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Research paper

Synthesis of a novel galactosylated lipid and its application to the hepatocyte-selective targeting of liposomal doxorubicin

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Abstract

This paper described the synthesis of a novel galactosylated lipid with mono-galactoside moiety, (5-Cholesten-3β-yl) 4-oxo-4-[2-(lactobionyl amido) ethylamido] butanoate (CHS-ED-LA), and the targetability of doxorubicin (DOX), a model drug, in liposomes containing 10% mol/mol CHS-ED-LA (galactosylated liposomes, GalL) to the liver was studied. The weighted-average overall drug targeting efficiency (Te^*) was used to evaluate the liver targetability of GalL DOX. The results showed that GalL DOX gave a relatively high $(Te^*)_{liver}$ value of 64.6%, while DOX in conventional liposome (CL DOX) only gave a $(Te^*)_{liver}$ value of 21.8%. In the liver, the GalL DOX was mainly taken up by parenchymal cells (88% of the total hepatic uptake). Moreover, preinjection of asialofetuin significantly inhibited the liver uptake of GalL DOX (from 70 to 12% of the total injected dose). It was suggested that liposomes containing such novel galactosylated lipid, CHS-ED-LA, had a great potential as drug delivery carriers for hepatocyte-selective targeting.

Keywords: Galactosylated lipid; Hepatocyte-selective targeting; Parenchymal cells; Liposomes; Doxorubicin

1. Introduction

Mammalian hepatocytes (parenchymal cells) have a large number of asialoglycoprotein receptors (ASGPr) that can bind deasialylated proteins from the serum and internalize them in the cell interior [1]. The ASGPr can recognize terminal β-D-galactose or *N*-acetylgalactosamine-residues [2]. ASGPr are considered a particularly attractive target in many drug carrier studies. The use of such nature molecules with galactosylated or lactosylated residues, i.e. asialofetuin [3], or synthetic compounds such as glycolipids [4], glycoprotein [5] or galactosylated polymers [6] in drug targeting carriers, has resulted in significant targeting efficacy to the liver.

In recent studies, liposome [7–9], nanopaticles [10], microspheres [11], emulsions [12] and polymer conjugates [13,14] containing galactosylated residues have been intensively investigated for liver targeting. Liposomes are now considered to be a mainstream drug delivery technology. Compared with polymer-drug conjugates, liposomes can offer various advantages. For example, drugs can be encapsulated into liposomes without any chemical modification, while multi-step reaction procedures are necessary to obtain suitable polymer conjugates. Due to the absence of covalent linkages, drugs in liposomes can be steadily released into the target cell, while the covalent bond of polymer-drug conjugates must be broken to allow the intracellular release of conjugated drugs in vivo [13].

During recent decades, different types of glycolipids with lipophilic anchor moieties have been synthesized for incorporation into liposomes [15,16]. Cholesterol, one of the lipid components used to form liposomes, is usually selected as the lipophilic anchor moiety for stably introducing the galactosyl moiety into liposomes [4,9,15].

Lactobionic acid (LA), bearing a galactosyl group, is usually used as a recognition moiety for the hepatocyte-targeting carrier [17–19]. In the present study, LA and cholesterol were chemically attached via the esterification and amidation reactions to produce a novel glycolipid,

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(5-Cholesten-3β-yl) 4-oxo-4- [2-(lactobionyl amido) ethylamido] butanoate (CHS-ED-LA).

It is well known that doxorubicin (DOX) can be efficiently encapsulated in liposomes according to transmembrane sulfate ammonium gradients and form a stable drug-sulfate gel in the liposome interior, which results in the greater stability of DOX liposomes in plasma and during storage [20]. Meanwhile the fluorescence property of DOX makes it easily quantifiable in vivo. In this study, DOX was selected as a model drug and encapsulated in galactosylated liposomes with hydrogenated soya phosphatidylcholine (HSPC), cholesterol and CHS-ED-LA. The hepatocyte targeting potential of the DOX entrapped in galactosylated liposomes (GalL DOX) was also evaluated.

2. Materials and methods

2.1. Chemicals

Cholesterol, *N*-hydroxysuccinimide (NHS), *N*, *N*′-dicyclohexylcarbodiimide (DCC), succinic anhydride, Dowex 50WX resins and asialofetuin were purchased from Sigma Chemical Co. (St Louis, MO, USA). Lactobionic acid calcium salt was purchased from Fluka Chemical Co. Ltd (Buchs, Switzerland). HSPC was purchased from Avanti Polar Lipids (Alabaster, AL, USA). Collagenase type I was purchased from Invitrogen Corporation (Grand Island, NY, USA). Doxorubicin (DOX) was obtained from Hisun Pharmaceutical Co. Ltd (Zhejiang, China). All other chemicals were of reagent grade.

2.2. Methods

2.2.1. Synthesis of 1-N -[O-β-D-Galactopyranosyl-(1,4)-D-Gluconamide]-2-N′-methylamine (LA-ED)

A solution of lactobionic acid calcium salt was passed through a cation-exchange resin column (Dowex 50WX8) to convert it to free lactobionic acid. The eluted free acid was lyophilized and converted to lactobionic lactone by repeated evaporation from methanol and ethanol [17].

A solution of lactobionic lactone (5 g, 14.7 mmol) in 50 mL methanol was added dropwise to a solution of ethylenediamine (8.8 g, 147 mmol) in 20 mL methanol. The reaction mixture was stirred at reflux for 6 h. When the reaction was completed, the solvent was removed. Then 300 mL dichloromethane was added to the residue and the precipitated white crystals were filtered and washed with ether and a small amount of cold methanol yielding 5.03 g (12.6 mmol, 85.7%) LA-ED. TLC: R_f = 0.5 (1-butanol: water: acetic acid = 2:1:1).

2.2.2. Synthesis of N-Hydroxysuccinimidly 5-Cholesten-3β-yloxy Succinate (CHS-NHS)

CHS-NHS was synthesized as previously described by Kempen et al [21]. Briefly, cholesterol (3.87 g, 10 mmol) was dissolved in 20 mL dichloromethane and 5 mL pyridine, and then succinic anhydride (1.03 g, 10 mmol) was added. After refluxing for 8 h, the mixture was evaporated to dryness. The residue was dissolved in warm acetone and recrystallized by cooling to −20 °C overnight to yield 4.05 g (8.32 mmol, 83.2%) cholesteryl hydrogen succinate (CHS). Then, DCC (5 g, 24.2 mmol) was added to a solution of CHS (4.0 g, 8.2 mmol) and NHS (1.9 g, 16.4 mmol) in 50 mL tetrahydrofuran (cooled to −20 °C). The mixture was stirred for 60 min at −20 °C, and then left at 4 °C over night. The precipitated *N*, *N*′-dicyclohexylurea (DCU) was removed by filtration, the filtrate was evaporated to dryness, and the residue was recrystallized from THF/2-propanol to yield 4.5 g (7.7 mmol, 93.0%) CHS-NHS. TLC: R_f = 0.5 ($CHCl_3$: acetone = 9:1)

2.2.3. Synthesis of (5-Cholesten-3β-yl) 4-oxo-4-[2-(lactobionyl amido) ethylamido] butanoate (CHS-ED-LA)

LA-ED (0.8 g, 2 mmol) was dissolved in DMSO/dichloromethane. A solution of CHS-NHS (1.2 g, 2 mmol) in the same solvent was added, and the mixture was stirred at 4 °C for 24 h. The dichloromethane was evaporated, and the precipitate was removed by filtration. Then, 20 mL water was added to the residue. The resulted suspension was dialyzed against distilled water for 48 h (10–12 KD cutoff dialysis membrane) to remove DMSO and LA-ED. The dialysate was lyophilized to yield 1.43 g (1.6 mmol, 80%) CHS-ED-LA. TLC: R_f = 0.3 (ethyl acetate: ethanol: water = 8:2:1), R_f = 0.7 ($CHCl_3$: methanol: water = 6:4:1).

The chemical structure was confirmed by IR, ^{1}H NMR, ^{13}C-NMR and ESI-MS. IR $\bar{\nu}$/cm^{-1}: 3377 (br, OH), 2937 (–CH_2–), 1732 (ester, C=O), 1651(amide, C=O), 1543 (C=C)^{1}H NMR (C_5D_5N) δ: 8.68 (2H, brt-like, –NH×2), 5.32 (1H, m, 6-H), 5.26 (1H, m, J = 3.18, 3′-H), 5.20 (1H, d, 1″-H), 5.19 (1H, d, 2′-H), 4.82 (1H, m, 3-H), 4.78 (1H, m, 4′-H), 4.70 (1H, m, 5′-H), [4.41 (1H, dd) & 4.26 (1H, dd, J = 4.4, 11.2), 6″-H], 4.12 (1H, dd, J = 3.0, 9.5, 3″-H), 4.07 (1H, m, 5″-H), 3.70 (2H, m, 6‴-H), 3.67 (2H, m, 5‴-H), 2.87 (2H, m, 3‴-H), 2.76 (2H, m, 2‴-H), [2.45 (1H, dd, J = 12.2, 3.5) & 2.40 (1H, brd, J = 12.2), 4-H], 1.94 (1H, m, 8-H), 0.96 (3H, d, 21-H), 0.95 (3H, s, 19-H), 0.89 (6H, d, J = 7.7, 26, 27-H), 0.63 (3H, s, 18-H). ^{13}C NMR (C_5D_5N) δ_C: 174.82 (1′-C), 172.70 (4‴-C), 172.24 (1‴-C), 140.03 (5-C), 122.75 (6-C), 106.58 (1″-C), 84.11 (4′-C), 77.54 (5″-C), 75.26 (3″-C), 74.14 (3-C), 73.66 (2′-C), 73.23 (5′-C), 72.99 (2″-C), 72.76 (3′-C), 70.26 (4″-C), 64.44 (6′-C), 62.57 (6″-C), 56.75 (14-C), 56.33 (17-C), 50.18 (9-C), 42.47 (13-C), 39.92 (16-C), 39.74 (5‴, 6‴-C), 39.60 (24-C), 38.46 (4-C), 37.19 (1-C), 36.78 (10-C), 36.49 (22-C), 36.05 (20-C), 32.13 (8-C), 31.99 (7-C), 31.04 (2‴-C), 30.27 (3‴-C), 28.52 (2-C), 28.27 (12-C), 28.08 (25-C), 24.50 (15-C), 24.16 (23-C), 22.97, 22.71 (26, 27-C), 21.25 (11-C), 19.36 (19-C), 18.95 (21-C), 11.99 (18-C). ESI-MS m/z: 891.6 $[M+Na]^+$.

2.2.4. Liposome preparation

Conventional liposomes (CL) and galactosylated liposomes (incorporated with 10% mol/mol of CHS-ED-LA, GalL) were prepared according to the method described by Lasic [22]. Briefly, lipid components were dissolved in ethanol at 60 °C. The ethanol solution was then hydrated with 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, MA, USA) at 11.6 kpsi for 5 cycles, and then extruded (10 times) through polycarbonate membranes of gradually decreasing pore size (0.2 and 0.1 μm). Untrapped ammonium sulfate was removed by dialysis of the liposome suspension against 10% sucrose solution (250-fold volumes) for 24 h, and then the DOX solution was added to the liposomes and incubated at 60 °C for 30 min. Non-entrapped DOX was removed by passing the liposome suspension through a 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 containing 0.075 mol/L HCL [23]. The particle size was determined by dynamic laser light scattering (Submicron Particle Sizer, NICOMP™ 380, Particle Sizing Systems, Santa Barbara, CA, USA). All measurements were conducted at 25 °C in triplicates.

2.2.5. Animal experiments

2.2.5.1. In vivo tissue distribution. Female KM mice (18–22 g) were injected with GalL DOX or CL DOX through the tail vein at a dose of 10 mg/kg. At a predetermined time, mice were injected with 50 mg/kg asialofetuin 1 min before the injection of liposomal DOX. This study used groups of 3 mice per liposome formulation per time point. At different time intervals, blood samples were collected via eye puncture; subsequently, the mice were humanely killed, and their hearts, livers, spleens, lungs and kidneys were recovered. Plasma was obtained by centrifuging whole-blood samples at $500\times g$ for 10 min. The plasma and tissue samples were kept at −20 °C until analysis.

A previous HPLC analysis of biological samples carried out by Bally et al. [24] indicated that >98% of the fluorescence detected was due to nonmetabolized DOX after mice receiving liposomal DOX. In our studies, the concentrations of DOX in plasma and tissue samples were assayed by a spectrofluorometric method described by Mayer et al [25]. Briefly, 5 mL 50% aqueous acidic methanol (0.3 mol/L HCL) was added to the frozen samples. After homogenization, the homogenates were incubated at 4 °C for 60 min in the dark and then centrifuged at $10{,}000\times g$ for 30 min. The supernatants were assayed in a spectrofluorometer (Hitachi 650-60, Japan), at an excitation wavelength of 472 nm and an emission wavelength of 591 nm.

2.2.5.2. Hepatic cellular localization. A dose of 10 mg/kg GalL DOX or CL DOX was injected via the tail vein into KM mice (25–30 g). One hour after the injection, the mice were anaesthetized. Parenchymal and nonparenchymal cells were isolated by the collagenase perfusion method [9,26]. Following cannulation of the vena porta, perfusion was started with Hanks' buffer (pH 7.2) containing EDTA 0.02% (w/v,), at 37 °C and the perfusion rate was maintained at 3–4 mL/min. As soon as the perfusion had started, the vena cava and aorta were cut off. After 10 min, perfusion was continued for another 10 min with Hanks' buffer containing collagenase type I 0.05% (w/v) 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 size 150 μm), followed by centrifugation at $50\times g$ for 1 min. The pellets containing parenchymal cells were washed twice with Hanks' buffer. The supernatant was centrifuged at $100\times g$ for 5 min, and the pellets containing nonparenchymal cells were washed twice with Hanks' buffer. Through the trypan blue exclusion method, the cell viability was found to be more than 90%. The amount of DOX in the cells was determined by the methods used for the assay of tissue samples.

2.2.5.3. Evaluation of liver targetability. According to Gupta et al. [27], the targeted delivery of DOX to the liver could be evaluated by the weighted-averag overall drug targeting efficiency (Te^*), which was based on the area under DOX amount - time curve (AUQ).

$$Te^* = \frac{AUQ_i}{\sum_{j=1}^{n}(AUQ)_j}\times 100$$

where i refers to the target tissue, and j refers to each tissue.

2.2.5.4. Statistical analysis. ANOVA was used to analyze data for statistical significance ($P<0.05$).

3. Results and discussion

3.1. Synthesis of (5-Cholesten-3β-yl) 4-oxo-4-[2-(lactobionyl amido) ethylamido] butanoate (CHS-ED-LA)

As far as the affinity of galactosides for the asialoglycoprotein receptors is concerned, there is a 'cluster effect': tetraantennary > triantennary ≫ biantennary ≫ monoantennary galactosides [28]. Sliedregt et al. demonstrated that liposomes containing synthetic

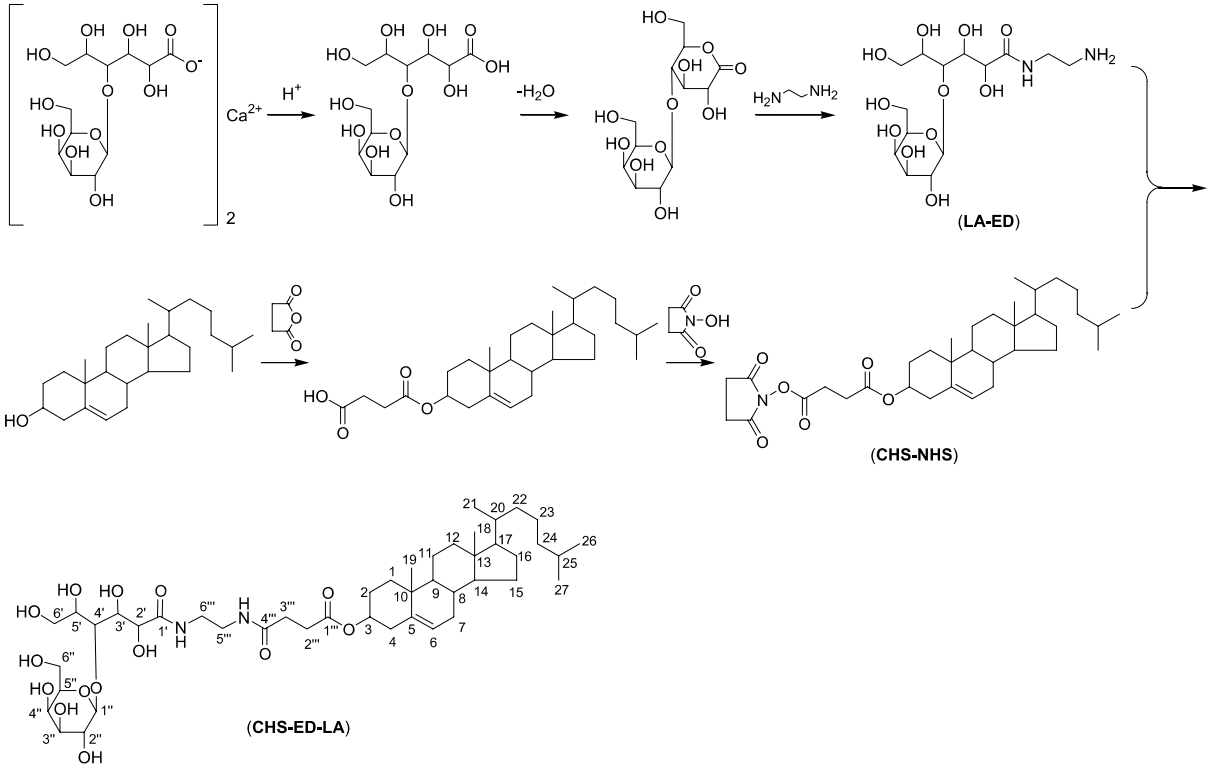

Fig. 1. Synthesis of (5-Cholesten-3β-yl) 4-oxo-4-[2-(lactobionyl amido) ethylamido] butanoate (CHS-ED-LA).

glycolipids with tris-galactosides were highly recognized by ASGPr [4]. However, the synthesis of glycolipids with a cluster galactoside moiety was too complicated for application in an industrial scale. Hashida's group had developed a glycolipid with mono-galactoside, cholesten-5-yloxy-*N*-(4-((1-imino-2-β-D-thiogalactosylethyl) amino) butyl) formamide (Gal-C4-Chol), and demonstrated the good liver targeting effect of liposomes containing Gal-C4-Chol for gene and lipophilic drug delivery [29–32].

In this study, a novel galactosylated lipid with a mono-galactoside moiety (CHS-ED-LA) was obtained in good yield by a multi-step synthetic procedure. The synthesis of CHS-ED-LA was initiated by coupling lactobionic acid with ethylenediamine to form LA-ED, yielding a terminal amine. The carboxylic acid group on the CHS was converted to a reactive NHS ester group. The NHS ester could be easily coupled with amines, leading to amide bonds. Thus, the LA-ED was covalently attached to CHS via the amide formation between the amine and carboxylic acid groups to produce CHS-ED-LA (Fig. 1).

3.2. Characterization of liposomes

For both liposomal formulations prepared, the drug entrapment efficiency was more than 95%, and the mean particle size was about 80 nm (Table 1). This suggested that the incorporation of CHS-ED-LA had no effect on the entrapment efficiency of DOX liposomes.

3.3. Tissue distribution

Lots of studies have demonstrated that galactocylated lipids can enhance the uptake of liposomes by hepatocytes [4,7,9]. In order to investigate whether galactocylated lipids can alter the distribution of the drug entrapped in liposomes, the in vivo fate of DOX was evaluated after administration of GalL DOX or CL DOX to mice.

Compared with the injection of CL DOX, the plasma concentration of DOX decreased significantly ($P<0.05$) after intravenous injection of GalL DOX (Fig. 2). In contrast, the liver accumulation of GalL DOX was up to $70.29\pm11.66\%$ of the total injected dose within 1 h, while the accumulation of CL DOX was relatively lower ($12.00\pm$

Table 1
Entrapment efficiency and particle size of freshly prepared liposomal suspensions

Type of liposomes	Lipid composition (HSPC: Cholesterol:CHS-ED-LA)	Drug % entraptment ± SD	Mean particle size (nm)
Conventional liposomes (CL)	60:40:0	96.20 ± 1.43	85
Galactosylated liposomes (GalL)	60:40:10	98.08 ± 2.41	79

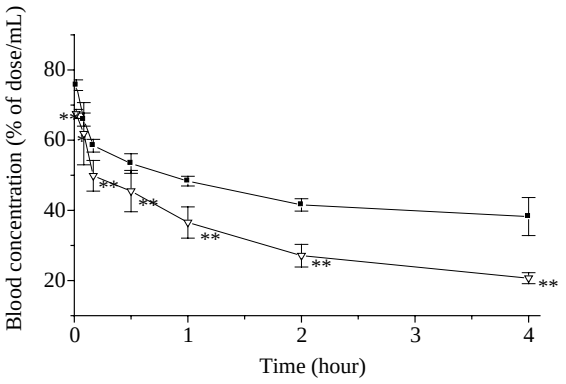

Fig. 2. Plasma concentration of DOX after intravenous injection of GalL DOX (-▽-) and CL DOX (-■-). Each point represents the mean percentage dose ± SD. $^{**}P<0.05$, $^{*}P<0.1$.

2.00%) (Fig. 3). The liver uptake of GalL DOX was significantly higher than that of CL DOX ($P<0.05$).

The liver targetability of GalL DOX could be estimated using Te^* values (Table 2). The Te^* value in the liver $(Te^*)_{liver}$ of CL DOX was only 21.8%, while GalL DOX gave a $(Te^*)_{liver}$ value of 64.6%. The $(Te^*)_{liver}$ values suggested that the liver targeting of GalL DOX was almost three times that of CL DOX. However, when compared with CL DOX, the Te^* values of GalL DOX in the spleen, plasma, lungs, heart and kidneys were reduced by 73, 55, 50, 48, and 45%, respectively.

3.4. Intrahepatic distribution and competitive inhibition study

To determine whether parenchymal cells or non-parenchymal cells were responsible for the hepatic accumulation of DOX, mice were injected with GalL DOX or CL DOX. One hour after the injection, the liver cell distribution of DOX was determined (Fig. 4). It was found that the distribution of CL DOX between parenchymal and non-parenchymal cells was at a ratio of 52:48. However,

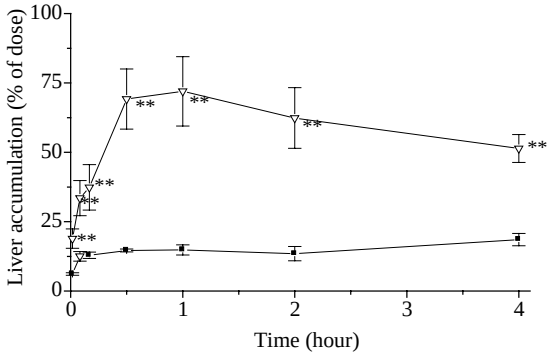

Fig. 3. Liver accumulation of DOX after intravenous injection of GalL DOX (-▽-) and CL DOX (-■-). Each point represents the mean percentage dose ± SD. $^{**}P<0.05$.

Table 2
The area under DOX amount - time curves ($AUQ_{(0–4\ h)}$) and the weighted - average drug targeting efficiency (Te^*) of in various tissues of mice, following after intravenous administration of 10 mg/kg in GalL DOX and CL DOX

Tissues	CL DOX		GalL DOX		r_d[a]
	AUQ (% dose·h)	Te^*	AUQ (% dose·h)	Te^*	
Plasma[b]	160	69.5	109	31.3	0.45
Liver	50.0	21.8	223	64.6	3.00
Heart	1.21	0.52	0.95	0.27	0.52
Lung	4.51	1.96	3.36	0.97	0.50
Kidney	7.44	3.23	6.15	1.78	0.55
Spleen	6.77	2.94	2.78	0.80	0.27
Total	230	100	345	100	

[a] $r_d=(Te^*)_{GalL}/(Te^*)_{CL}$.
[b] Total plasma volume of the mouse was considered as 0.9 mL per 20 g body weight.

the incorporation of 10% mol/mol CHS-ED-LA in liposomes resulted in a greater amount of DOX accumulation in parenchymal cells, and the ratio of drug uptake by parenchymal to non-parenchymal cells was found to be 88:12.

To determine whether ASGPr is responsible for the enhanced uptake by the parenchymal liver cells, asialofetuin was used as a specific competitor. Preinjection of asialofetuin inhibited the uptake of GalL DOX significantly and only 12% of the injected dose was recovered from the liver, while 70% was recovered without preinjection of asialofetuin (Fig. 5). For CL DOX, preinjection of asialofetuin had no obvious effect on the accumulation of DOX in liver.

It was supposed, just like other galactosylated liposomes [4,7,29–32] that the recognition of galactosylated residues of CHS-ED-LA by ASGPr on the surface of parenchymal cells may also be responsible for the liver targeting of GalL DOX l.

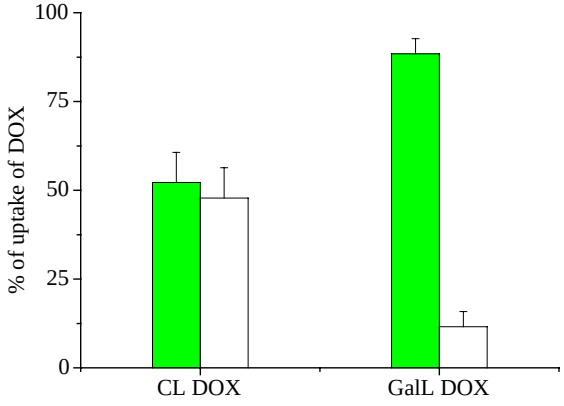

Fig. 4. Hepatic cellular localization of DOX after 1 h of intravenous injection of GalL DOX and CL DOX, respectively. Hatched bars, parenchymal cells; open bars, non-parenchymal cells.

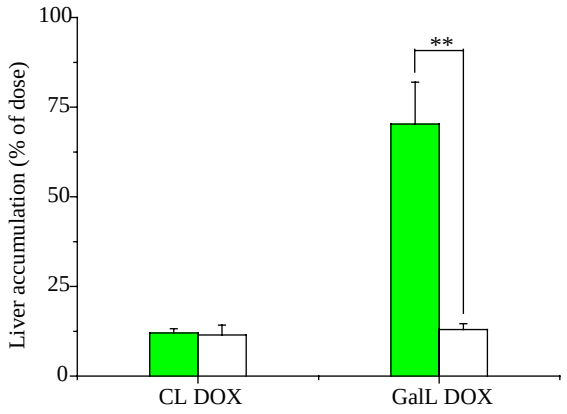

Fig. 5. Inhibition of liver uptake of DOX by preinjection of asialofetuin. Liver accumulation was determined after 1 h of intravenous injection of GalL DOX and CL DOX, respectively. Hatched bars, without preinjection of asialofetuin; open bars, with preinjection of asialofetuin. $^{**}P<0.05$.

4. Conclusion

In this study, we have synthesized a novel galactosylated lipid with a mono-galactoside moiety, (5-Cholesten-3β-yl) 4-oxo-4-[2-(lactobionyl amido) ethylamido] butanoate (CHS-ED-LA). The incorporation of CHS-ED-LA (10% mol/mol) into liposomes enhanced the liver targetability of liposomal DOX. Moreover, the results of intrahepatic distribution and competitive inhibition studies provided evidence that the recognition of galactosylated residues of CHS-ED-LA by ASGPr on the surface of parenchymal cells accounted for the liver accumulation of GalL DOX. These results suggested that liposomes containing CHS-ED-LA could be a useful drug carrier system for hepatocyte-selective targeting.

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