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Preparation, characterization and pharmacokinetics of N-palmitoyl chitosan anchored docetaxel liposomes

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Abstract

The objective of this work was to investigate the preparation, characterization and pharmacokinetics of N-palmitoyl chitosan anchored docetaxel liposomes. To decrease toxic effects and improve anti-tumour efficacy of the drug, docetaxel has been incorporated in liposomes; the formulation, stability and pharmacokinetics of plain docetaxel liposomes (PDLs), PEGylated docetaxel liposomes (PEGDLs) and N-palmitoyl chitosan anchored docetaxel liposomes (NDLs) were compared. NDL was more stable than PDL and PEGDL in-vitro, especially in the presence of serum at 37°C. The concentration of docetaxel in the plasma of rats after intravenous administration of docetaxel injection, PDL, PEGDL and NDL was studied by RP-HPLC. The pharmacokinetic behaviour of docetaxel injection, PDL, PEGDL and NDL were significantly different. These findings suggest that anchored liposomes could increase the stability of docetaxel in-vivo, as compared with plain liposomes, but the improvement was not more significant than PEGylated liposomes. N-Palmitoyl chitosan as a new polymeric membrane to anchor liposome was useful to stabilize liposomes containing anti-tumour drug.

Introduction

Liposomes have been widely investigated as potential drug delivery systems for anti-tumour drugs (Poste et al 1984; Zee-Cheng & Cheng 1989; Zamboni 2005). They have become a valuable experimental and commercially important drug delivery system for anti-tumour drugs owing to their biodegradability, biocompatibility, low toxicity and ability to control the time and the amount of drug release, reduce the toxicity of encapsulated drugs and target the tumour (Senior 1987; Namba & Oku 1993; Rolland 1993; Harashima et al 1999; Gabizon et al 2006). However, the unfavorable physico-chemical stability profile of liposomes in-vitro and in-vivo remains a major problem for the controlled release and tumour targeting of anti-tumour drugs. The stability of liposome is a major consideration in all steps of their production and administration. To increase the stability of liposomes, the formation of polymeric membranes around the liposome has been studied using natural components, such as amylopectin and glycolipids (Takada et al 1984; Miyazaki et al 1992; Allen 1994; Cheng et al 2006), or synthetic polymers, such as polyethylene glycol and polyvinyl alcohol (Klibanov et al 1990; Bakker-Woudenberg et al 2001; Hariqai et al 2001; Takeuchi et al 1999, 2000; Mu & Zhong 2006).

Chitosan, a deacetylated derivative of chitin, is an abundant, renewable, nontoxic and biodegradable carbohydrate polymer, available largely in the exoskeletons of shellfish and insects, that was widely used in chitosan-based particles as a controlled drug delivery system (Felt et al 1998; Janes et al 2001; Kim et al 2003b; Martinac et al 2005; Prego et al 2005). The application of chitosan is limited by its low solubility in acid-free aqueous media. So, low substitution ratio N-palmitoyl chitosan, a chitosan-based polymeric surfactant, was synthesized, which destroys the crystalline structure of chitosan and improves the solubility capacity in aqueous solution (Lee et al 2005). N-Palmitoyl chitosan was chemically modified with a hydrophobic anchor and subsequently integrated with the lipid bilayer membranes. N-Palmitoyl chitosan was more suitable for anchoring the liopsomes than chitosan. A series of investigations focused on the chitosan-coated liposomes (Takeuchi et al 1996, 2003, 2005; Filipovic-Grcic et al 2001; Guo et al 2003). However, most of the studies to date cover in-vitro stability aspects of chitosan-coated liposomes that

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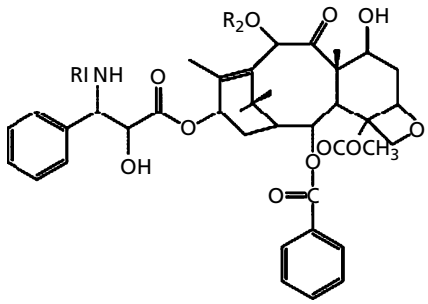

Figure 1 The chemical structures of docetaxel and paclitaxel.

carried peptide and protein drugs and there is a lack of studies of hydrophobic anti-tumour drugs existing in lipid bilayer membranes. Moreover, in-vivo stability studies for chitosan-coated liposomes are fairly few and there are no detailed evaluations of pharmacokinetic parameters. We tried to deal with both in-vitro and in-vivo stabilities of chitosan-coated liposomes.

In this research, N-palmitoyl chitosan (NPC) was synthesized and characterized. Then, the NPC was used to anchor liposomes containing docetaxel, which is used in clinical trials against ovarian carcinoma and breast, lung and head/neck cancer. Paclitaxel and docetaxel (Figure 1), belonging to the taxane class of anti-cancer agents, are perhaps the most important chemotherapeutic agents against cancer that have emerged over the past several decades (Earhart 1999). Docetaxel is formulated using Tween 80 and ethanol, which may be responsible for some toxic effects. To decrease these toxic effects and improve the anti-tumour efficacy of the drug, docetaxel has been incorporated in liposomes (Immordino et al 2003). However, the stability of liposomes was poor; NPC used to anchor the docetaxel liposomes was found to increase the stability of docetaxel liposomes significantly in-vitro, and it will help to further increase the drug concentration in the lipid bilayer without affecting the liposome stability. There has been no detailed evaluation of pharmacokinetic parameters for docetaxel injections, doxetaxel liposomes or chitosan-coated liposomes in rats; comparison of pharmacokinetic profiles of four different docetaxel preparations is given in our paper. The characterization of plain docetaxel liposomes (PDLs) and PEGylated docetaxel liposomes (PEGDLs) was compared with NPC anchored docetaxel liposomes (NDLs), including particle size, zeta-potential, morphological shape, in-vitro stability and in-vivo pharmacokinetic profiles.

Materials and Methods

Chemicals and instrumentation

Docetaxel was a gift from Jiangsu Hengrui Medicine Co., Ltd (Jiangsu, China). Egg yolk phosphatidylcholine (EPC) and PEG-DSPE was obtained from Lipoid (Ludwigshafen, Germany). Cholesterol was purchased from Sigma (St Louis, MO). Palmitic anhydride was obtained from Aldrich (Milwaukee, WI). Chitosan (degree of deacetylation: 90%, viscosity average molecular weight: 65 000 Da) was from Nantong Suanglin Biochemical Co. Ltd (Jiangsu, China). The cellulose membrane tubing was purchased from Shanghai Chemical Reagent Co. Ltd (Shanghai, China), and had a molecular weight cut-off of 8000–10 000. All other reagents and solvents used were of analytical grade or better. Preparation, analysis and characterization were carried out using APV homogenizer (APV2000, Denmark), FD2.5 Freeze Dryer (Heto, Denmark), 3K30 refrigerated centrifugation (Sigma, Germany), Zetasizer 3000 (Malvern Instruments, Malvern, UK), H-7000 model transmission electron microscope (Hitachi, Japan), FT-IR (Shimadzu, Japan) and an HPLC system consisting of a pump (Model LC-20A, Shimadzu, Japan), a lichrosphere C18 column, 4.6 mm × 15 cm, 5 μm (Hanbang Analytical Instrument Co. Ltd, China) and a UV detector (Model SPD-20A, Shimadzu).

Synthesis of N-palmitoyl chitosan

The mechanism is that the unshared electron pair on the nucleophile attacks the positive carbonyl carbon leading to displacement of a leaving group. Each 1 g of chitosan was dissolved in 25 mL of 20% (v/v) aqueous acetic acid solution, with mechanical stirring, and then diluted with 50 mL of methanol. Molar equivalent 0.1 of palmitic anhydride was dissolved in 100 mL of methanol, and then added to the chitosan solution. The solution mixture was conducted at 80°C overnight and then mixed vigorously with 200 mL of acetone. The precipitated NPC was centrifuged and sequentially washed with acetone and diethyl ether to remove water and unreacted reagent. The NPC was freeze-dried before being used (Lee et al 2005).

Preparation of liposomes (PDL, PEGDL and NDL)

PDLs were prepared by the film dispersion method (Bangham et al 1965). Chloroform solutions of docetaxel, cholesterol and EPC (mass ratio = 1:3:25) were mixed and dried under vacuum. A thin film of dry lipid deposited on the inner wall of the flask was hydrated at 40°C with pH 7.4 phosphate-buffered saline (PBS). Then the liposome suspensions were passed through the homogenizer for five cycles at 1500 bar pressure to decrease the particle size. PEGDLs were prepared by the same method (mass ratio of docetaxel, cholesterol, EPC and PEG-DSPE = 1:3:25:5) NDLs were prepared by anchoring NPC on the surface of PDLs. NPC was dissolved with phosphate buffer (pH 6.5), then NPC of various concentrations was added dropwise to PDLs under magnetic stirring at room temperature (28°C) for 1 h. Coating parameters were optimized by measuring the change of zeta-potential of the liposomal suspension with a Zetasizer 3000. The 1-h incubation time of optimized lipid:NPC was determined in the same way; the ratio of lipid:NPC used for optimizing incubation times was 15%. The superfluous NPC was removed by centrifugation (18 000 rev min^{-1}, 4°C, 15 min). The NDLs for the following experiments were prepared according to the optimal coating parameters.

Characterization of the liposomes

Particle size and zeta-potential of liposomes were measured by a Zetasizer 3000 instrument. Samples for transmission electron microscopy (TEM) were prepared at room temperature by conventional negative staining methods using 0.3% phosphotungstic acid buffer (pH 6). Samples were viewed on an H-7000 model transmission electron microscope.

The concentration of docetaxel was determined by HPLC. The HPLC system was maintained at 28°C and detection was made by the UV detector at 229 nm. The composition of the mobile phase was acetonitrile–0.02 M ammonium acetate buffer (60:40 v/v). The mobile phase was delivered at a flow rate of 1 mL min^{-1} (Garg & Ackland 2000). The injection volume was 20 μL and relative retention time was found to be 7.9 min.

The entrapment efficiency of the various liposomes was measured by dialysis (Immordino et al 2003). The initial docetaxel concentration used for drug entrapment was 10 mg mL^{-1}. Free drug after dialysis was detected by HPLC. The liposomal suspension was diluted with acetonitrile for HPLC assay to get the total drug content. The drug content within the liposomes was calculated as the total drug content of the suspension minus the free drug. The entrapment efficiency was calculated as the ratio of drug content within the liposomes to the total drug content of the suspension. The entrapment efficiency upon dilution in HCL solution (pH 1.2) and phosphate buffer (pH 6.9) was also studied.

Physical stability of liposomes

Stability in PBS and FCS

Dialysis was used to evaluate the physical stability of liposomes. The 1-mL sample containing 1 mg docetaxel of PDLs, NDLs, PEGDLs or docetaxel injection was diluted 50-fold with pH 7.4 PBS or fetal calf serum (FCS), the 50-mL sample of the diluted liposomal suspension or docetaxel injection was placed outside of a cellulose membrane tubing, whose molecular weight cut-off was 8000–10000. The cellulose membrane tubing was pretreated by soaking in water overnight and washed with de-ionized water. Then the two ends of the tubing were tightened and 2 mL pH 7.4 PBS or FCS was placed in the bag. Finally the bag was soaked in 50 mL diluted sample. Experiments were carried out at 37°C for 48 h. At scheduled intervals, 25 μL of the release medium in the bag was collected for HPLC assay. The same volume of blank medium at the same temperature was added immediately. The docetaxel release percentage was calculated according to the equation:

$$\text{Drug release } (\%) = 52\ C_t / D_0 \times 100\% \quad (1)$$

where C_t and D_0 indicate the concentration (mg mL^{-1}) of drug released from liposomal suspension at each interval and the total amount of drug in liposomal suspension, respectively.

Long-term stability

The PDLs, PEGDLs and NDLs were kept in amber glass ampoules flushed with nitrogen and stored at 4°C for a period of 12 weeks. At scheduled intervals, the leakage profile was determined according to the measurement for entrapment efficiency, as mentioned above. The particle size and zeta-potential were measured simultaneously.

Pharmacokinetics

The animal study was approved by the China Pharmaceutical University. Experiments followed an approved protocol from the China Pharmaceutical University Animal Care and Use Committee. Pharmacokinetic studies were performed using male Sprague-Dawley rats. The PDLs, NDLs, PEGDLs or docetaxel injection was injected into the rats via the tail vein at a dose of 2.5 mg docetaxel per kg body weight. Less than 400 μL blood samples were obtained at various intervals after administration (5, 10, 15, 20, 30, 40, 50, 180, 240, 360 and 720 min). To each 200 μL of plasma sample, 3 mL ethyl acetate was added. Extraction was conducted by vortexing for 15 min and centrifugation (6000 rev min^{-1}, 4 °C, 10 min). The organic phase was transferred to a glass tube and evaporated to dryness under a gentle stream of nitrogen at 45 °C; eventually the residue was dissolved in 200 μL acetonitrile for HPLC assay. The standard curves ranging from 0.05 to 5 μg mL^{-1} were linear (r=0.9991). The precision and accuracy of this method were also satisfactory.

Pharmacokinetic parameters were determined using a log-linear trapezoidal method 3P97 (Mathematic Pharmacological Committee, Chinese Pharmacological Society, China). Systemic plasma clearance (Cl) was calculated as dose/$AUC_{t0\text{-}tinf}$. The volume of distribution (V_C) was the distribution volume of central compartment. Mean residence time (MRT) was derived from the equation $AUMC_{t0\text{-}tinf}$ /$AUC_{t0\text{-}tinf}$. The data were analysed for statistical significance by the *t*-test. All results were expressed as mean ± standard deviation (s.d.).

Statistical methods

In this study the difference between mean values was statistically examined using either the analysis of variance (one-way or repeated-measures) or the Kruskal–Wallis test (whenever n = 3). Following this, individual differences between means were identified using a post-hoc test (*t*-test or Dunn's test). In all cases $P < 0.05$ denoted significance.

Results and Discussion

Preparation and characterization of docetaxel liposomes

The structural characterization of NPC was determined by measuring FTIR. From FTIR spectra, there was no peak at 1740 cm^{-1}, which was indicative of an ester group confirming O-acylation. This could be explained by the fact that only N-acylation occurred in the reaction condition. The peaks at 2920 and 2850 cm^{-1} and 1650 and 1555 cm^{-1} were attributed to the alkyl chain and amide bond, respectively.

To optimize the procedure of NPC coating, the parameters studied included optimum incubation time and lipid-to-NPC ratio. The incubation time for coating was optimized by measuring the surface potential of the liposomes at different incubation times. It could be seen from Table 1 that the

Table 1 Optimization of NPC-to-lipid ratio (w/w) and NPC incubation time

NPC:lipid (w/w)	Zeta-potential (mV)	Incubation time (min)	Zeta-potential (mV)
0%	−23.4 ± 0.32	0	−7.31 ± 0.144
5%	10.2 ± 0.098	20	10.31 ± 0.087
10%	15.6 ± 0.192	40	18.3 ± 0.359
15%	18.5 ± 0.367	60	18.5 ± 0.401
25%	18.5 ± 0.359	90	18.6 ± 0.379
40%	18.7 ± 0.301	120	18.5 ± 0.391

The first measuring point is the value of zeta-potential measured as soon as possible after mixing NPC with PDL. Each point represents the mean ± s.d., n = 3.

change in surface potential after 1 h was negligible to nil ($P < 0.05$) and the optimal incubation time was 1 h. The NDLs for lipid-to-NPC ratio were prepared by incubating NPC with PDLs for 1 h; the zeta-potential increased as the NPC concentration increased to 15% (NPC:lipid) ($P < 0.05$), then it settled to a relatively constant value. The optimal ratio of lipid to NPC was 15%.

Addition of NPC to PDLs made the milky PDL suspensions change to filemot suspensions. Morphologically, the PDL was spherical in shape; however, the NDL was observed as irregularly shaped and opaque due to the NPC anchoring. The mean particle size of PDLs, PEGDLs and NDLs was 219 nm, 223 nm and 231 nm, respectively.

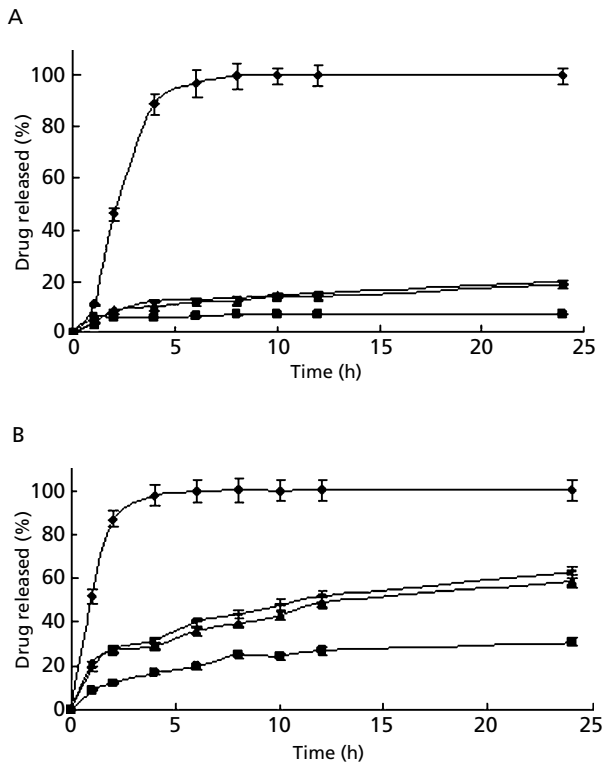

Figure 2 Dynamic release profiles of docetaxel injection (◆), PDLs (−), PEGDLs (▲) and NDLs (■) in pH 7.4 PBS (A) and FCS (B). Each point represents the mean ± s.d., n = 3.

Docetaxel entrapment efficiency

The docetaxel entrapment efficiency in pH 7.4 PBS was 96.4 ± 0.21% and 96.1 ± 0.48% with respect to PEGDL and PDL. However, the addition of NPC decreased the entrapment efficiency to 94.2 ± 0.11% ($P < 0.05$); the entrapment efficiency of liposomes in pH 6.9 PBS and pH 1.2 HCl had the same trend. Relatively high entrapment efficiency could be attributed to the lipophilic nature of docetaxel – it tends to interact with the hydrophobic domain of lipid membranes. According to the hydrophobicity of docetaxel, the presence of a hydrophobic and rigid molecule, i.e. the palmityl chain from NPC, in the docetaxel liposomes had a negative effect on entrapment efficiency.

Stability study in PBS and FCS

PDLs, PEGDLs and NDLs were evaluated for physical stability in PBS and FCS at 37°C over 24 h. The released drug was separated by dialysis and release properties were measured as the accumulative release percentages. In the experimental protocol, the docetaxel was released promptly from docetaxel injection either in PBS or in FCS. This showed that the docetaxel could pass through the cellulose membrane tubing freely.

PDLs and PEGDLs released nearly 20% of their initial drug content over the period; release of docetaxel from NDLs was less than 10% (Figure 2A) ($P < 0.05$). These data showed that NDLs retarded the release of entrapped drug effectively in comparison with PEGDLs and PDLs in PBS. Release of docetaxel from NDLs, PEGDLs and PDLs in FCS at 37°C was faster than in PBS, but the drug release profiles followed the same trend in the FCS (Figure 2B) as in PBS; the mean amount of docetaxel released from NDLs after 24 h was 31 ± 1.52%, which is obviously lower than 59 ± 2.18% from PEGDLs and 63 ± 1.98% from PDLs ($P < 0.05$). From the two figures, the release record of NDL was significantly lower than PEGDL or PDL.

It is known that docetaxel is insoluble in water, so docetaxel existed in phospholipid bilayer membranes. NPC may interact with the polar head groups on the phospholipid bilayers via electrostatic attraction and hydrophobic interaction. The structured absorbed polymer film was supposed to stabilize the particles. Through the analysis of measured released profiles, the comparatively lower release of docetaxel in the case of NDLs could be ascribed to the more stable outer surface of the particles.

Long-term stability

Long-term stability of the liposomes was examined by measuring the entrapment efficiency, particle size and zeta-potential as a fuction of time at 4°C.

PEGDLs and PDLs retained 85.5 ± 1.11% and 84.4 ± 0.59% of their initial drug content, respectively, after 2 weeks of storage (Figure 3A), while NDLs retained 94.1 ± 1.64% ($P < 0.05$). At the end of 12 weeks of storage at 4°C, PEGDLs

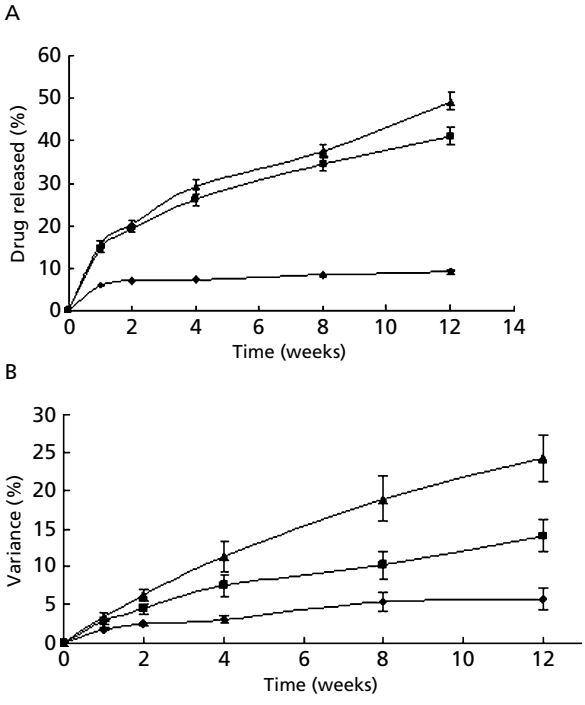

Figure 3 The leakage of docetaxel in PDLs (▲), PEGDLs (■) and NDLs (◆) (A) and variance profiles of vesicle size to PDLs (▲), PEGDLs (■) and NDLs (◆) (B) during long-term storage. Each point represents the mean ± s.d., n = 3.

and PDLs recorded 41.1 ± 1.45% and 49.2 ± 0.98% leakage, respectively, whereas NPDs only showed 9.1 ± 0.69% leakage ($P < 0.05$).

During the long-term stability experiments, the particle size was observed to have changed (Figure 3B); a similar trend was found for NDLs. During storage no appreciable variation of the NDL zeta-potential was observed, but the zeta-potential of PEGDLs and PDLs changed appreciably (data not shown). These results were well anticipated and appreciated due to the stability of the lipid bilayer membranes interpolated with NPC; the structured adsorbed polymer film was supposed to stabilize the particles against particle–particle interaction presumably by the mechanism of steric stabilization (Kellaway & Najib 1981). From these results it was hoped that NPC, as a new polymeric membrane to anchor liposomes, would help to solve the stability problems of antitumour liposomes.

Pharmacokinetics in rats

After the intravenous injection of docetaxel, a rapid clearance of the drug from the systemic circulation was observed during the first 20 min (Figure 4). This was followed by a slower, more steady, decrease; the docetaxel concentration in the plasma was below the lowest determination line (0.05 μg) after 240 min. Encapsulation of docetaxel in liposomes produced a significant change in drug pharmacokinetic parameters. After bolus administration, the docetaxel liposome formulations were more slowly removed from the circulation. NDLs

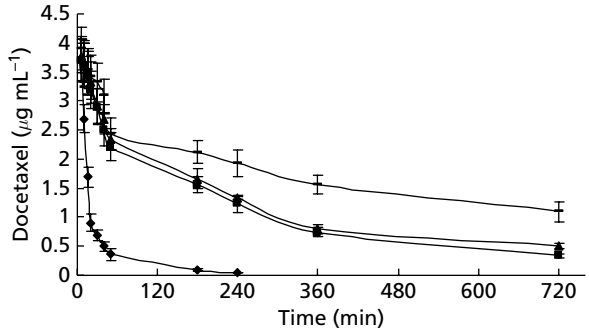

Figure 4 Plasma concentration–time profiles in rats after intravenous administration of docetaxel injection (◆), PDLs (■), PEGDLs (—) and NDLs (▲). Each point represents the mean ± s.d., n = 5.

exhibited concentrations higher than PDLs, but noticeably lower than those obtained after the administration of PEGDLs. Results of the pharmacokinetic data showed that an open two-compartment model was fitted to all administrations: docetaxel injection, PDLs, PEGDLs and NDLs.

The main pharmacokinetic parameters of intravenous administration of different docetaxel formulations are shown in Table 2. Encapsulation of docetaxel in liposomes was applied as an approach to decrease the elimination clearance. Docetaxel liposomes resulted in a significant increase in the AUC, MRT, $t½_{\alpha}$ and $t½_{\beta}$ ($P < 0.05$), which correlated with a significant decrease in the Cl ($P < 0.05$). These results were ascribed to the protection of the lipid bilayer membranes and slow drug release from liposomes. In addition, the V_C of liposomes was larger than that of free docetaxel, although statistically not significant ($P > 0.05$). The volume of distribution showed that docetaxel encapsulated in liposomes was restricted to the systemic circulation, the larger V_C indicating the initial low drug concentration of liposome groups. The initial low drug concentration in the liposome group could be due to the uptake of liposomes by the mononuclear phagocyte system (Zhixuan et al 2006).

The increments of AUC, MRT, $t½_{\beta}$ and Cl could be obtained from NDLs or PEGDLs as compared with PDLs ($P < 0.05$), but the increments for PEGDLs were significantly higher than for NDLs ($P < 0.05$). Inhibition of the rapid uptake of liposomes by the reticulo-endothelial system (RES) and reduction of the rate of drug leakage have resulted in PEGDLs with valuable pharmacologic properties, which are typically related to the so-called long-circulating or stealth liposomes (Oku & Namba 1994; Gabizon et al 1997; Kim et al 2003a). While NDLs only reduced the rate of docetaxel leakage from the liposomes in-vivo by anchoring NPC to change docetaxel pharmacologic properties, the mechanism of prolonged circulation time of docetaxel in NDLs is to stabilize the lipid bilayer membranes, which is similar to the effect of O-palmitoyl amylopectin anchored liposomes (Cheng et al 2006).

Conclusion

In this paper, we describe the feasibility of using N-palmitoyl chitosan as a new polymeric membrane to anchor docetaxel

Table 2 Mean pharmacokinetic parameters of docetaxel after intravenous administration of docetaxel injection, PDLs, PEGDLs and NDLs

Parameters	Injection	PDLs	NDLs	PEGDLs
AUC ($(\mu g\ mL^{-1}\ min)$)	115.563 ± 12.143	913.790 ± 122.347*	1117.557 ± 115.143*#	2185.336 ± 328.581*#†
MRT (min)	50.069 ± 2.12	328.840 ± 29.95*	446.759 ± 48.98*#	816.045 ± 121.54*#†
V_C (L)	0.0752 ± 0.0178	0.120 ± 0.0168	0.124 ± 0.012	0.119 ± 0.0189
Cl ($L\ min^{-1}$)	0.00456 ± 0.000337	0.000563 ± 0.0000609*	0.000455 ± 0.0000528*	0.000227 ± 0.0000320*#†
$t^1/_{2\alpha}$ (min)	5.853 ± 0.931	20.694 ± 3.043*	34.080 ± 3.919*#	23.042 ± 2.097*†
$t^1/_{2\beta}$ (min)	66.863 ± 10.899	253.632 ± 35.762*	361.996 ± 54.659*#	598.708 ± 74.341*#†

Each value is the mean ± s.d., n = 5. *$P < 0.05$, compared with docetaxel injection; #$P < 0.05$, compared with PDLs; †$P < 0.05$, compared with NDLs.

liposome. In in-vitro studies, we demonstrated that the NPC anchored liposomes could increase the stability of docetaxel in dispersal medium and biofluid as compared with plain liposomes and PEGylated liposomes, especially during long-term storage. However, the stability profiles of NDLs, PEGDLs and PDLs in-vivo were not in accordance with this. These findings suggest that anchored liposomes could increase the stability of docetaxel in-vivo as compared with plain liposomes, but that the change was not more significant than for PEGylated liposomes. These results were correlated with reduction in the rate of docetaxel leakage from the liposomes. Subsequent to these observations, investigations on NPC anchored liposomes are ongoing to elucidate the cytotoxicity of NPC and the mechanism of stabilization. N-Palmitoyl chitosan as a new polymeric membrane to anchor liposomes will help to solve the stability problem of liposomes containing anti-tumour drugs.

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