Targeting of Liposomes Surface-Modified with Glycyrrhizin to the Liver. I. Preparation and Biological Disposition

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We consider glycyrrhizin to be a new ligand for liposomes to the liver because it is known that about 80% of glycyrrhizin is excreted into the bile after intravenous administration in rats. In order to modify the liposomal surface with glycyrrhizin, 30-stearyl glycyrrhizin (GLOSt), one of the lipophilic glycyrrhizin derivatives, was synthesized. The structure of this new compound was identified by nuclear magnetic resonance (NMR), infrared (IR) and mass spectra (MS). Sonicated liposomes were prepared from hydrogenated egg phosphatidylcholine–cholesterol–GLOSt or dicetyl phosphate (DCP) (4:4:1) and were labelled with [^{3}H]inulin as an aqueous marker. It was confirmed by measuring the encapsulation efficiencies and the mean diameters that GLOSt-containing sonicated liposomes (GLOSt-SUV) were SUV-type as well as DCP-containing control liposomes (control-SUV). Four hours after intravenous injection into rats at a dose of 90 μmol as total lipid per kg of rat body weight, GLOSt-SUV showed 4-fold more accumulation (42.4%) in the liver than control-SUV. Therefore, glycyrrhizin is considered to be a useful new ligand on liposomes for targeting to the liver.

Keywords targeting; drug delivery system; liposomes; glycyrrhizin; 30-stearyl glycyrrhizin; liver

Introduction

Liposomes are thought to be the drug carrier for selective delivery to specific tissues or cells. For more than a decade numerous investigations using many kinds of ligands, (glycolipids, lectins, monoclonal antibodies, *etc.*) have been carried out in an attempt to navigate liposomes to target sites.[1] However, it is difficult to apply liposomes *in vivo* since in most tissues the continuous barrier of the vascular endothelial cells prevents liposomes from moving from the intravascular space to the parenchymal cells. The use of liposomes as a tool for active targeting is, therefore, confined to several organs or tissues, such as the liver which has fenestrated endothelium,[2] cancer lesions where the permeability of vasculature is increased in comparison with normal tissues[3] and cells of the reticuloendothelial system resident in the vascular space.[4]

The liver is one of the most important organs in the body but there is to date no effective chemotherapeutic agent to counteract potentially fatal diseases such as chronic virus hepatitis and hepatoma. Several kinds of drugs, for example, cytokines like interferons and interleukin-2, antiviral agents such as adenine arabinoside, and biological response modifiers have been used in clinical trials. But many problems of low therapeutic activity and serious side effect(s) of these drugs remain to be solved. For these reasons, paralleling with development of new drugs, an adequate technique to target drugs to the liver is also necessary.[5]

The liver contains specialized blood capillaries called "sinusoids" are developed. Since these sinusoids are lined with a discontinuous basement membrane and the endothelial lining has many pores called "fenestrations", blood components have easy access to the parenchymal cells through the vascular barrier. The mean diameter of the fenestrations has been estimated at 106 nm and the maximum at 200—300 nm.[6] From this morphological point of view, the liver is also one of the most suitable organs for a drug delivery system such as liposomes.

We selected glycyrrhizin (GL), which is a component of licorice (*Glycyrrhiza glabra*) roots, as the ligand of liposomes for targeting to the liver. It is known that GL demonstrates high accumulation in the liver, and Ichikawa *et al.* reported that when given intravenously to rats, about 80% of the injected dose is excreted into bile.[7] Therefore, it can be assumed that the uptake of GL by the liver is substantial.

In the present study, we examined the ability of liposomes surface-modified with GL to target the liver. We synthesized 30-stearyl glycyrrhizin (GLOSt), which is a novel compound as one of the lipophilic GL derivatives. Small unilamellar liposomes containing GLOSt (GLOSt-SUV) and surface-modified with GL were prepared, and the fate of the liposomes after intravenous injection into rats was investigated.

Experimental

Materials Hydrogenated egg phosphatidylcholine (PC) was a gift from Nippon Fine Chemicals Co., Ltd. (Osaka). Cholesterol (CH) was obtained from Wako Pure Chemicals Co., Ltd. (Osaka), and recrystallized from ethanol. Dicetyl phosphate (DCP) was purchased from Nacalai Tesque Inc. (Kyoto). GL was from Maruzen Chemicals Co., Ltd. (Osaka). All other synthetic chemicals were commercial products of reagent grade or better. [^{3}H(G)]inulin was purchased from New England Nuclear (Boston, MA).

Spectral Analysis Spectral data were obtained with the following instruments: infrared (IR) spectra with a Perkin-Elmer 1720 infrared Fourier transform spectrometer; mass spectra (MS) with a JEOL DX 303 mass spectrometer; ^{1}H- and ^{13}C-nuclear magnetic resonance (^{1}H- and ^{13}C-NMR) spectra with a JEOL GSX-400 spectrometer (400 and 100 MHz, respectively).

Synthesis of GLOSt A solution of *O*-benzyl-*N*,*N*′-dicyclohexylisourea (23.2 g, 73.8 mmol), prepared by the method of Vowinkel,[8] in dimethylformamide (DMF) (40 ml) was added to a solution of GL (30.3 g, 36.8 mmol) in DMF (60 ml) with stirring on an ice-water bath. After continued stirring overnight, the precipitate (dicyclohexylurea) was filtered off and the filtrate was concentrated *in vacuo* to yield a syrup. The syrup was purified by silica gel column chromatography with stepwise elution to obtain 6′,6″-dibenzylglycyrrhizin (I) (10.3 g, 27.8%) with $CHCl_3$–MeOH (8:1, v/v). FAB-MS (NaI) *m/z*: 1025 $[M+Na]^+$.

A solution of I (4.01 g, 4.00 mmol) in DMF (20 ml) was added to melted *O*-*n*-stearyl-*N*,*N*′-dicyclohexylisourea containing CuCl, prepared from *n*-stearylalcohol (1.71 g, 6.32 mmol), dicyclohexylcarbodiimide (1.24 g, 6.05 mmol) and CuCl (5 mg) at 85 °C,[9] and the mixture was stirred at 85 °C for 4 h. The precipitate was filtered off and washed with ethyl acetate and then the filtrate was concentrated *in vacuo* to give a pale green syrup. The syrup was separated by silica gel column chromatography with $CHCl_3$–MeOH (93:7, v/v) to give 6′,6″-dibenzyl-30-stearyl glycyrrhizin (II) (2.90 g, 56.9%). FAB-MS (NaI) *m/z*: 1277 $[M+Na]^+$. ^{1}H-NMR ($CDCl_3$–methanol-d_4 1:1) δ: 1.28 (brs, stearyl-H), 5.27 (m, 4H, Ph-C$\underline{H}_2$–), 5.68 (s, 1H, 12-H), 7.36 (m, 5H, Ph-H).

A solution of II (1.50 g, 1.18 mmol) in 20 ml of isopropanol–AcOEt

(5:1, v/v) was catalytically hydrogenated over 5% Pd–carbon (1 g) at atmospheric pressure for 4 h. Pd–carbon was filtered off and the filtrate was evaporated and dried *in vacuo* to give GLOSt (III) (1.20 g, 99%), which was thin-layer-chromatographically pure. No other signal for impurity was observed in nuclear magnetic resonance (NMR) charts of GLOSt. FAB-MS (NaI) *m/z*: 1097 $[M+Na]^+$, 705 $[M-C_{12}H_{17}O_{13}]^+$. IR ν_{max}^{KBr} cm^{-1}: 3431 (OH), 2926, 2854 (–$(CH_2)_n$–), 1729, 1661 (C=O). ^1H- and ^{13}C-NMR data are compared in Table I with those of authentic GL.

Preparation and Characterization of Liposomes Small unilamellar vesicles (SUV) consisting of PC, CH, and DCP or GLOSt in a molar ratio of 4:4:1 were prepared as follows. A homogeneous lipid solution in chloroform was evaporated to form a dried lipid film in a round-bottomed flask. The lipid film was hydrated with 12 mM NaH_2PO_4–51 mM Na_2HPO_4–77 mM NaCl (pH 7.4, 280 mOsm/kg) containing 0.1 mM [^{3}H]inulin as an aqueous space marker. The total lipid concentration was 45 μmol/ml in the liposomal suspension. The mixture was voltexed and sonicated in a bath type sonicator (Branson ultrasonic cleaner, type-5200, Yamato Science Co., Ltd., Tokyo) until a homogeneous suspension was obtained. The suspension was further sonicated with a probe-type sonicator (ultrasonic disruptor, UR-200P, Tomy Seikou Co., Ltd.) for about 1 h to give SUV.

The vesicles were dialyzed in a flow-type dialysis cell using polycarbonate membranes with a pore size of 0.03 μm against a 200-fold volume of 140 mM phosphate-buffered saline (pH 7.4) at room temperature for 4 d. The phosphate-buffered saline was replaced twice a day.

The encapsulation ratio of [^{3}H]inulin was calculated from the radioactivity in the suspension after dialysis, which was counted with a liquid scintillation counter (Aloka LSC-703, Tokyo) in an emulsifier scintillation cocktail (Scintisol EX-H, Wako Pure Chemicals Co., Ltd., Osaka), and expressed as percentage of the initial radioactivity in the suspension before dialysis.

The incorporation ratio of GLOSt was calculated by measuring the content of GLOSt in the suspension with high performance liquid chromatography (HPLC) after dialysis and expressed as a percentage of the initial content before dialysis. HPLC conditions were as follows: column, μ-Bondapak-C18 (Waters, 15 cm × 3.9 mm i.d.); column temperature, room temperature; flow rate, 1.0 ml/min; detector, UV 254 nm; mobile phase, 2% acetic acid solution (v/v)–MeOH (1:9, v/v).

The mean diameter of the vesicles was measured with a submicron particle analyzer (Coulter N4, Coulter Electronics Inc., U.S.A.).

Animal Experiments Male albino Wistar rats (body weight 300 ± 50 g) anesthetized with ether were cannulated in the femoral vein, femoral artery and bladder and treated as described.[10] Two cannulae were inserted into the bladder, one for sampling and the other for washing. Each operated rat was placed in a Bollman cage. After recovering from anesthesia, liposome suspension was injected into the rat through the femoral vein cannula. At appropriate times after injection, blood and urine samples were collected from the cannulae inserted into the artery and bladder, respectively, without disturbing or anesthetizing the animals. Four hours after injection, rats were anesthetized with pentobarbital and sacrificed by removing blood from the cannula inserted in the femoral artery. Then liver, lung, spleen and kidney were excised and rinsed with 140 mM phosphate-buffered saline (pH 7.4). The radioactivity of each sample was measured as follows.

Determination of Radioactivity Radioactivity in tissues, blood and urine samples after intravenous injection of liposomes encapsulating [^{3}H]inulin as an aqueous marker was determined as described previously with slight modification.[11]

The liver was homogenized in water and the volume was adjusted to 50 ml with water. The urine was diluted with water to 10 ml. Blood samples (0.1 ml), urine samples (0.5 ml) or samples of the liver homogenate (0.5 ml) were treated with a 0.2 ml aliquot of 30% H_2O_2 and 0.2 ml of 2 N KOH solution in isopropanol in a liquid scintillation vial to decolorize and solubilize. The mixture was stirred gently but sufficiently and then left at room temperature overnight. After 0.4 ml of 10% acetic acid was added for neutralization, 10 ml of scintillation cocktail (Scintisol EX-H) was added.

Whole spleen, lung or kidney was solubilized and decolorized by treatment with 2 ml of 2 N KOH solution in isopropanol and 1 or 2 ml of 30% H_2O_2 during incubation at 37 °C overnight. Then, 2.4 ml of 10% (v/v) acetic acid solution was added for neutralization and the volume was adjusted to 10 ml with water. One ml of the mixture was collected in a liquid scintillation vial and then 10 ml of Scintisol EX-H was added.

The mixed scintillator solutions were allowed to stand at room temperature to dissipate chemiluminescence and counted with a liquid scintillation counter (Aloka LSC-703, Tokyo).

Stability of Liposomes in Rat Plasma In this experiment, 5(6)-carboxyfluorescein (CF, Eastman Kodak, N.Y.) solution prepared as described before[12] was used as an aqueous marker of liposomes instead of [^{3}H]inulin. A 0.1 ml aliquot of liposomal suspension (4.5 μmol as total lipid) was incubated with 0.9 ml of fresh rat plasma at 37 °C. At appropriate times after incubation, a 10 μl aliquot was transfered into 5 ml of cold 140 mM phosphate-buffered saline (pH 7.4). A 1 ml aliquot of the diluted mixture was well mixed with 1 ml of 5% Triton X-100 solution and then 2 ml of water was added. The latency of CF in the liposomes was calculated from the fluorescence intensity with and without Triton X-100 treatment and expressed as a percentage of the initial latency.

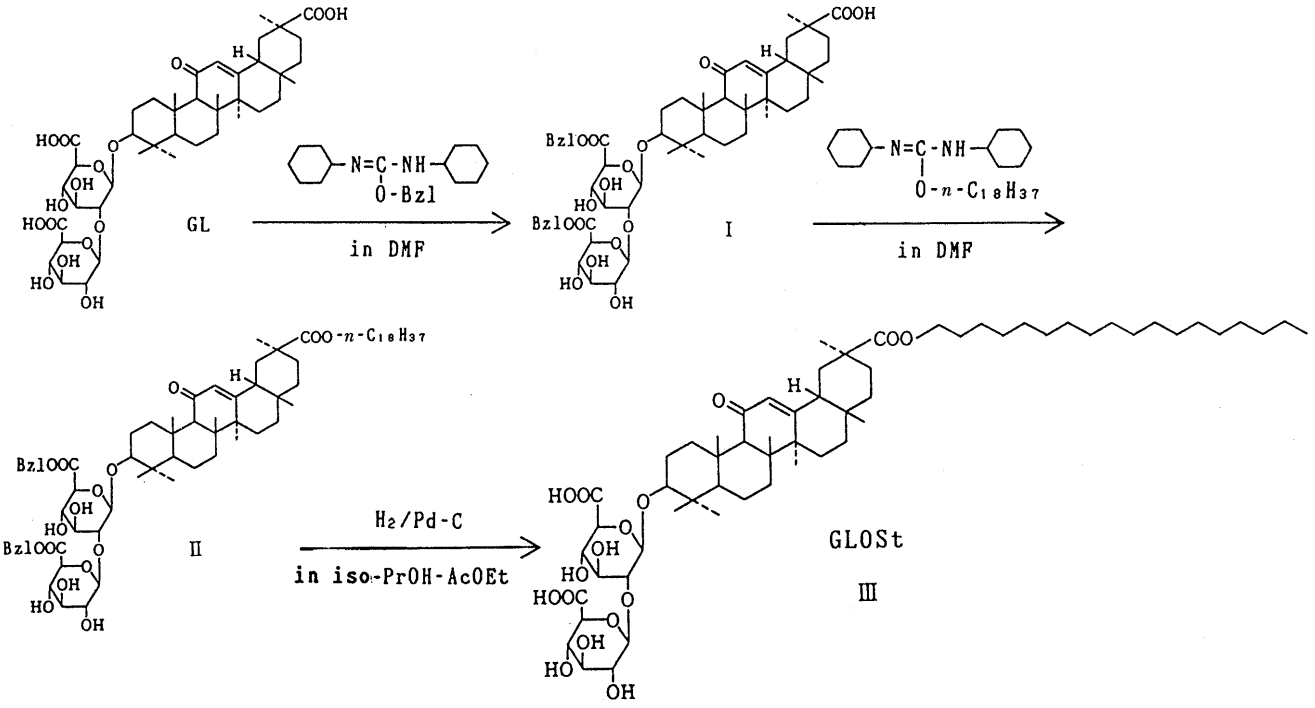

Chart 1. Synthetic Route of GLOSt (Bzl = –CH_2–C_6H_5)

Results

Synthesis of GLOSt GLOSt, a novel compound, was synthesized successively by esterification employing isoureas[8,9] according to the process shown in Chart 1. The structure of the new compound was identified by several spectra, *e.g.* NMR, IR, and mass spectra. ^{1}H- and ^{13}C-NMR spectral data of GLOSt are shown in Table I in comparison with those of authentic GL. δ values of GLOSt except for signals based on the stearyl group agreed with those of GL perfectly. No other signal of impurity was observed in NMR charts of GLOSt.

Preparation and Characterization of Liposomes In order to have access to liver parenchymal cells, it is necessary for liposomes to be a size which is smaller than the pore size of the fenestrations, the mean diameter of which has been estimated as 106 nm and the maximum as 200—300 nm.[6] Therefore, SUV-type liposomes were prepared by sonication with a probe-type sonicator for 1 h. CH equimolar with PC was incorporated into liposomes to enhance the *in vivo* stability.[13] DCP was incorporated into control-SUV as an alternative to GLOSt to give a negative charge. The characterization of these liposome preparations is listed in Table II. The encapsulation ratio of [^{3}H]inulin was 2.25% and 2.06% (0.50 and 0.46 μl/μmol as encapsulated volume) for GLOSt-containing liposomes and control-SUV, respectively. The mean diameter of GLOSt-containing liposomes and control-SUV was 60 ± 30 and 54 ± 30 nm, respectively. All these values of GLOSt-containing liposomes were compatible with those of control-SUV. Therefore, it was confirmed that GLOSt-containing sonicated liposomes formed SUV-type liposomes (GLOSt-SUV) as well as control-SUV. Values shown in this table are those of a typical example; deviations in the values of other preparations were less than 5% of each value.

The incorporation ratio of GLOSt obtained by measuring its content in the suspension sampled before and after dialysis was 99.7% (Table II). This result shows that GLOSt was quantitatively incorporated into the liposomal membrane after dialysis.

Blood Disappearance and Urinary Excretion Figure 1 shows the dispositions of [^{3}H]inulin after intravenous injection of the liposomes at a dose of 90 μmol per kg rat body weight. The blood disappearance of [^{3}H]inulin entrapped in liposomes up to 4 h is shown in panel A. The incorporation of GLOSt into SUV induced a substantial increase of the elimination rate. Total body clearances of GLOSt-SUV and control-SUV were 47.4 and 6.6 ml/h/kg,

Table I. Spectral Data of ^{1}H- and ^{13}C-NMR for GLOSt Compared with GL

Compound	GLOSt	GL
^{1}H-NMR		
H-1′ (1H, d)	4.50 (J=7.2 Hz)	4.50 (J=7.3 Hz)
H-1″ (1H, d)	4.64 (J=7.2 Hz)	4.61 (J=7.3 Hz)
H-12 (1H, s)	5.63	5.63
CH_3 (3H, s)	0.83, 0.83, 1.06, 1.14, 1.14, 1.19, 1.40	0.83, 0.84, 1.05, 1.13, 1.13, 1.19, 1.40
Stearyl-H	1.27 (–(CH_2)$_n$–, br s) 4.12 (–(CH_2–OCO–, 2H, m)	
^{13}C-NMR		
CH_3	16.3, 16.6, 19.0, 23.6, 27.8, 28.6, 28.9	16.2, 16.5, 18.9, 23.5, 27.6, 28.5, 28.7
CH_2	17.7, 26.3, 26.8, 26.9, 31.5, 33.2, 38.2, 39.6, 41.5	17.6, 26.2, 26.7, 26.8, 31.3, 33.1, 38.1, 39.6, 41.5
CH	48.9, 55.8, 62.3, 72.0, 72.0, 75.0, 75.0, 75.8, 76.0, 76.4, 82.3, 90.3, 104.3, 104,7, 128.6	48.9, 55.7, 62.3, 71.9, 72.0, 75.0, 75.2, 76.0, 76.1, 76.4, 82.9, 90.1, 104.3, 105.2, 128.3
C	32.2, 37.2, 39.8, 43.8, 44.5, 46.0, 171.1, 172.0, 172.3, 177.5, 201.7	32.2, 37.1, 39.8, 43.7, 44.1, 45.9, 171.2, 171.6, 171.7, 179.7, 202.0
Stearyl-C	14.2 (CH_3) 23.0, 26.4, 29.1, 29.5, 29.7, 29.9, 30.0 (overlapped), 32.3, 43.8, 65.1 (CH_2)	

^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) spectra were recorded on a JEOL GSX-400 spectrometer using $CDCl_3$–methanol-d_4=1:1 as a solvent with tetramethylsilane as an internal standard. Values are chemical shifts given on the δ (ppm) scale with the internal standard. Values of characteristic signals for ^{1}H-NMR and all signals for ^{13}C-NMR are represented in this table. The abbreviations are as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad; J, coupling constant.

Table II. Characterization of Control-SUV and GLOSt-SUV

Liposome	Mean diameter (nm)	Encapsulation ratio of [^{3}H]inulin (%)	Incorporation ratio of GLOSt (%)
Control-SUV	54 ± 30	2.25	—
GLOSt-SUV	60 ± 30	2.06	99.7

Liposomes were composed of hydrogenated egg PC, CH and GLOSt or DCP in a 4:4:1 molar ratio and prepared with 140 mM phosphate-buffered saline (pH 7.4, 280 mOsm/kg) containing 0.1 mM [^{3}H]inulin as an aqueous space marker and using a probe-type sonicator to form SUV-type liposomes. Mean diameter was measured with a submicron particle analyzer (Coulter N4) and incorporation of GLOSt was measured with HPLC.

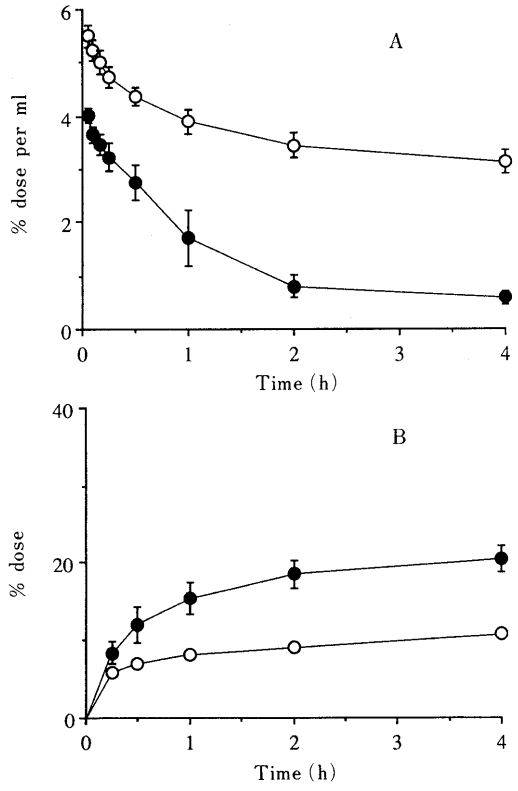

Fig. 1. Time Courses of Blood Levels (Panel A) and Cumulative Urinary Excretions (Panel B) of [^{3}H]Inulin Encapsulated in Control-SUV (○) and GLOSt-SUV (●) after Intravenous Injection into Rats

Liposomes were injected at a dose of 90 μmol as total lipid per kg body weight. Results are expressed as percentage of the injected dose per 1 ml of blood volume (A) and of the injected dose per total urine (B). Values are means±S.D. of three animals.

as calculated by area under the plasma concentration (AUC) and dose, respectively (Table IV). Panel B shows the time courses of the cumulative urinary excretion of [^{3}H]inulin which is an aqueous marker of liposomes. The 4 h excretion of [^{3}H]inulin was 20.5% of injected dose after administration of GLOSt-SUV, which was about 2-fold higher than in the case of control-SUV (10.7%). Since the radioactivity found in the urine is considered to be free [^{3}H]inulin, it is concluded that the aqueous marker of GLOSt-SUV, rather than that of control-SUV, tends to leak to some extent *in vivo*.

Stability in Rat Plasma The time courses of leakage of CF, as an aqueous marker, from liposomes during incubation with fresh rat plasma at 37 °C were examined (Fig. 2). Control-SUV was very stable in plasma and the latency of CF was 96%, whereas that of GLOSt-SUV was 70% after 2 h incubation. The instability of GLOSt-SUV in plasma might be one of the reasons that the urinary excretion of GLOSt-SUV was higher than that of control-SUV *in vivo*. In 140 mM phosphate-buffered saline (pH 7.4) no leakage of CF from either SUV preparation was observed during incubation at 37 °C. The latency of control-SUV and GLOSt-SUV respectively was 98% and 99% 2 h after incubation. Therefore, the leakage of [^{3}H]inulin from GLOSt-SUV must be attributable some factor(s) in the plasma.

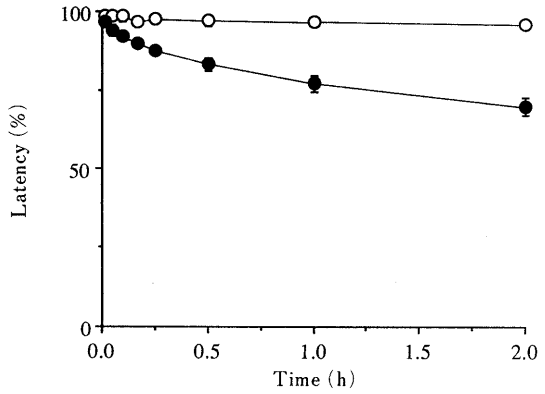

Fig. 2. Time Courses of Latency of CF Encapsulated in Control-SUV (○) and GLOSt-SUV (●) in Fresh Rat Plasma

0.1 ml of liposomal suspension (4.5 μmol as total lipid) was incubated with 0.9 ml of fresh rat plasma at 37 °C. The extent of latency was determined as described in Materials and Methods. Values are means ± S.D. of three animals.

TABLE III. Tissue Distribution of [^{3}H]Inulin Entrapped in Control-SUV and GLOSt-SUV 4 h after Intravenous Injection into Rats

Liposome	Control-SUV	GLOSt-SUV
Blood	51.7 ± 3.0	11.4 ± 2.1[c]
Liver	10.4 ± 0.7	42.4 ± 1.4[c]
Spleen	4.3 ± 0.6	2.0 ± 0.4[a]
Lung	0.5 ± 0.1	0.2 ± 0.1[a]
Kidney	0.9 ± 0.2	0.8 ± 0.1
Urine	10.7 ± 0.5	20.5 ± 1.8[b]
Recovery	78.5 ± 4.1	77.2 ± 3.2

Liposomes were injected at a dose of 90 μmol as total lipid per kg body weight. Values are means ± S.D. of three animals and are expressed as percentage of the initial dose per whole tissues. Blood volume was estimated as 6.5% of total body weight. Significant difference from control-SUV using Student's *t*-test: *a*) $p<0.05$, *b*) $p<0.01$, *c*) $p<0.001$.

TABLE IV. Pharmacokinetic Parameters of [^{3}H]Inulin Entrapped in Control-SUV and GLOSt-SUV

Parameter	Control-SUV	GLOSt-SUV
$AUC^{0\to\infty}$ (% dose·h/ml)	60.5 ± 5.8	7.4 ± 1.1
$AUC^{0\to 4h}$ (% dose·h/ml)	14.8 ± 0.9	5.5 ± 1.0
CL_{total} (ml/h/kg)	6.6 ± 0.5	47.4 ± 6.8
CL_{liver} (ml/h/kg)	2.8 ± 0.2	27.6 ± 5.6
CL_{spleen} (ml/h/kg)	1.2 ± 0.2	1.3 ± 0.04
CL_{lung} (ml/h/kg)	0.1 ± 0.01	0.1 ± 0.02
CL_{kidney} (ml/h/kg)	0.2 ± 0.04	0.5 ± 0.04
CL_{renal} (ml/h/kg)	2.9 ± 0.1	13.3 ± 2.5

Parameters present here were calculated from the values used in Fig. 1 and Table III. AUC was estimated by the trapezoidal rule. Total body clearance (CL_{total}) was calculated by $AUC^{0\to\infty}$ and injected dose. Organ clearance (CL_{organ}) was obtained by dividing the uptake amount for 4 h by $AUC^{0\to 4h}$. Renal clearance (CL_{renal}) was calculated by dividing total urinary excretion for 4 h by $AUC^{0\to 4h}$. Values are mean ± S.D. of three animals.

Tissue Distribution and Organ Clearances Tissue distribution and organ clearances of the liposomes 4 h after intravenous injection are shown in Tables III and IV, respectively. Organ clearance is considered to be a more appropriate parameter than the percent dose distribution commonly used, because clearance represents the ability of tissue uptake independent of blood concentration. In the present study, organ clearance was expressed as the uptake amount for 4 h divided by $AUC^{0\to 4h}$ because the efflux of [^{3}H]inulin from organs can be neglected. A substantial increase was found in the liver uptake as a result of GLOSt incorporation in the SUV. The distribution of GLOSt-SUV to the liver was 42.4% of injected dose, almost 4-fold higher than that of control-SUV. The liver clearance was 10-fold higher. On the other hand, the uptake of GLOSt-SUV by spleen and lung, which are organs rich in reticuloendothelial cells, and by kidney were even lower than that by the liver, confirming that GLOSt-SUV was selectively taken up by the liver.

Discussion

In order to modify the liposomal surface with GL, this ligand was converted into a lipophilic derivative possessing an affinity for liposomal membranes. In this study, GLOSt was synthesized in anticipation of its high incorporation into the liposomal membrane. GLOSt is a single-chain amphiphile having diglucuronide moiety as a hydrophilic head group, a rigid glycyrrhetinate moiety and a flexible tail of alkyl chain as a lipophilic group. It is a favorable structure for incorporation into liposomal membranes.[14] Actually, as shown in Table II, it was confirmed that SUV-type liposomes were also formed in the incorporation of GLOSt as well as control-SUV, and GLOSt was tightly incorporated into the liposomal membrane. It is therefore concluded that GLOSt is appropriate to use for modification of the liposomal surface with GL. In this case, it is assumed that diglucuronide moiety of the GL molecule protrudes from the liposomal surface.

The use of solid liposomes assures maximum efficacy of ligand incorporated into the liposomal membrane.[15] Yoshioka and his colleagues reported that the uptake of lactosylceramide-containing liposomes by hepatocytes was enhanced by decrease in the fluidity of the liposomal membrane.[16] Therefore, in this study we chose hydrogenated egg PC as a liposomal base to maximize the effect of the ligand.

The elimination of GLOSt-SUV from blood after intravenous injection into rats was far more rapid than

that of control-SUV (Fig. 1A), and concomitantly, the GLOSt-SUV showed significantly higher uptake in the liver than the control-SUV (Tables III and IV). The liver uptake of GLOSt-SUV was almost 4-fold that of control-SUV and the liver clearance was 10-fold. The uptake by other organs was low, so that it was confirmed GLOSt-SUV were selectively taken up by the liver. The renal clearance of the GLOSt-SUV was 4-fold higher than that of control-SUV, as calculated from renal excretion of inulin released from liposomes in the body. However, the renal clearance does not reflect the uptake of the liposomes by the kidney but the degradation in the body. Therefore the uptake by the liver is dominant in the biological dispositions of these liposomes. Since the morphology of GLOSt-SUV was the same as that of control-SUV, the difference of biological dispositions is considered to be based on the surface modification with GL. It was also found that the blood clearance and the liver accumulation were dependent on the GLOSt content in liposomal membrane (unpublished data). These results suggest that GLOSt-SUV are accumulated through the affinity site(s) for GL moiety located in the liver.

The urinary excretion data showed that GLOSt-SUV tends to release more of an entrapped aqueous marker than control-SUV *in vivo* (Table II). The results of the latency in plasma suggested that plasma component(s) participates in the instability of GLOSt-SUV *in vivo* (Fig. 3). Cell-surface-binding protein causing instability of liposomes as reported[17] might also take part in the destabilization of the liposomes *in vivo*. It seemed that such instability *in vivo* reduced the targeting efficiency of the liposomes to the liver. The uptake into the liver would be further enhanced if the stability of the liposomes could be enhanced. Indeed, 4 h after intravenous injection to rat, 67% of GLOSt-SUV labeled with [^{3}H]cholesteryl hexadecyl ether, which is a lipophilic and undegradable marker,[18] was found in the liver (urinary excretion was less than 1%). and this was 1.6-fold higher than that of GLOSt-SUV labeled with [^{3}H]inulin (unpublished data). This result shows that GLOSt-SUV leaking an aqueous marker is also taken up by the liver.

In recent years, a number of investigators have attempted to target liposomes to liver parenchymal cells *in vivo*.[2] To increase the uptake into these cells, most of them have exploited the receptor for galactosyl residues on the plasma membrane of liver parenchymal cells[19] and used galactosyl residue as a ligand.[2a,e] However, it has been reported that considerable amounts of galactose-bearing liposomes were also found in Kupffer cells.[20] Therefore, a new ligand for the liver is desired, and GL is one of the candidates. Targeting efficiency of the GLOSt-SUV to the liver was comparable to those of conventional galactose liposomes. The significance of this study is the proposal of a possibility of GL as a targetable ligand on the liposomal surface to liver cells, although the mechanisms of the affinity are not clear at present. The diglucuronide moiety of GL molecule is assumed to protrude from the liposomal surface and to contribute to the affinity of the liposomes to the liver. It is impossible, however, to neglect the contribution of glycyrrhetinate moiety and/or membrane characteristics to the affinity.

From the present study, it is not clear which cell subfraction contributes to the *in vivo* liver uptake of GLOSt-SUV. Since the size of GLOSt-SUV is small enough to pass the fenestrations of liver sinusoids and it is presumed that the uptake of free GL by liver parenchymal cells is very high, it is expected that GLOSt-SUV are preferably taken up by these cells; yet the participation of Kupffer cells cannot be excluded at this time. Surface modification of liposomes may enhance their association with Kupffer cells. Intrahepatic distribution after their intravenous injection is the next subject to be resolved for these liposomes.

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