis (Figure 4)

Table 5: Death rate of PTX-ACPNP treated mice (n=10)

Dosage	D1	D2	D3	D14	%
235mg/kg	10	0	0	0	100%
200mg/kg	8	1	0	0	90%
170mg/kg	5	2	0	0	70%
145mg.kg	3	2	0	0	50%
123mg/kg	1	1	0	0	20%
105mg/kg	0	0	0	0	0%

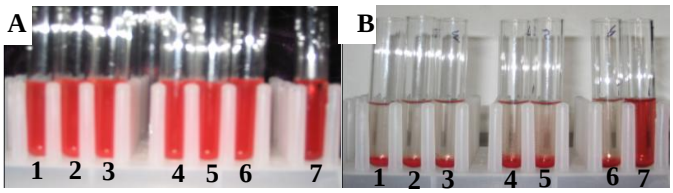


Figure 4: A and B shown red cells were mixed with different doses of PTX-ACPNPs (tube 1-5) for 15 minutes and 3h respectively Tube 6: red cells+salin. Tube 7: red cells+water

4 CONCLUSIONS

Clearly, ACPNP s-based PTX has achieved better therapeutic effect with no Cremophor EL was added compared with Taxol, particularly when prepared using the incorporation technique.

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