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Research Article

Sustained Liver Targeting and Improved Antiproliferative Effect of Doxorubicin Liposomes Modified with Galactosylated Lipid and PEG-Lipid

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Abstract. In this study, a cleavable PEG-lipid (methoxypolyethyleneglycol 2000-cholesteryl hemisuccinate, PEG_{2000}-CHEMS) linked via ester bond and galactosylated lipid ((5-cholesten-3β-yl) 4-oxo-4-[2-(lactobionyl amido) ethylamido] butanoate, CHS-ED-LA) were used to modify doxorubicin (DOX) liposome. DOX was encapsulated into conventional liposomes (CL), galactosylated liposomes (modified with CHS-ED-LA, GalL), pegylated liposomes (modified with PEG_{2000}-CHEMS, PEG-CL), and pegylated galactosylated liposomes (modified with CHS-ED-LA and PEG_{2000}-CHEMS, PEG-GalL) using an ammonium sulfate gradient loading method and then intravenously injected to normal mice. Both PEG-GalL DOX and GalL DOX gave relatively high overall drug targeting efficiencies to liver ($(T_e)_{liver}$) and were mainly taken up by hepatocyte. However, PEG-GalL DOX showed unique "sustained targeting" characterized by slowed transfer of DOX to liver and reduced peak concentrations in the liver. The biodistribution and antitumor efficacy of various DOX preparations were studied in hepatocarcinoma 22 (H22) tumor-bearing mice. The inhibitory rate of PEG-GalL DOX to H22 tumors was up to 94%, significantly higher than that of PEG-CL DOX, GalL DOX, CL DOX, and free DOX, although the tumor distribution of DOX revealed no difference between PEG-GalL DOX and PEG-CL DOX. Meanwhile, the gradual increase in the liver DOX concentration due to the sustained uptake of PEG-GalL DOX formulations resulted in lower damage to liver. In conclusion, the present investigation indicated that double modification of liposomes with PEG_{2000}-CHEMS, and CHS-ED-LA represents a potentially advantageous strategy in the therapy of liver cancers or other liver diseases.

KEY WORDS: antitumor; cleavable PEG-lipid; doxorubicin; galactosylated lipsomes; sustained liver targeting.

INTRODUCTION

The liver deals with most of the chemicals entering the body and therefore, it is in high risk of damage. Hepatocytes (liver parenchymal cells) are the main functional cells of the liver and constitute 60~80% of the mass of the liver tissue. The liver diseases are mainly developed from hepatocytes, such as viral hepatitis, cirrhosis, and hepatocellular carcinoma (HCC). The asialoglycoprotein receptors (ASGPr), exclusively expressed on the surface of hepatocyte, can bind asialoglycoproteins and subsequently internalize them into the cell interior (1), and ASGPr can specially recognize terminal β-D-galactose or *N*-acetylgalactosamine residues (2). Liposomes modified with β-D-galactose or *N*-acetylgalactosamine residues may target to hepatocytes via ASGPr-mediated way, thus provide significant therapeutic benefits to hepatic disease.

Many studies, especially those carried out by Hashida's group, verified that liposomes modified with galactosylated lipid achieved effective target to hepatocytes (3–6). Our group has been dedicated to the studies on the targeted drug delivery of galactosylated liposomes in recently years. Our previous studies indicated that doxorubicin (DOX) liposomes modified with a novel galactosylated lipid, (5-cholesten-3β-yl) 4-oxo-4-[2-(lactobionyl amido) ethylamido] butanoate (CHS-ED-LA), were rapidly eliminated from blood circulation and delivered to liver via the ASGPr-mediated mechanism (7). However, from the pharmacokinetic point of view, the rapid accumulation of drugs in liver may not be a desired result. As pointed out by Levy (8), the elimination of drug from the site of action following its targeted delivery would be much more rapid than that of a conventionally administered dose, and the duration of action of a targeted "bolus" dose would be shorter. Takino *et al.* (9) reported that the rapid uptake of probucol incorporated emulsion into the liver resulted in a low pharmacological effect. In addition, the rapid uptake of anticancer agents may induce liver damage due to its narrow therapeutic window. While a

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Table I. The Entrapment Efficiencies and Particle Sizes of Different DOX Liposomal Formulations

Formulation	Freshly prepared		1 month after stored at 4°C	
	Entrapment efficiency (%)	Mean particle size (nm)	Entrapment efficiency (%)	Mean particle size (nm)
CL DOX	98.45	84	95.43	81
GalL DOX	96.16	82	15.26*	**
PEG-CL DOX	97.65	78	96.54	80
PEG-GalL DOX	95.43	71	96.27	75

*The entrapment efficiency was determined on the seventh day after preparation. Aggregation was found in GalL DOX after stored at 4°C for more than 7 days; **not determined

longer blood circulation time may be essential to assure sustained interaction of liposomes with the target tissue, yielding better targeting and lower toxicity.

It is generally recognized that PEG creates a so-called "steric stabilization" effect: the PEG molecules form a protective hydrophilic layer on the surface of the liposomes; the dense "conformational clouds" prevent other macromolecules in the surrounding solution from interaction with the liposome surface even at low concentration of protecting polymer (10). As a result, liposomes modified with PEG-lipid have proved to have prolonged blood circulation time and reduced mononuclear phagocyte system uptake (11). While coupling targeting ligand (such as antibody and folate) to the distal end of PEG chain make liposomes possess both long-circulating and active-targeting properties (12–14). However, Shimada *et al.* (15) reported that galactosylated liposomes failed to achieve significant targeting to ASGPr on the hepatocytes when galactose residue was attached to the distal end of PEG chain.

One alternative strategy to fulfill effective targeting is to coimmobilize a targeting ligand together with cleavable PEG-lipid on the surface of liposomes (16). Terada *et al.* (17) developed a novel metalloproteinase-2 (which was overexpressed in HCC) cleavable PEG-lipid (PEG-PD) and incorporated it in galactosylated liposomes (Gal-PEG-PD-liposomes). The *in vitro* experimental results showed that Gal-PEG-PD-liposomes completely masked the galactose ligands and inhibited its uptake by HepG2 cells. However, pretreatment with MMP2 led to an MMP2 concentration-dependent higher uptake, which gave a new clue for hepatocytes targeting.

Based on the fact that ester bond is susceptible to hydrolysis by esterase widely distributed in the plasma and tissues, a novel cleavable PEG-lipid, i.e., methoxypolyethyleneglycol 2000-cholesteryl hemisuccinate (PEG_{2000}-CHEMS), was developed in our laboratory recently (18). In this study, DOX liposomes were modified with CHS-ED-LA and PEG_{2000}-CHEMS to obtain doubly modified DOX liposomes (PEG-GalL DOX). We expect that with the cleavage of PEG-CHEMS in blood circulation and the dissociation of PEG chains from the surface of the liposomes, the targeting ligand, galactose residues, will gradually be exposed to ASGPr, which may sustain the distribution rate of PEG-GalL DOX to liver without losing its targetability. The antitumor effect of PEG-GalL DOX was tested after intravenous injection to hepatocarcinoma 22 (H22)-bearing mice to evaluate its potentials in HCC targeting therapy.

EXPERIMENTAL METHODS

Materials

Cholesterol (Chol) was purchased from Sigma Chemical Co. (St. Louis, Missouri, USA). HSPC was purchased from Avanti Polar Lipid (Alabaster, Alabama, USA). DOX was obtained from Hisun Pharmaceutical Co. Ltd. (Zhejiang, China). CHS-ED-LA and PEG2000-CHEMS were synthesized in our laboratory as reported previously (7,18). All other chemicals were of reagent grade.

Female KM mice (18∼22 g) were obtained from Animal Breeding House of Shenyang Pharmaceutical University. Mice were housed five per cage under 12 h/12 h light–dark circadian cycle at room temperature with free access to food and water. The experimental procedures were in accordance with institutional guidelines of Shenyang Pharmaceutical University.

Preparation of DOX-Loaded Liposomes

An ammonium sulfate gradient loading method (19) was used to encapsulate DOX into conventional liposomes (HSPC/Chol = 60:40, CL), galactosylated liposomes (HSPC/Chol/CHS-ED-LA = 60:30:10, GalL), pegylated liposomes

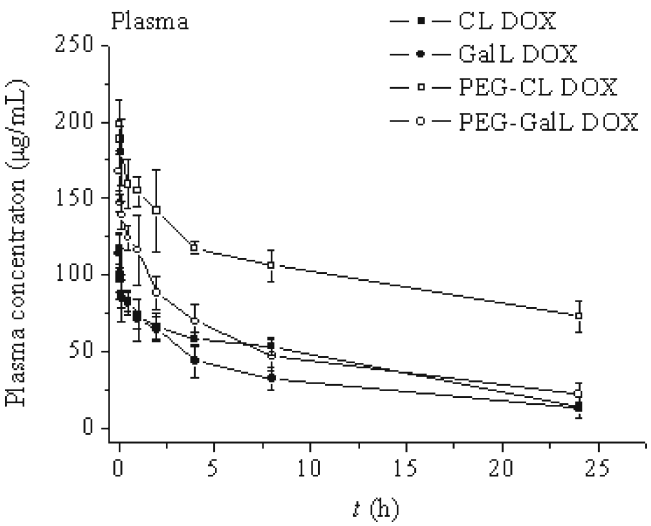

Fig. 1. Plasma concentrations of DOX in normal mice after intravenous injection of CL DOX, GalL DOX, PEG-CL DOX, and PEG-GalL DOX at a dose of 10 mg/kg

Table II. The Pharmacokinetic Parameters of DOX after Intravenous Injection of 10 mg/kg DOX in Different Formulations (Mean±SD, n=3)

	CL DOX	GalL DOX	PEG-CL DOX	PEG-GalL DOX
AUC_{0-24} (μg·h/mL)	1,035.07 ± 101.13	774.99 ± 152.60	2,454.28 ± 127.87	1,173.89 ± 67.72
$t_{1/2}$ (h)	9.55 ± 0.92	10.80 ± 3.26	25.73 ± 2.53	12.38 ± 5.03
Cl (mL/min)	0.137 ± 0.018	0.174 ± 0.047	0.032 ± 0.005	0.106 ± 0.018
Vss (L)	0.100 ± 0.006	0.146 ± 0.030	0.071 ± 0.002	0.102 ± 0.028

(HSPC/Chol/PEG_{2000}-CHEMS = 60:40:2, PEG-CL), and pegylated galactosylated liposomes (HSPC/Chol/CHS-ED-LA/PEG_{2000}-CHEMS = 60:30:10:2, PEG-GalL). Briefly, lipid components were dissolved in ethanol at 60°C. The ethanol solution was then hydrated with a 250 mmol ammonium sulfate buffer at the same temperature for 30 min. The liposome suspensions were passed through a microfluidizer (Microfluidizer M-110L, Microfluidics, Newton, Massachusetts, USA) at 11.6 kpsi for five circles, and then extruded (ten times) through polycarbonate membranes of gradually decreasing pore size (0.2 and 0.1 μm). Untrapped ammonium sulfate was removed by dialysis the liposome suspension against 10% sucrose solution (250-fold volumes) for 24 h. The free DOX was then added to the liposome suspensions and incubated at 60°C for 30 min. Nonentrapped DOX was removed by passing the liposome suspensions through cation-exchange resin column (Dowex 50WX4). DOX concentrations were determined by measurement of absorbance at 480 nm (U-2800 UV–vis Spectrophotometer, Hitachi, Japan) after dissolving the liposomes in 90% isopropyl alcohol solution containing 0.075 mol/L HCL. Particle size was determined by dynamic laser light scattering (Submicron Particle Sizer, NICOMP™ 380, Particle Sizing Systems, Santa Barbara, California, USA). All measurements were conducted at 25°C in triplicates.

Pharmacokinetics and Biodistribution in Normal Mice

In Vivo Tissue Distribution

Mice were injected with CL DOX, GalL DOX, PEG-CL DOX, or PEG-GalL DOX through tail vein at a dose of 10 mg of DOX/kg of mice. Groups of three mice per liposome formulation per time point were used in this study. At different time intervals, blood samples were collected from the orbital venous sinus; subsequently, the mice were killed by cervical dislocation, the hearts, livers, spleens, lungs, and kidneys were recovered. Plasma was obtained by centrifuging whole-blood samples at 500 g for 10 min. The plasma and tissue samples were kept at −20°C until analysis.

The concentrations of DOX in plasma and tissue samples were assayed on a spectrofluorometer (Hitachi 650-60, Japan) according to the method described by Mayer *et al.* (20). Pharmacokinetic parameters were calculated using the non-compartment model with TOPFIT® software. The area under the concentration–time curve from time zero to 24 h (AUC_{0-24}) was calculated by trapezoidal rule. The C_{max} and T_{max} of liver were determined by a visual inspection of the experimental data.

Evaluation of Liver Targetability

According to Gupta *et al.* (21), the targeted delivery of DOX to the liver could be evaluated by the overall drug targeting efficiency (T_e) based on the area under the concentration of DOX–time curve (AUC).

$$T_e = \frac{AUC_i}{\sum_{j=1}^{n} (AUC)_j} \times 100$$

Hepatic Cellular Localization

Mice were injected with CL DOX, GalL DOX, PEG-CL DOX, or PEG-GalL DOX through tail vein at a dose of 10 mg of DOX/kg of mice. After 24 h of injection, the mice were anesthetized. Following cannulation of the vena porta,

Table III. The Area under the Concentration–Time Curve (AUC_{0-24}) and Overall Drug Targeting Efficiency (T_e) Value of Various Tissues of Mice after Intravenous Injection of 10 mg/kg DOX in Different Formulations through Tail Vein (n = 3)

	CL DOX		GalL DOX		PEG-CL DOX		PEG-GalL DOX	
tissues	AUC (μg·h/g)	T_e	AUC (μg·h/g)	T_e	AUC (μg·h/g)	T_e	AUC (μg·h/g)	T_e
Plasma	1,035.07	27.71	774.99	22.73	2,454.28	50.34	1,173.89	30.22
Liver	571.69	15.30	1463.55	42.92	665.16	13.64	1,627.30	41.90
Heart	63.17	1.69	47.61	1.40	64.66	1.33	67.57	1.74
Lung	336.32	9.00	246.35	7.22	341.14	7.00	252.09	6.49
Kidney	249.33	6.67	246.69	7.23	280.96	5.76	282.11	7.26
Spleen	1,479.93	39.62	630.69	18.50	1,068.98	21.93	481.21	12.39
Total	3,735.51	100.00	3,409.88	100.00	4,875.18	100.00	3,884.17	100.00

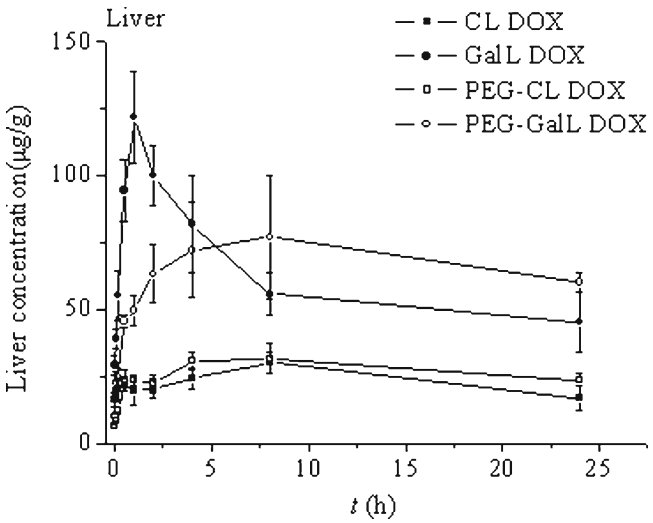

Fig. 2. Liver concentrations of DOX in normal mice after intravenous injection of CL DOX, GalL DOX, PEG-CL DOX, and PEG-GalL DOX at a dose of 10 mg/kg

perfusion was started with Hanks' buffer (pH 7.2) containing 0.02% (*w*/*v*) EDTA at 37°C, the perfusion rate was maintained at 3∼4 mL/min. As soon as the perfusion started, the vena cava and aorta were cut off. After 10 min, perfusion was continued for another 10 min with Hanks' buffer containing 0.05% (*w*/*v*) collagenase type I and 1 mmol Ca^{2+} (pH 7.4). The liver was subsequently excised, and the capsular membrane was removed. The liver was cut into pieces in ice-cold medium, transferred to a plastic beaker, and slowly stirred with a magnetic stirring bar in Hanks' buffer containing 0.1% BSA (without collagenase) at 0°C. This temperature was maintained during the further isolation procedure. After 5 min, the suspension was filtered through nylon gauze (mesh width 150 μm), followed by centrifugation at 50 g for 1 min. The pellets containing parenchymal cells were washed twice with Hanks' buffer. The supernatant was centrifuged at 100 g for 5 min, the pellets containing nonparenchymal cells were washed twice with Hanks' buffer. The amounts of DOX in

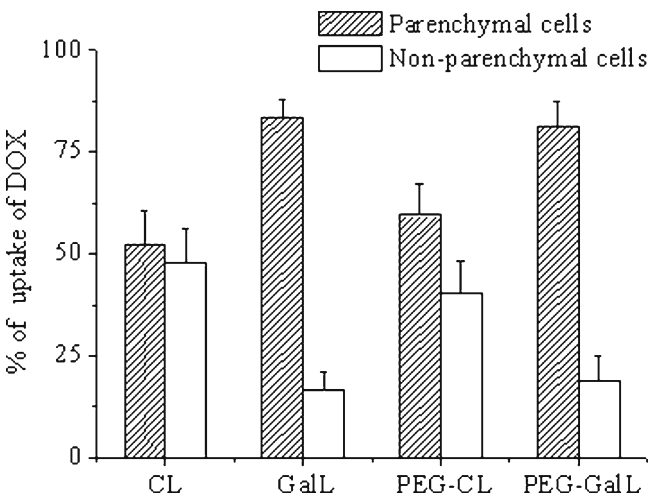

Fig. 3. Hepatic cellular localization of DOX after 24 h of intravenous injection of CL DOX, GalL DOX, PEG-CL DOX, and PEG-GalL DOX at a dose of 10 mg/kg

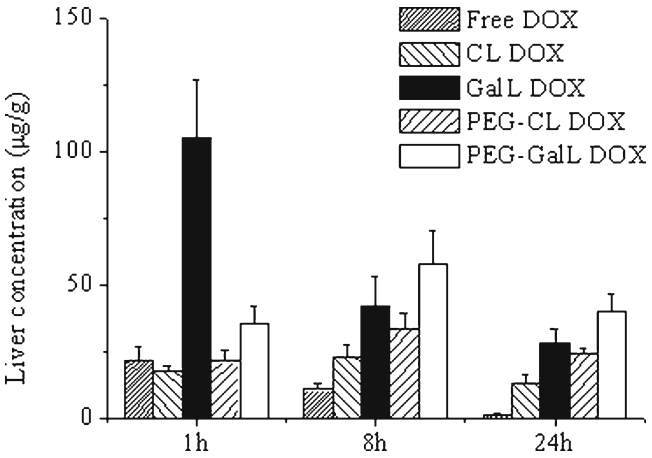

Fig. 4. Liver concentrations of DOX in H22 tumor-bearing mice at 1, 8, and 24 h after intravenous injection of free DOX, CL DOX, GalL DOX, PEG-CL DOX, and PEG-GalL DOX at a dose of 10 mg/kg

parenchymal or nonparenchymal cells were determined by the methods used for the assay of tissue samples.

Distribution and Antitumor Effect in Hepatoma Tumor-Bearing Mice

The H22 mouse liver cancer cells were kindly donated by Division of Pharmacology, Shenyang Pharmaceutical University. Mice were subcutaneously injected 0.1 mL of cell suspensions containing 1.8×10^7 cells/mL at the anterior part of the shoulder and used for the following studies.

Distribution in Liver and Tumor

When tumor diameters reached 0.5∼1 cm (5 days after inoculation), the mice were injected with free DOX (in sterile normal saline), CL DOX, GalL DOX, PEG-CL DOX, or PEG-GalL DOX through tail vein at a dose of 10 mg/kg. At 1, 8, and 24 h after injection, mice were sacrificed; livers and tumors were recovered. The concentrations of DOX were determined as described in "*In Vivo* Antitumor Effect" section.

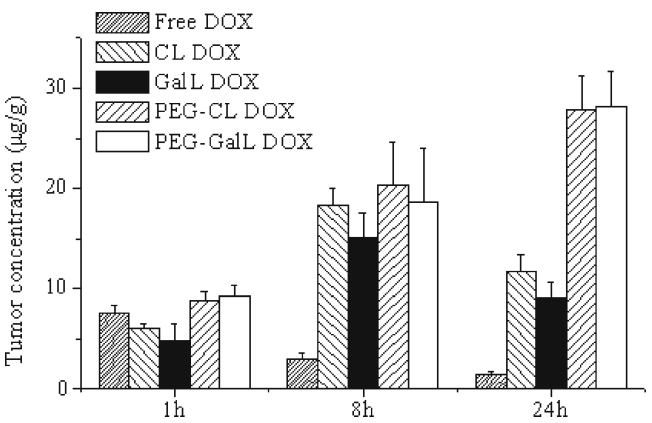

Fig. 5. Tumor concentrations of DOX in H22 tumor-bearing mice at 1, 8, and 24 h after intravenous injection of free DOX, CL DOX, GalL DOX, PEG-CL DOX, and PEG-GalL DOX at a dose of 10 mg/kg

Fig. 6. Photograph of tumor excited from H22-bearing mice after 9-day treatment of different DOX preparations. H22 cells were inoculated in the anterior part of the shoulder of mice on day 0. Free DOX, CL DOX, GalL DOX, PEG-CL DOX, or PEG-GalL DOX was intravenously injected on day 3 and 6 at a dose of 10 mg/kg. The control group received sterile normal saline

After inoculated with H22 cells on day 0, mice were randomly assigned into six groups (ten mice per group). The treated groups were intravenously injected with free DOX, CL DOX, GalL DOX, PEG-CL DOX, or PEG-GalL DOX on days 3 and 6 at a dose of 10 mg/kg. The control group received sterile normal saline. On day 9, mice were sacrificed; tumors were dissected and weighted. Meanwhile, livers were excised and fixed in formalin for further pathological experiments. The inhibitory ratio (IR) was calculated as IR (%)=(1−Wt/Wc)×100, here Wt and Wc were the tumor weights of the mice of treated and control groups, respectively.

RESULTS

Characterization of Liposomes

As shown in Table I*, the mean particle sizes of all liposomal formulations* were decreased below 100 nm by passing the prepared liposomal suspensions though microfluidizer and microporous membranes. The drug entrapment efficiencies were more than 95%. The liposomes remained stable for at least 1 month at 4°C except GalL DOX. After stored at 4°C for 7 days, GalL aggregated and DOX leaked out with obvious decrease of entrapment efficiency (to 15%). For *in vivo* experiments, all the liposomal formulations were used immediately after preparation.

Pharmacokinetics and Biodistribution in Normal Mice

PEG-CL DOX and CL DOX

The plasma concentrations of DOX after intravenous administration of various liposomal preparations were shown in Fig. 1, and the pharmacokinetic parameters were listed in Table II. The results showed that both the $t_{1/2}$ value and (AUC_{0-24}) value of PEG-CL DOX were higher than those of CL DOX with significant difference ($P<0.01$), which indicated prolonged blood circulation period due to the incorporation of PEG_{2000}-CHEMS in DOX liposomes.

PEG-GalL DOX and PEG-CL DOX

The incorporation of CHS-ED-LA in the liposome bilayer dramatically affected the *in vivo* fates of the liposomes. As shown in Table II, $t_{1/2}$ and (AUC_{0-24}) value of PEG-GalL DOX were about half of PEG-CL DOX. On the

Table IV. Comparison of Tumor Weight after Intravenous Administration of Different DOX Preparations a Dose of 10 mg/kg to H22 Tumor-Bearing Mice *In Vivo* (n=10)

Groups	Numbers of animal (end/start)	Tumor weight (g)	Inhibitory ratio (%)	P_1	P_2	P_3	P_4	P_5
NS	8/10	1.039 ± 0.419						
DOX solution	6/10	0.362 ± 0.127	65.19	<0.01				
CL DOX	10/10	0.198 ± 0.112	80.90	<0.01	<0.05			
GalL DOX	8/10	0.221 ± 0.129	78.76	<0.01	<0.05	>0.05		
PEG-CL DOX	10/10	0.114 ± 0.048	89.06	<0.01	<0.05	<0.05		
PEG-GalL DOX	10/10	0.062 ± 0.033	94.00	<0.01	<0.05	<0.05	<0.05	<0.05

P_1 compared to NS, P_2 compared to DOX solution, P_3 compared to CL DOX, P_4 compared to GalL DOX, P_5 compared to PEG-CL DOX

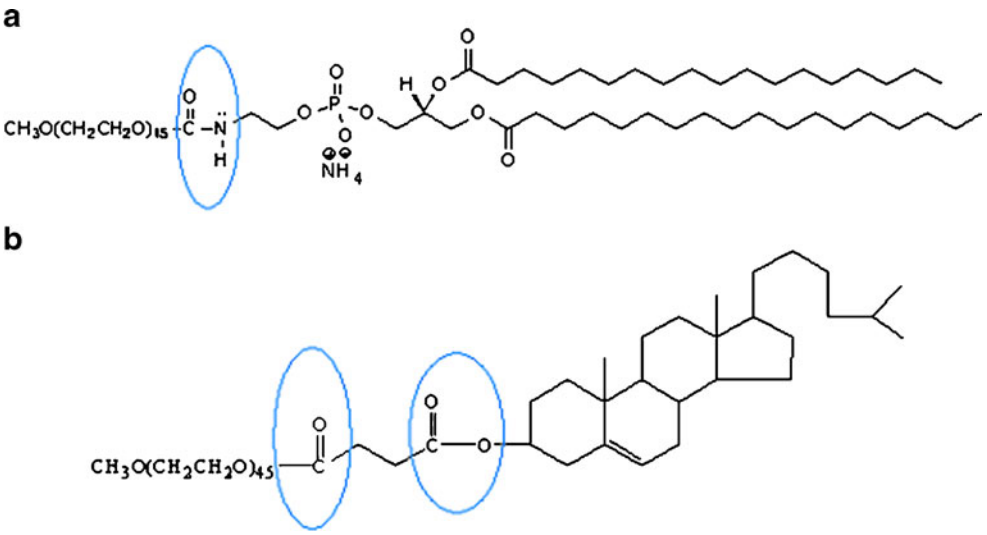

Fig. 7. Structures of **a** PEG_{2000}-DSPE and **b** PEG_{2000}-CHEMS

contrary, the accumulation of PEG-GalL DOX in liver increased with a $(AUC_{0-24})_{liver}$ value up to 1,627.30 μg·h/g (Table III), which was significantly higher than that of PEG-CL DOX ($P<0.05$). The overall drug targeting efficiency value (T_e) data showed that $(T_e)_{liver}$ of PEG-GalL DOX was 41.90%, almost three times of PEG-CL DOX (13.64%), which indicated better liver targetability of PEG-GalL DOX.

PEG-GalL DOX and GalL DOX

The liver targetability of DOX liposomes modified with CHS-ED-LA (GalL DOX) was identified previously (7). In this study, the results showed that the $(T_e)_{liver}$ of PEG-GalL DOX (41.90%) was similar to that of GalL DOX (42.92%), i.e., the incorporation of PEG_{2000}-CHEMS did not decrease the $(T_e)_{liver}$ of GalL DOX. However, PEG-GalL DOX exhibited a different concentration–time profile in liver to Gal DOX (see Fig. 2). After 1 h of injection, the concentration of GalL DOX peaked at 121.73 μg/g, followed by a rapid elimination phase. While the concentration of PEG-GalL DOX peaked at 77.02 μg/g after 8 h of administration. Compared to the rapid accumulation of Gal DOX in liver, PEG-GalL DOX showed a gradual uptake by the liver.

Intrahepatic Distribution

The intrahepatic distribution of DOX at 24 h after intravenous injection of different liposomal preparations was shown in Fig. 3. Gal DOX and PEG-GalL DOX exerted significantly higher ratio (83:17 and 81:19, respectively) between parenchymal and nonparenchymal cells than that of CL DOX and PEG-CL DOX (58:42 and 60:40, respectively; $P<0.05$), whereas, no significant difference was observed between Gal DOX and PEG-GalL DOX ($P>0.1$). The results showed that most of the PEG-GalL DOX and Gal DOX were uptaken by parenchymal cells, i.e., the incorporation of PEG_{2000}-CHEMS did not affect the uptake of liposomes into parenchymal cells.

Distribution of DOX Preparations in Liver and H22 Tumor

To compare the distribution of galactosylated liposomes between liver and hepatoma tumor, H22 tumor was transplanted in a heterotopic subcutaneous position in the anterior part of the shoulder of mice. The mice with a tumor diameter of about 0.5~1 cm received intravenous injection of DOX preparations at a dose of 10 mg/kg. The distribution profile (Fig. 4) of DOX in liver obtained from tumor-bearing mice was found to resemble to that obtained from normal mice (Fig. 2). At 1 h postadministration, the drug level of GalL DOX in liver was up to 104.9 μg/g, which was about 6-folds of CL DOX, 5-folds of PEG-CL DOX, and 3-folds of PEG-GalL DOX. The liver concentrations of PEG-GalL DOX at 1, 8, and 24 h postadministration were significantly higher than that of PEG-CL DOX and CL DOX ($P<0.05$). While, the highest concentration of PEG-GalL DOX (58.01 μg/g, 8 h) recorded was only about half of GalL DOX.

The DOX concentrations in tumor were illustrated in Fig. 5. The concentrations of liposomal DOX in tumor were higher than those of free DOX with the exception of GalL DOX and CL DOX at 1 h postadministration ($P<0.05$). GalL DOX exhibited slightly lower concentrations compared to CL DOX with no statistical significance The concentrations of PEG-GalL DOX were no difference with PEG-CL DOX ($P>0.1$), but higher than CL DOX.

In Vivo Antitumor Effect

The antitumor efficacy of DOX preparations could be manifested via direct observation of tumors excised from H22-bearing mice (as shown in Fig. 6). It was clear that the groups of mice treated with DOX preparations all showed significant inhibition to the tumor growth. Compared to free DOX, the inhibitory rate of liposomal DOX was greatly increased ($P<0.01$), and PEG-GalL DOX exhibited the highest inhibitory effect with an inhibitory rate up to 94% (Table IV). However, no difference of inhibitory rate was found between GalL DOX and CL DOX.

During the experiment duration, death of mice was observed in control group (two mice), free DOX group (four mice), and GalL DOX group (two mice). Obvious ascites was only observed in control group after dissection of died mice. This result suggested that the toxicity of DOX rather than tumor metastasis attributed to the death of mice in free DOX and GalL DOX groups. We examined pathological change of liver in mice treated with DOX preparations. Severer necrosis was viewed in GalL DOX group, while only cloudy swelling parenchymal cells were found in PEG-GalL DOX group.

DISCUSSION

HCC comprises clinically chemotherapy resistant tumors with a low response rate to DOX treatment. Compared with the free drug, pegylated liposomal DOX exhibited improved safety, but failed in providing better therapeutic activity for the treatment of HCC (22,23). Recently, many studies demonstrated that galactosylated polymeric prodrugs made DOX an effective drug against HCC (24,25). In this study, the antitumor efficacies of DOX containing galactosylated liposomes were investigated. To do that, DOX liposomes modified with (GalL DOX, PEG-GalL DOX) or without (CL DOX, PEG-CL DOX) CHS-ED-LA were intravenously injected to mice bearing hepatoma tumor (H22 tumor).

Managit previously reported that PEG_{2000}-DSPE completely inhibited receptor recognition of Gal-liposomes due to its long PEG chain (26). A cleavable pegylated lipid which can gradually liberate PEG chain and expose the target ligand may be ideal for the receptor recognition. In our previous study, we investigated the cleavage of PEG chain from liposomes modified with various PEG-lipids derivatives, and the results showed that PEG was readily liberated from PEG_{2000}-CHEMS-liposomes, while the liberation of PEG from PEG2000-DSPE-liposomes was less than 1% even after incubation in 75% FBS for 24 h (18). For PEG_{2000}-DSPE (Fig. 7a), DSPE and methoxypolyethylene glycol (2000) were linked by amide linkage which was very stable to (non) enzymatic hydrolysis and usually used as a choice to make permanent conjugates (27). For PEG_{2000}-CHEMS (Fig. 7b), succinic anhydride was selected to link cholesterol and methoxypolyethylene glycol (2000) via ester bond. Compared to amide bond, ester bond was more liable to hydrolysis and often selected as degradable linkages of prodrugs (28). In this study, we designed a new liposomal formulation (PEG-GalL DOX) by modifying DOX liposomes with a cleavable PEG-lipid (PEG_{2000}-CHEMS) and galactosylated lipid (CHS-ED-LA) to avoid the rapid accumulation of GalL DOX in liver with high drug level. The *in vivo* tissue distribution in normal and H22 tumor-bearing mice showed that PEG-GalL DOX can successfully sustain the delivery of DOX to liver and decrease the peak concentration (Figs. 2 and 4).

The results of antitumor effect in hepatoma tumor-bearing mice showed that GalL DOX exhibited the equal antitumor effect and more liver toxicity compared with CL. The exorbitant accumulation of GalL DOX in liver (Fig. 4) was the probable reason for high liver toxicity of GalL DOX and subsequently led to severe liver damage and the death of two mice. Due to the known decreased level of ASGPr expression in hepatoma compared with normal liver (29), the hepatoma may fail to compete with normal hepatocytes in uptaking GalL DOX via ASGPr-mediated mechanism. Consequently, the abundant accumulation of GalL DOX in liver may decrease the assignment of GalL DOX in tumor and lead to similar antitumor effect to CL.

Excitingly, the inhibitory rate of PEG-GalL DOX to H22 tumors was up to 94%, significantly higher than that of PEG-CL DOX, GalL DOX, and CL DOX. Meanwhile, the gradual accumulation avoided excess PEG-GalL DOX in liver, which resulted in lower damage to liver. Additionally, we found PEG-GalL DOX blocked tumor growth more effectively than PEG-CL DOX although both exhibited similar degree of drug accumulation in the tumor site (Fig. 5). The reason could be ASGPr-mediated intracellular uptake of GalL containing liposomes. Accordingly, we considered that the modification with PEG made both PEG-CL DOX and PEG-GalL DOX benefit "enhanced permeability and retention (EPR)" effect, in which long-circulating agents undergo preferential extravasation through the leaky vasculature of tumors, resulting in higher accumulation in tumor site (30). After the extravasation, only pegylated liposomes enter tumor interstitium (31). During the retention, ester hydrolysis made PEG cleaved and galactose residue exposed in the physical environment. Through ASGPr-mediated endocytosis, more PEG-GalL DOX than PEG-CL DOX accumulated in H22 tumor cells leading to a significantly improved antitumor effect. These results are in agreement with the literature (32,33) stating that anti-HER2 immunoliposomes did not increase tumor tissue accumulation in HER2-overexpressing breast cancer xenografts in nude mice, but did increase internalization in cancer cells via receptor-mediated endocytosis when compared with nontargetted liposomes.

In conclusion, PEG-GalL DOX cannot only control the delivery rate of DOX to hepatocytes, but also conserve the ASGPr-mediated targeting capability; the consequent therapeutic benefits of PEG-GalL modified liposomes are decreased liver damage and enhanced therapeutic efficacy to HCC. The present investigation indicate that double modification of liposomes with novel cleavable PEG-lipid (PEG_{2000}-CHEMS) linked via ester bond and galactosylated lipid (CHS-ED-LA) represents a potentially advantageous strategy in the therapy of liver cancers or other liver diseases.

REFERENCES

1. Ashwell G, Harford J. Carbohydrate-specific receptors of the liver. Annu Rev Biochem. 1982;51:531–54.
2. Wu J, Nantz MH, Zern MA. Targeting hepatocytes for drug and gene delivery: emerging novel approaches and application. Front Biosci. 2002;7:d717–25.
3. Mandal AK, Das S, Basu MK, Chakrabarti RN, Das N. Hepatoprotective activity of liposomal flavonoid against arsenite-induced liver fibrosis. Pharmacol Exp Ther. 2007;320:994–1001.
4. Sato A, Takagi M, Shimamoto A, Kawakami S, Hashida M. Small interfering RNA delivery to the liver by intravenous administration of galactosylated cationic liposomes in mice. Biomaterials. 2007;28:1434–42.
5. Managit C, Kawakami S, Yamashita F, Hashida M. Effect of galactose density on asialoglycoprotein receptor-mediated uptake of galactosylated liposomes. J Pharm Sci. 2005;94:2266–75.
6. Murao A, Nishikawa M, Managit C, Wong J, Kawakami S, Yamashita F, *et al*. Targeting efficiency of galactosylated lip-

osomes to hepatocytes *in vivo*: effect of lipid composition. Pharm Res. 2002;19:1808–14.
7. Wang SN, Deng YH, Xu H, Wu HB, Qiu YK, Chen DW. Synthesis of a novel galactosylated lipid and its application to the hepatocyte-selective targeting of liposomal doxorubicin. Eur J Pharm Biopharm. 2006;62:32–8.
8. Levy G. Targeted drug delivery—some pharmacokinetic considerations. Pharm Res. 1987;4:3–4.
9. Takino T, Koreeda N, Nomura T, Sakaeda T, Yamashita F, Takakura Y, *et al*. Control of plasma cholesterol-lowering action of probucol with various lipid carrier systems. Biol Pharm Bull. 1998;21:492–7.
10. Torchilin VP, Omelyanenko VG, Papisov MI, Bogdanov Jr AA, Trubetskoy VS, Herron JN, *et al*. Poly(ethylene glycol) on the liposome surface: on the mechanism of polymer-coated liposome longevity. Biochim Biophys Acta. 1994;1195:11–20.
11. Woodle MC, Lasic DD. Sterically stabilized liposomes. Biochim Biophys Acta. 1992;1113:171–99.
12. Maruyama K. PEG-immunoliposome. Biosci Rep. 2002;22:251–6.
13. Gosselin MA, Lee RJ. Folate receptor-targeted liposomes as vectors for therapeutic agents. Biotechnol Annu Rev. 2002;8:103–31.
14. Cattel L, Ceruti M, Dosio F. From conventional to stealth liposomes: a new frontier in cancer chemotherapy. Tumori. 2003;89:237–49.
15. Shimada K, Kamps JA, Regts J, Ikeda K, Shiozawa T, Hirota S, *et al*. Biodistribution of liposomes containing synthetic galactose-terminated diacylglyceryl-poly(ethyleneglycol)s. Biochim Biophys Acta. 1997;1326:329–41.
16. Gabizon A, Shmeeda H, Horowitz AT, Zalipsky S. Tumor cell targeting of liposome-entrapped drugs with phospholipid-anchored folic acid-PEG conjugates. Adv Drug Deliv Rev. 2004;56:1177–92.
17. Terada T, Iwai M, Kawakami S, Yamashita F, Hashida M. Novel PEG-matrix metalloproteinase-2 cleavable peptide-lipid containing galactosylated liposomes for hepatocellular carcinoma-selective targeting. J Control Release. 2006;111:333–42.
18. Xu H, Deng Y, Chen D, Hong W, Lu Y, Dong X. Esterase-catalyzed dePEGylation of pH-sensitive vesicles modified with cleavable PEG-lipid derivatives. J Control Release. 2008;130:238–45.
19. Lasic DD, Frederick PM, Stuart MCA, Barenholz Y, McIntosh TJ. Gelation of liposome interior: a novel method for drug encapsulation. FEBS Lett. 1992;312:255–8.
20. Mayer LD, Tai LC, Ko DS, Masin D, Ginsberg RS, Cullis PR, *et al*. Influence of vesicle size, lipid composition, and drug-to-lipid ratio on the biological activity of liposomal doxorubicin in mice. Cancer Res. 1989;49:5922–30.
21. Gupta PK, Hung CT. Quantitative evaluation of targeted drug delivery systems. Int J Pharm. 1989;56:217–26.
22. Valle JW, Dangoor A, Beech J, Sherlock DJ, Lee SM, Scarffe JH, *et al*. Treatment of inoperable hepatocellular carcinoma with pegylated liposomal doxorubicin (PLD): results of a phase II study. Br J Cancer. 2005;92:628–30.
23. Lind PA, Naucler G, Holm A, Gubanski M, Svensson C. Efficacy of pegylated liposomal doxorubicin in patients with advanced hepatocellular carcinoma. Acta Oncol. 2007;46:230–3.
24. Hopewel JW, Duncan R, Wilding D, Chakrabarti K. Preclinical evaluation of the cardiotoxicity of PK2: a novel HPMA copolymer-doxorubicin-galactosamine conjugate antitumour agent. Hum Exp Toxicol. 2001;20:461–70.
25. Fiume L, Baglioni M, Bolondi L, Farina C, Di Stefano G. Doxorubicin coupled to lactosaminated human albumin: a hepatocellular carcinoma targeted drug. Drug Discov Today. 2008;13:1002–9.
26. Managit C, Kawakami S, Nishikawa M, Yamashita F, Hashida M. Targeted and sustained drug delivery using PEGylated galactosylated liposomes. Int J Pharm. 2003;266:77–84.
27. D'Souza AJ, Topp EM. Release from polymeric prodrugs: linkages and their degradation. J Pharm Sci. 2004;93:1962–79.
28. Lee BS, Yuan X, Xu Q, McLafferty FS, Petersen BA, Collette JC, *et al*. Stimuli-responsive antioxidant nanoprodrugs of NSAIDs. Int J Pharm. 2009;372:112–4.
29. Virgolini I, Müller C, Klepetko W, Angelberger P, Bergmann H, O'Grady J, *et al*. Decreased hepatic function in patients with hepatoma or liver metastasis monitored by a hepatocyte specific galactosylated radioligand. Br J Cancer. 1990;61:937–41.
30. Gabizon A, Martin F. Polyethylene glycol-coated (pegylated) liposomal doxorubicin. Rationale for use in solid tumours. Drugs. 1997;54(S4):15–21.
31. Wu NZ, Da D, Rudoll TL, Needham D, Whorton AR, Dewhirst MW. Increased microvascular permeability contributes to preferential accumulation of stealth liposomes in tumor tissue. Cancer Res. 1993;53:3765–70.
32. Park JW, Hong K, Kirpotin DB, Meyer O, Papahadjopoulos D, Benz CC. Anti-HER2 immunoliposomes for targeted therapy of human tumors. Cancer Lett. 1997;118:153–60.
33. Kirpotin DB, Drummond DC, Shao Y, Shalaby MR, Hong K, Nielsen UB, *et al*. Antibody targeting of long-circulating lipidic nanoparticles does not increase tumor localization but does increase internalization in animal models. Cancer Res. 2006;66:6732–40.