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Effect of surface ligand density on cytotoxicity and pharmacokinetic profile of docetaxel loaded liposomes

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ABSTRACT

Various biotin-modified liposomes incorporated with docetaxel (DTX) were prepared to study the effect of surface biotin density on the pharmacokinetic profile of the liposome. Four types of liposomes such as PEG modified liposome (PDL), 0.5% (mol) biotin modified liposome (0.5BDL), 1% (mol) biotin modified liposome (1BDL) and 2% (mol) biotin modified liposome (2BDL) were prepared using thin film dispersion method. The prepared liposomes were characterized by measuring encapsulation efficiency (EE), particle size, Zeta-potential, physical stability and drug release profiles *in vitro*. MTT assay was performed to elevate the cytotoxicity of liposomes on MCF-7 cells. *In vivo* evaluation was further performed to investigate the effect of biotin surface density on the pharmacokinetic profiles. All the prepared liposomes exhibited high encapsulation efficiency, small particle size, narrow particle distribution and sustained release profiles *in vitro*. In MTT assay, 0.5BDL showed largest tumor cell toxicity, compared with DTX solution. All liposomes containing DTX showed prolonged blood circulation *in vivo*, and 0.5BDL showed the longest circulation time among the biotin modified liposome. Surface modification of liposome had a negative impact on the circulation of liposomes in the blood, which needs to be considered when designing the ligand mediated targeting delivery systems. A proper amount of biotin liposome with 0.5% molar ratio is expected to produce the best anti-tumor effect.

1. Introduction

Docetaxel, a second-generation semi-synthetic taxane derivative has shown dramatic antitumor activities, mostly against various human cancers such as ovarian carcinoma, advanced breast cancer, lung cancer and head/neck cancer [1]. Currently, docetaxel is formulated using Tween80 and ethanol (50:50,v/v) as solvent; however, its clinical use is limited due to its side effects related to formulation, such as neutropenia,

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peripheral neuropathy and hypersensitivity reactions [1,2]. Therefore, there is a strong rationale for reformulating docetaxel using a safer vehicle than Tween80.

Recently, many carriers have been studied, such as nanoparticle-aptamer, bioconjugates [3], DTX loaded liposomes [4], pegylated liposomes [4,5], N-palmitoyl chitosan anchored DTX liposomes [6], and pegylated immunoliposomes [7]. Liposomes with spherical lipid bilayer structures are one of the most successful drug carriers in drug delivery systems; however, they are prone to be taken up by reticuloendothelial system (RES) cells in liver and spleen [8,9]. As is well known that PEG modified liposomes exhibit a long circulation property in the blood and accumulate in tumor *via* passive targeting [4,10,11], increasing evidence has suggested that the selectivity of PEG modified liposomes is far from satisfaction. Therefore, many researchers have been focusing on developing active targeting drug delivery systems, in which many ligands have been introduced to the surface of drug carrier, such as folic acid [12], antibody [13] and integrin $\alpha_v\beta_3$ [14]. Biotin, a member of the vitamin family (vitamin H), is a growth promoter of cells. Its ligand in cancerous tumors is higher than in normal tissue [15] because rapid proliferation of cancer cells requires extra biotin. Some cancer cells including JC, Colo-26, P815 and MCF-7 overexpress biotin-specific receptors [16,17], which are responsible for the uptake of essential nutrients such as biotin, lipoate, pantothenate [18] and peptides [19]. Biotin has been considered as a promising ligand for active targeting [16,20–22]; Yang et al. prepared biotin–dendrimer conjugate, which exhibited much higher cellular uptake into Hela cells than the dendrimer without biotin modification [23]. Biotinylated pullulan acetate nanoparticles was prepared by Na, which has been shown strong adsorption to the HepG2 cells [24]. Biotin-conjugated polymeric micelles could effectively release doxorubicin in acidic tumor cells compared to that without biotin [25].

However, the effect of biotin modification and density on cytotoxicity and pharmacokinetic profiles of the carriers has not been explored to date. Previous studies have shown that the cellular uptake increased with the increase of ligand density on the surface of particles [26–28]. However, dense surface coverage of ligand may not produce expected improvements in cellular uptake [29,30]. Moreover, some studies have shown that the insertion of ligand resulted in faster clearance of liposomes from plasma, which compromised the accumulation of liposomes in tumor via EPR effect [31,32]. It was reported that the 2.56 mol% NGR (asparagine–glycine–arginine amino acid sequence) resulted in lower total tumor accumulation than the formulation with only 0.64 mol% NGR [33].

As known, the success of an active targeting strategy relied heavily on the accumulation of the carriers at tumor site via passive targeting. If the drug loaded carriers were quickly eliminated *in vivo*, the total tumor accumulation of carriers will be decreased. The pharmacokinetic profiles of the active targeting formulation are important factors that need to be considered to achieve successive active targeting.

The researches on the effect of biotin density of liposome on cytotoxicity and pharmacokinetic profiles were absent. In this study, biotin was conjugated to PEG chains on the surface of liposomes containing DTX, in an attempt to improve cancer targeting. DTX loaded liposomes modified with different biotin density were prepared to investigate the effect of biotin density on the cytotoxicity and the pharmacokinetic profiles of liposomes in blood. This study will lay a foundation for optimization of liposome formulation for further *in vivo* evaluation.

2. Materials and methods

2.1. Materials

Docetaxel was purchased from Jiansu Hengrui Medicinal Co., Ltd (Jiangsu, China). Cholesterol (Chol) was purchased from Tianjin Bodi Chemical Industry Co. (Tianjin, China). Soy phosphatidylcholine (Spc) was purchased from Shanghai Taiwei Pharmacetical Co. (Shanghai, China). Dichloromethane was purchased from Tianjin Jingxi Chemical Industry Co. (Tianjin, China). Disteroylphophatidyl ethanolamine methoxypolyethylene glycol conjugate (DSPE-PEG_{2000}) was purchased from Nippon Fine Chemical Co., Ltd (Kobe, Japan) and Biotin-PEG_{2000}-DSPE was purchased from Creative PEG Works Co. (Winston-Salem, NC, USA). Methanol, acetonitrile and tert-butyl methyl ether were of high-performance liquid chromatography (HPLC) grade, all other regents and solvents used were of analytical grade. Sprague–Dawley rats (200 ± 20 g) were supplied by the Laboratory Animal Center of Shenyang Pharmaceutical University (Shenyang, China). All animal procedures were approved by the Animal Ethics Committee of Shenyang Pharmaceutical University.

2.2. Preparation of liposomes

Liposomes were prepared by thin-film hydration method. Briefly, to prepare PEG modified liposome (PDL), Spc Chol (Spc: Chol, 9:2, molar ratio) and DSPE-PEG (4% mol) as well as DTX (1:18, mass ratio) were first dissolved in dichloromethane solutions. The mixture was placed in a round-bottomed flask and the solvent was removed by rotary evaporation at 37 °C under vacuum to obtain the dry film. Afterward, the film was hydrated with pH 7.4 PBS buffer in a water bath at 40 °C with stirring for 20 min until a homogenous liposome suspension was obtained. The suspension was then sonicated with a ultrasonic cell disruptor, and the pulse function was 220w, on 1 s and off 1 s for 2 min. Polycarbonate filters, with a pore size of 220 nm were used to decrease the size of liposomes. The liposomes were purified by centrifugating at 15 000 × *g* for 15 min. Biotin modified liposomes (0.5BDL, 1BDL, 2BDL) were prepared using the same method mentioned above except that 0.5%, 1% and 2% (mol) of DSPE-PEG_{2000} were used respectively.

2.3. Characterization of liposomes

The DTX concentration was determined by high performance liquid chromatography (HPLC). In detail, the analysis was performed with a Hitachi HPLC system (UV Detector L-2400, Pump L-2130, Hitachi, Tokyo, Japan), equipped with a 20 μl loop and a reversed-phase column (Hypersil, ODS, 4.6 × 250 mm, 5 μm). The mobile phase was made up of acetonitrile and water (60:40; v/v) at a flow rate of 1 ml/min, and the DTX was detected at a wavelength of 228 nm. Methanol was used before analysis as a demulsifier. The encapsulation efficiency (EE%)

and drug loading content (LC%) were calculated using equation 1 and equation 2:

$$EE\% = \left(\frac{W_e}{W_{add}}\right) \times 100\% \quad (1)$$

$$LC\% = \left(\frac{W_e}{W_t}\right) \times 100\% \quad (2)$$

where, W_e is the amount of drug encapsulated, W_{add} is the amount of drug used and W_t is total amount of encapsulated drug and liposome.

The diameter and Zeta potential of the liposomes were determined by Zetasizer3000 (Malven Instruments Ltd., UK). Intensity autocorrelation was measured at a scattering angle of 90 degrees at room temperature.

The morphological feature of the liposomes was observed with a transmission electron microscope (TEM) (JEM-1200EX, JEOL Ltd., Japan) at 80 kV. A drop of liposomal sample was placed on a copper grid, excess water was blotted with a piece of filter paper and a drop of 1% phosphotungstic acid was added for negative staining.

Initial stability of liposomes was evaluated by detecting leakage of DTX from liposomes at 4 °C. Encapsulation efficiency of four liposomes was detected by HPLC at 1, 2, 3, 4, 5, 7, 10, 13 and 15 days after preparation. The leakage rate (*LR%*) was calculated using equation 3:

$$LR\% = 1 - \left(\frac{EE_n}{EE_1}\right) \times 100\% \quad (3)$$

where EE_n is the encapsulation efficiency detected at n day, and EE_1 is the encapsulation efficiency detected at the first day.

In vitro release of DTX from liposomes was determined using dialysis method with a pharmaceutical dissolution tester at 37 °C. The samples were put into dialysis bags (COMW: 8000–14,000), and PBS (80 ml, PH7.4) containing 0.5% (v/v) Tween80 was used as the release medium. Tween80 was added to ensure the sink condition. The stirring speed is 50 rpm. At each time point, 2 ml release medium was withdrawn and replaced with fresh PBS. The concentration of DTX in the release medium was determined using HPLC. The mobile phase was made up of acetonitrile and water (52:48, v/v) at a flow rate of 1 ml/min.

2.4. Cytotoxicity in vitro

The human breast cell line MCF-7 (purchased from Shanghai Fuleibao Bio-Tech Co., Ltd) was maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and 0.1% antibiotics (penicillin streptomycin) in a 5% CO_2 humidified atmosphere at 37 °C. The MTT assay was used to test the cytotoxicity *in vitro*; the cells were seeded at 8×10^3 cells/well in 96 well plates and incubated for 24 h. The cells were then incubated in 96 well plates at 37 °C for 24 h, 48 h, and 72 h in the presence of a series of concentration of formulations, including DTX solution (FD), PDL, 0.5BDL, 1BDL, and 2BDL. The cells incubated in medium without any drug or liposomes were used as controls. 20 μl of MTT (5 mg/ml) were added at the end of incubation period. The plates were incubated for an additional 4 h. DMSO (150 μl) were added to dissolve the formazan crystals and the absorbance value was determined at wavelengths of 490 nm.

2.5. Pharmacokinetic studies

SD rats were divided into five groups randomly (three rats per group). PDL, 0.5BDL, 1BDL, and 2BDL were i.v. administration at a dose of 10 mg/kg and DTX in tween80 (FD) was chosen as control group. Blood samples were collected at 5 min, 10 min, 15 min, 30 min, 45 min, 1 h, 2 h, 4 h, 8 h, 12 h and 24 h. The samples were then centrifuged at 5000 rpm for 10 min to separate the plasma; 10 μl of paclitaxel (10 μg/ml) used as internal standard was added into 200 μl of plasma. The mixture was vortexed for 30 s twice to mix well, and then 2 ml of tert-butyl methyl ether was added. Then the mixture was sonicated for 3 min, and vortexed for 5 min. Clear supernatant was obtained by centrifugating at 10,000 rpm for 5 min. The supernatant was dried with nitrogen gas and reconstituted in 100 μl of methanol. The concentration of DTX in blood was measured using HPLC. A reversed-phase column (Hypersil, ODS, 4.6 × 250 mm, 5 μm) was used. The mobile phase was made up of acetonitrile and water (57:43, v/v) at a flow rate of 1 ml/min. The DTX detection was performed at a wavelength of 228 nm. Pharmacokinetic parameters were determined using the software DAS2.0. The significance of the difference was analyzed by ANOVA models with Statistical Program for Social Sciences (SPSS 11.0) and the significant level was set at 0.05.

3. Results and discussion

3.1. Characterization of liposomes

Encapsulation efficiency (EE%), drug loading content (LC%), mean diameter with poly dispersion index (PDI) and zeta potential of four liposomes were displayed in Table 1. It could be seen that incorporation of Biotin-PEG_{2000}-DSPE into the membrane increased the mean diameter and slightly decreased the Zeta potential of the liposomes. Morphology of four liposomes observed by TEM is shown in Fig. 1. The double

Table 1 – Characterization of four kinds of liposomes (n = 3).

	PDL	0.5BDL	1BDL	2BDL
EE (%)	94.60 ± 1.889	96.19 ± 2.515	91.92 ± 2.604	88.11 ± 2.079
LC (%)	3.950 ± 0.237	4.001 ± 0.321	3.808 ± 0.425	3.613 ± 0.385
Diameter (nm)	97.99 ± 14.32	133.2 ± 4.644	135.1 ± 3.570	145.0 ± 5.015
PDI	0.266 ± 0.048	0.280 ± 0.007	0.323 ± 0.003	0.264 ± 0.032
Zeta potential (mv)	−30.00 ± 1.150	−25.23 ± 0.473	−24.20 ± 1.212	−20.40 ± 0.361

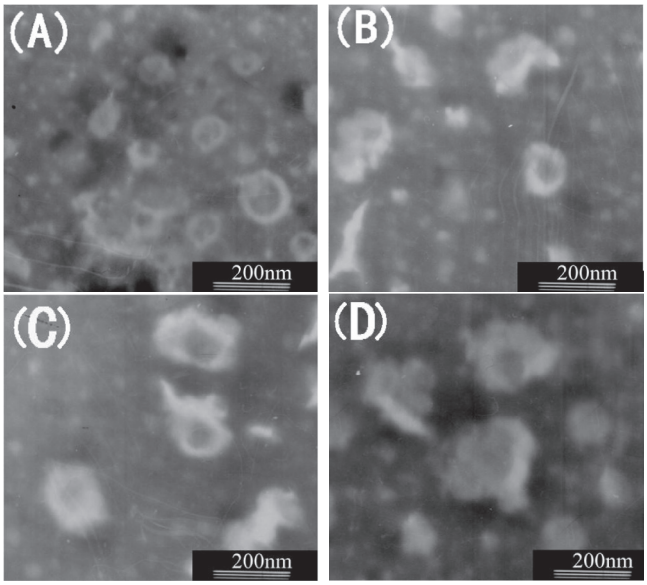

Fig. 1 – Transmission electron microscopy of PDL (A); 0.5BDL (B); 1BDL (C) and 2BDL (D) (×40,000).

membrane of the liposomes could be seen clearly and no drug crystal was visible. The leakage rate (Fig. 2) of all liposomes was less than 25% at 4 °C in the hydrated state for 15 days. The release behavior of DTX from four liposomes and free DTX in the release medium were shown in Fig. 3. The four liposomes (PDL, 0.5BDL, 1BDL, 2BDL) released 69.12 ± 9.98%, 57.44 ± 0.37%, 54.49 ± 6.58% and 52.37 ± 3.62% of DTX respectively at 96 h in comparison with free DTX (96.15 ± 0.48% at 6 h), indicating that four liposomes had a sustained release profile.

Docetaxel is a potent anticancer drug and its use is restricted by its poor aqueous solubility where addition of Tween80 to enhance DTX solubility was associated with its side effects [1]. Liposomes are capable of increasing the aqueous solubility of DTX. Moreover, biotin was used to modify the liposomes with an aim to increase the DTX accumulation in tumor site. All the prepared liposomes had high encapsulation efficiency and drug loading rate. Mean diameter of liposomes increased with the incorporation of Biotin-PEG_{2000}-DSPE due to

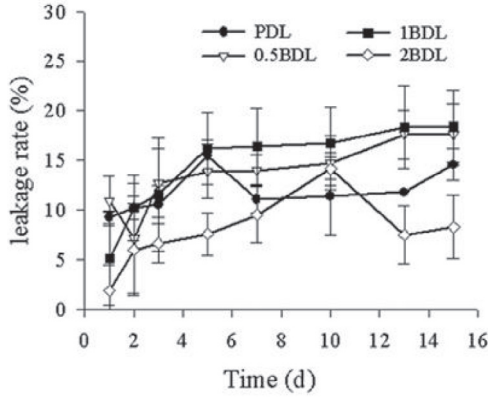

Fig. 2 – Leakage rate of four kinds of liposomes at 4 °C (n = 3).

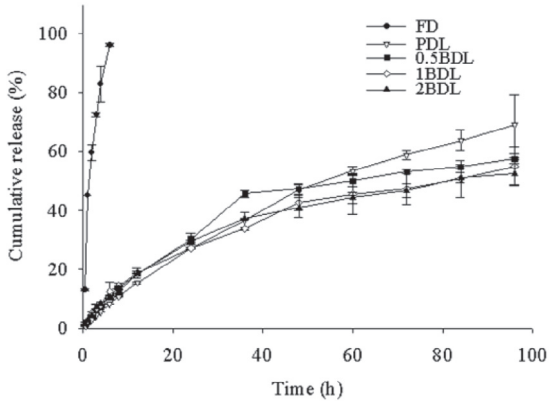

Fig. 3 – Drug release from FD and four kinds of liposomes in PBS (PH 7.4) containing 0.5% Tween80 (n = 3).

the interaction of Biotin at the end of Biotin-PEG_{2000}-DSPE and the swelling resulting from the hydrophilic property of biotin [24]. The particulate carrier systems with a diameter larger than 200 nm are known to induce nonspecific scavenging by monocytes and the reticuloendothelial system (RES) [34,35]. It was reported that some tumor vessels could cause extravasation of particulates with a diameter less than 400 nm [5,36]. The liposomes with a diameter around 100 nm extravasated much easier than bigger ones with a diameter ranging from 200 to 400 nm [37,38]. The liposomes prepared in this study were found to have a diameter between 97.99 ± 14.32 nm and 145.0 ± 5.015 nm, which were not expected to be removed by the RES. Biotinylation of liposome did not markedly change the Zeta potential; although biotin is known to possess a positive charge, the result is in agreement with an earlier study [39].

The therapeutic effect of drug in carriers is highly dependent on the release rate of the drug from the carrier. If the drug leaks from the carrier too rapidly, the carrier will lose most of the loaded drug before it reaches the diseased site, leading to the compromised therapeutic effect. In our study, we compared the DTX release behavior of FD and all liposomes. As shown in Fig. 3, four liposomes released DTX slower than FD. The sustained release of DTX from liposomes was probably attributed to the encapsulation by the bilayer membrane of liposomes.

3.2. *MTT assay*

Cell viability of 24 h, 48 h, and 72 h after adding empty liposome (EL), FD, PDL, 0.5BDL, 1BDL and 2BDL are shown in Fig. 4(A, B, C). IC_{50} was calculated and the results were shown in Table 2. The IC_{50} of liposomes were higher than FD ($P < 0.01$) after 24 h and 48 h incubation. However, 0.5BDL showed lower IC_{50} than FD at 72 h ($P < 0.01$). Compared with 0.5BDL, both 1BDL and 2BDL showed significantly higher IC_{50} ($P < 0.01$) and there is no significant difference between 1BDL and 2BDL ($P > 0.05$). The result suggested that empty liposome has no inhibition effect on cell growth, and cell viability decreased with increasing of the concentration of DTX. For all liposomes and FD, IC_{50} decreased with the increasing of incubation time.

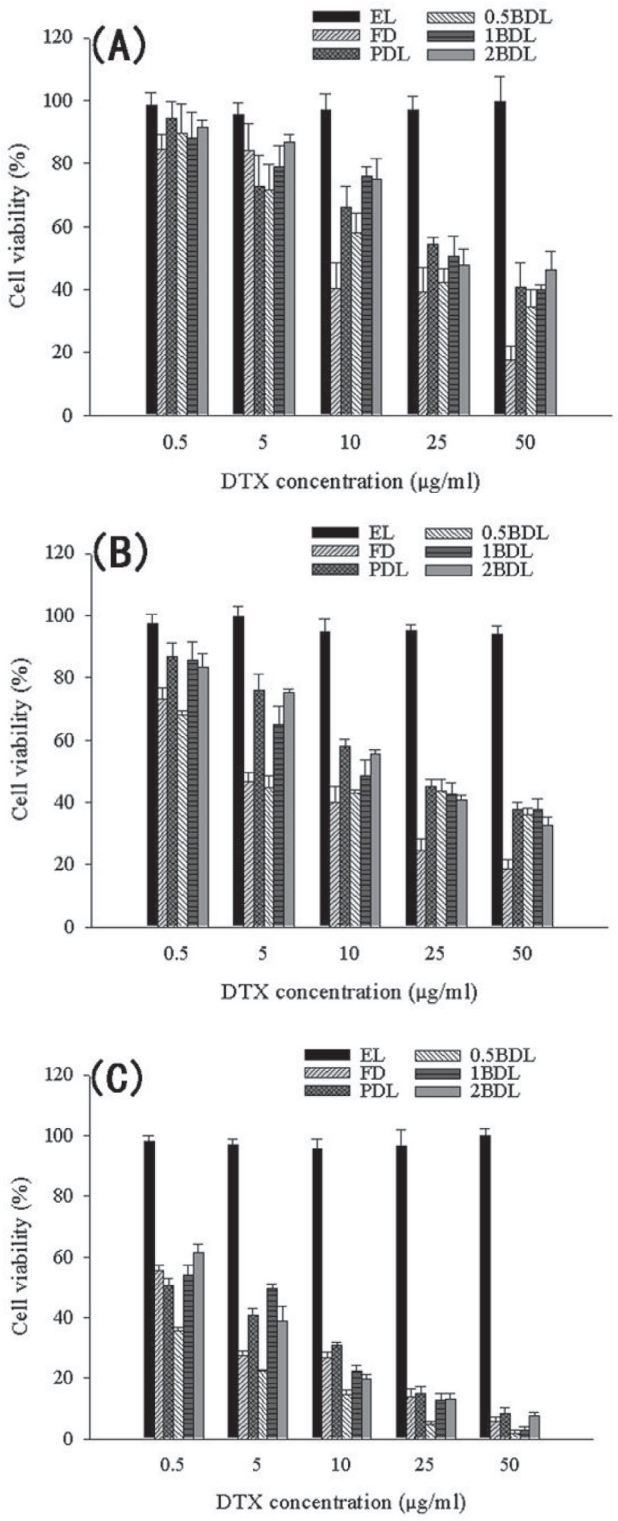

Fig. 4 – Viability of MCF-7 after incubation with FD and four kings of liposomes. Data are presented as Mean ± SD (n = 5–6).

It was found that drug concentration and exposure time were closely related to cytotoxicity of all formulations. This result was in agreement with previous research [40,41]. The higher cell viability of liposomes than FD at 24 h and 48 h is probably related to the double membrane of liposome and the steric effect of PEG chains, which, first, can inhibit the release of drug from carriers, and second, can prevent liposomes from interacting with cells. In addition, liposomes may delay internalization of drug in cells due to the negative charge on the surface of liposomes. Liposomes with negative charge generally exhibit stronger binding than neutral ones because of the existence of a membrane receptor recognizing negatively charged particles [42]. Fig. 3 suggested that among liposomes, PDL had the highest drug release rate, which induced the highest cytotoxicity at 24 h. The biotin receptor on the surface of MCF-7 may play a role in uptaking the liposomes into the cells and causing the cytotoxicity. It was found that IC_{50} of 1BDL and 2BDL showed no significant difference in the cytotoxicity. But IC_{50} value of 0.5BDL is nearly two times and three times lower. Higher biotin density on the liposomes failed to show higher cytotoxicity. This is consistent with the findings reported by other research groups [43,44]. This might be attributed to the internalized ligand molecules leading to a down-regulation or 'shut-off' of the receptor recycling system. The ligands conjugated liposomes may contribute to the intracellular ligand concentration and are therefore responsible for the saturation and 'shut-off' of the receptor uptake pathway. Liposomes with more targeting ligands would lead to more intracellular ligand content than those with less targeting ligands. This could result in a decreased cytotoxicity when more ligands are utilized [43]. An alternative explanation is the possible existence of DSPE-PEG_{2000}-Biotin micelles formed at higher number of targeting ligand. The biotin modified micelles would compete with the receptors and prevent biotin modified liposomes binding to the receptors.

3.3. *Pharmacokinetic studies*

Mean plasma concentration–time profiles of DTX after i.v. administration of DTX solution and four liposomes at a dose of 10 mg/kg was shown in Fig. 5. The main pharmacokinetic parameters were summarized in Table 3. When DTX was encapsulated in liposomes, the pharmacokinetic parameters were clearly different from those of FD. The AUC and the MRT were significantly increased, and the plasma clearance (CL) was reduced significantly. The AUC of liposomes (PDL; 0.5BDL; 1BDL and 2BDL) were 10.86 times, 4.456 times, 2.689 times and 3.976 times higher than FD (3.791 ± 1.375 mg/l·h). The MRT of liposomes (PDL,0.5BDL, 1BDL and 2BDL) were 14.89 times, 4.755 times, 1.973 times and 1.851 times higher than FD (3.573 ± 2.121h). In comparison with FD, CL of liposomes were 0.087 times, 0.208 times, 0.234 times and 0.352 times smaller. Among liposomes PDL presented the longest MRT, the largest AUC and the lowest CL, indicating a long circulation time. Compared with PDL, the biotin modified liposomes with different density exhibited a relatively smaller AUC, MRT and larger CL. Among the biotin modified liposomes, 0.5BDL showed best stability in blood circulation and a higher chance to exert biotin mediated endocytosis.

The pharmacokinetic data are shown in Table 3. It can be observed from Table 3 that the biotin modified liposomes presented shorter circulation time and smaller AUC and MRT. Among the biotin modified liposomes, 0.5BDL showed relatively longer circulation time, larger AUC and MRT. Biotin-PEG_{2000}-DSPE chains may cover parts of DSPE-PEG_{2000}, leading to less protection effect of PEG. In addition, biotin as a ligand at the end

Table 2 – IC_{50} value of FD and four kinds of liposomes (n = 5–6).

IC_{50}(μg/ml)					
Time(h)	FD	PDL	0.5BDL	1BDL	2BDL
24	11.04 ± 2.335	27.25 ± 1.634[a,b]	17.38 ± 2.989[a,b]	36.87 ± 3.805[a,b,c]	43.89 ± 2.980[a,b,c]
48	3.595 ± 0.350	21.51 ± 2.293[a,b]	5.278 ± 0.530[b]	14.53 ± 2.322[a,b,c]	16.07 ± 1.328[a,b,c]
72	0.964 ± 0.086	0.981 ± 0.080[b]	0.411 ± 0.062[a,b]	1.385 ± 0.137[a,b,c]	1.338 ± 0.189[a,b,c]

[a] $P < 0.01$ (compared with FD).
[b] $P < 0.05$ (compared with PDL).
[c] $P < 0.05$(compared with 0.5BDL).

Table 3 – Summary of the pharmacokinetic parameters of FD and four kinds of liposomes after i.v. administration in rats at dosage of 10 mg/kg (n = 3).

Parameters	FD	PDL	0.5BDL	1BDL	2BDL
$t_{1/2z}$ (h)	2.492 ± 1.996	40.52 ± 8.143[a]	14.36 ± 6.636[a,b]	6.409 ± 0.394[a,b,c]	5.911 ± 2.105[a,b,c]
AUC(0-t)(mg/l·h)	3.179 ± 0.532	16.07 ± 3.937[a]	12.35 ± 1.683[a]	14.13 ± 1.202[a]	9.515 ± 1.426[a,b]
AUC(0-∞)(mg/l·h)	3.791 ± 1.375	40.396 ± 9.329[a]	16.57 ± 1.484[a,b]	14.76 ± 1.111[a,b,c]	10.00 ± 1.873[a,b,c]
MRT(0-t) (h)	2.298 ± 0.154	8.379 ± 1.348[a]	6.649 ± 0.657[a,b]	5.866 ± 0.293[a,b]	5.307 ± 0.438[a,b]
MRT(0-∞) (h)	3.573 ± 2.121	53.22 ± 13.86[a]	16.98 ± 6.219[a,b]	7.051 ± 0.089[a,b,c]	6.614 ± 1.323[a,b,c]
Vz (l/kg)	8.720 ± 4.055	14.99 ± 4.475	12.49 ± 5.852	6.311 ± 0.886	8.442 ± 1.848
CLz (l/h/kg)	2.911 ± 0.909	0.256 ± 0.054[a]	0.606 ± 0.052[a,b]	0.680 ± 0.005[a,b]	1.025 ± 0.204[a,b,c]
Cmax (mg/l)	2.497 ± 0.371	13.23 ± 3.158[a]	13.69 ± 2.105[a]	13.86 ± 4.260[a]	13.36 ± 2.463[a]

[a] $P < 0.05$ (compared with FD).
[b] $P < 0.05$ (compared with PDL).
[c] $P < 0.05$ (compared with 0.5BDL).

of the PEG chains may be recognized by cell membrane receptor in blood and result in faster elimination from blood. Previous studies suggested that nanoparticles should be small, slightly negatively charged and covered with a protective PEG layer to achieve passive targeting [11]. Our study further indicates that the surface density of ligand will have a negative impact on the blood circulation of nanocarriers *in vivo*. Studies on tumor-bearing rat need to be further investigated to elucidate the active targeting efficiency.

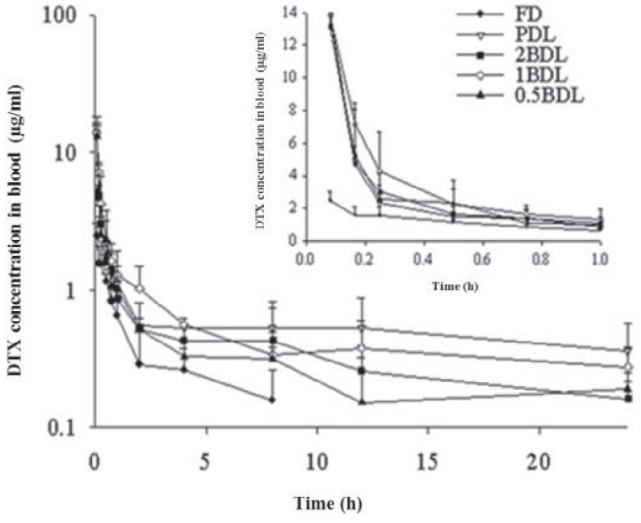

Fig. 5 – Mean plasma concentration–time profiles of DTX after i.v. administration of DTX solution and four liposomes at a dose of 10 mg/kg (n = 3).

4. Conclusions

The biotin ligand density on the surface of liposome has an impact on the cytotoxicity and pharmacokinetic of liposomes. The cytotoxicity of biotin conjugated liposomes decreased with an increase in biotin surface density. The elimination of biotin conjugated liposomes from blood was increased with increasing of biotin surface density. The ligand density of the active targeting liposomes needs to be optimized to achieve successful targeting.

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