Synthesis and Application of Neoglycolipids for Liposome Modification

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We synthesized various glycolipid derivatives and examined the *in vivo* behaviors of liposomes modified with these novel glycolipid derivatives. Gal-t-psa (1, {8-(2-hexadecyloctadecanoylamido)-3,6-dioxaoctyl}-β-D-galactoside), Lac-t-psa (3, 8-(2-hexadecyloctadecanoylamido)-3,6-dioxaoctyl β-D-lactoside) and GalNAc-t-psa (4, 8-(2-hexadecyloctadecanoylamido)-3,6-dioxaoctyl 2-acetamido-β-D-galactopyranoside) modified liposomes were recognized by the liver. Lac-t-psa (3) modified liposome was accumulated to the highest degree, followed by GalNAc-t-psa (4) modified liposome and then Gal-t-psa (1) modified liposome. The intrahepatic distributions of Gal-t-psa (1), GalNAc-t-psa (4), Glc-t-psa (2, 8-(2-hexadecyloctadecanoylamido)-3,6-dioxaoctyl β-D-glucopyranoside) and Lac-t-psa (3) modified liposomes were investigated. GalNAc-t-psa (4) and Lac-t-psa (3) modified liposome were accumulated to greater extents than Gal-t-psa (1) modified liposome in hepatic parenchymal cells. The intrahepatic distribution of these liposomes showed that Lac-t-psa (3) and GalNAc-t-psa (4) were preferable to Gal-t-psa (1) for the selective delivery of liposomes to hepatic parenchymal cells.

Key words liposome; glycolipid; targeting; rat

Liposomes can encapsulate hydrophilic and lipophilic drugs. Moreover, the *in vivo* behavior of liposomes can be controlled by modification of the surface. Many reseachers have attempted to develop medical applications for liposomes as drug carriers for selective delivery.[2,3)]

We synthesized various ligands with galactose as a homing device to the asialoglycoprotein receptors of liver parenchymal cells,[4)] and evaluated the relationship between the structure of the ligand and liposome accumulation in the liver. The effects of anchors, spacers and branching of the saccharide structure of neogalactosyllipid on the accumulation of liposomes in the liver have been examined. Neogalactosyllipids with a straight chain anchor bind to serum albumin in plasma and are released from liposomes. Branched aliphatic chains as an anchor have been found to be appropriate.[5,6)] In addition, the spacer between the anchor and the sugar should be longer than triethylene glycol in order to expose the sugar as the recognition element on the liposome surface.[7)] Furthermore, we have described the synthesis of glutamic acid branched galactose derivatives for liposome modification and the *in vivo* behavior of liposomes modified with these derivatives.[8)] When liposomes were modified with neogalactosyllipids, the galactose density on the liposome surface seemed to be more important than the branching structure of the ligand. Thus, we decided to use a neoglycolipid with a branched aliphatic chain anchor, a triethyleneglycol spacer and a non-branched saccharide structure for the surface modification of liposomes.

In this study, we synthesized several neoglycolipids and evaluated the *in vivo* behaviors and intrahepatic distribution of liposomes modified with these ligands.

MATERIALS AND METHODS

Materials L-α-Phosphatidylcholine dipalmitoyl (DPPC), dicetyl phosphate (DCP), cholesterol (CH) and inulin were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Collagenase was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Phosphate buffered saline was purchased from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan) and [^{3}H]Inulin from DuPont-NEN Research Products (Boston, MA, U.S.A.). All other chemicals were of reagent grade or better. Galactosyllipid, {8-(2-Hexadecyloctadecanoylamido)-3,6-dioxaoctyl}-β-D-galactoside (Gal-t-psa, **1**) was prepared as described previously.[5)]

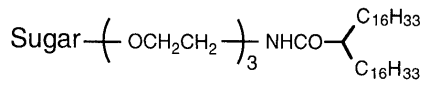
Syntheses of Neoglycolipids The structures of neoglycolipids are shown in Fig. 1 and the synthetic route of the ligands is outlined in Charts 1 and 2. The ^{1}H-NMR data are presented in Table 1. The characteristics of the ligands are described below.

8-(2-Hexadecyloctadecanoylamido)-3,6-dioxaoctyl β-D-glucopyranoside (Glc-t-psa, **2**): $[\alpha]_D^{21} = -7.1°$ ($c = 1.02$, $CHCl_3$–MeOH 1:1 v/v). IR (KBr): 3319, 1643 cm^{-1}. FAB-MS m/z: 802 $(M^{\cdot} + H)^+$.

8-(2-Hexadecyloctadecanoylamido)-3,6-dioxaoctyl β-D-lactoside (Lac-t-psa, **3**): colorless amorphous solid, $[\alpha]_D^{22} = -3.0°$ ($c = 1.01$, $CHCl_3$–MeOH 2:1 v/v). IR (KBr): 3400 (br), 1643 cm^{-1}. FAB-MS m/z: 964 $(M^{\cdot} + H)^+$.

8-(2-Hexadecyloctadecanoylamido)-3,6-dioxaoctyl 2-acetamido-β-D-galactopyranoside (GalNAc-t-psa, **4**): colorless amorphous solid, $[\alpha]_D^{25} = -5.9°$ ($c = 0.99$, $CHCl_3$–MeOH 1:1 v/v). IR (KBr): 3284, 1643 cm^{-1}. FAB-MS m/z: 843 $(M^{\cdot} + H)^+$.

Preparation of the Neoglycolipid Modified Liposomes



1 : Sugar = Galβ1 (Gal-t-psa)
2 : Sugar = Glcβ1 (Glc-t-psa)
3 : Sugar = Galβ1-4Glcβ1 (Lac-t-psa)
4 : Sugar = GalNAcβ1 (GalNAc-t-psa)

Fig. 1. Structure of Neoglycolipids

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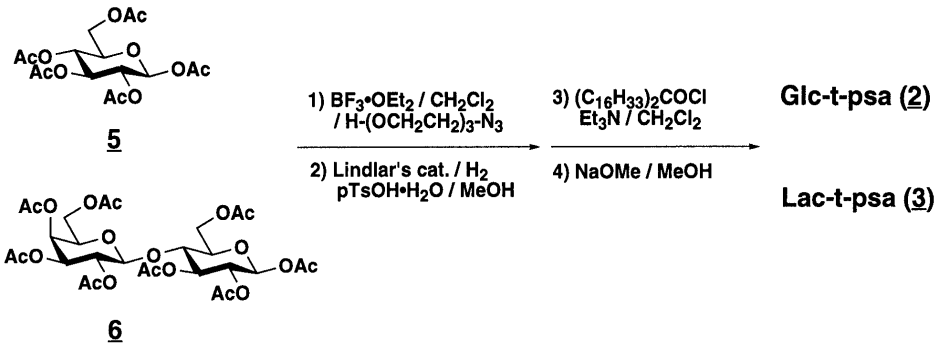

Chart 1. Synthesis of Neoglycolipids

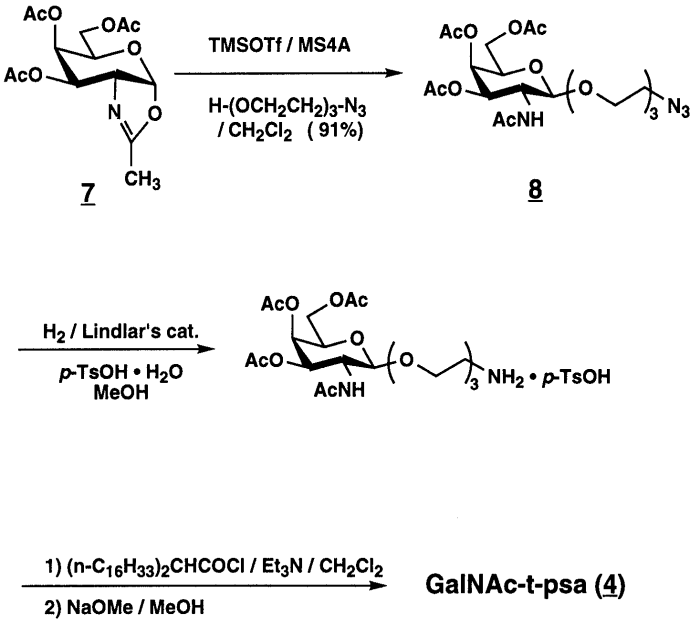

Chart 2. Synthesis of GalNAc-t-psa

Table 1. ^{1}H-NMR Spectral Data

Compd. No.	^{1}H-NMR chemical shifts (ppm)
2	0.88 (6H, t, J=6.8 Hz, $CH_3(CH_2)_{15}$), 1.22—1.60 (58H, m, $CH_3(CH_2)_{14}CH_2$, $CH_3(CH_2)_{14}CH_2$), 1.92—1.99 (2H, m, $CH_3(CH_2)_{14}CH_2$), 2.51—2.58 (1H, m, $(n\text{-}C_{16}H_{33})_2CH$), 3.59—3.67 (4H, m, ethyleneglycol moiety), 3.68—3.78 (6H, m, ethyleneglycol moiety), 3.90—3.95 (2H, m, Glc 5-H, Glc 1β-OCH_2), 4.00 (1H, t, J=7.8 Hz, Glc 2-H), 4.15—4.27 (3H, m, Glc 3-H, 4-H, Glc 1β-OCH_2), 4.32 (1H, dd, J=5.5, 11.7 Hz, Glc 6-H_a), 4.51 (1H, d, J=11.7 Hz, Glc 6-H_b), 4.85 (1H, d, J=7.8 Hz, Glc 1-H), 8.78 (1H, t, J=5.0 Hz, CONH)
3	0.88 (6H, t, J=6.8 Hz, $CH_3(CH_2)_{15}$), 1.20—1.61 (58H, m, $CH_3(CH_2)_{14}CH_2$, $CH_3(CH_2)_{14}CH_2$), 1.90—2.00 (2H, m, $CH_3(CH_2)_{14}CH_2$), 2.50—2.58 (1H, m, $(n\text{-}C_{16}H_{33})_2CH$), 3.59—3.82 (11H, m, ethyleneglycol moiety), 3.84—3.91 (2H, m, Glc 4-H, Glc 1β-OCH_2), 3.99 (1H, br t, Glc 2-H), 4.10—4.14 (2H, m, Glc C5-H, Gal 5-H), 4.18—4.26 (3H, m, Glc 3-H, Gal 3-H, Glc 1β-OCH_2), 4.34 (1H, dd, J=5.0, 11.0 Hz, Glc 6-H_a), 4.40—4.50 (5H, m, Glc 6-H_b, Gal 2-H, 4-H, 6-H), 4.77 (1H, d, J=7.5 Hz, Glc 1-H), 5.06 (1H, d, J=8.0 Hz, Gal 1-H), 8.82 (1H, br t, CONH)
4	0.88 (6H, t, J=7.0 Hz, $CH_3(CH_2)_{15}$), 1.19—1.41 (52H, m, $CH_3(CH_2)_{13}CH_2CH_2$), 1.43—1.62 (6H, m, $CH_3(CH_2)_{13}CH_2CH_2$, $CH_3(CH_2)_{13}CH_2CH_2$), 1.92—2.00 (2H, m, $CH_3(CH_2)_{13}CH_2CH_2$), 2.13 (3H, s, acetyl), 2.52—2.59 (1H, m, $(n\text{-}C_{16}H_{33})_2CH$), 3.63—3.80 (10H, m, ethyleneglycol moiety), 3.91 (1H, dt, J=11.0, 5.1 Hz, GalNAc 1β-OCH_2), 3.99—4.01 (1H, br t, GalNAc 5-H), 4.19 (1H, dt, J=11.0, 4.6 Hz, GalNAc 1β-OCH_2), 4.33 (1H, dd, J=3.2, 10.6 Hz, GalNAc 3-H), 4.37—4.42 (2H, m, GalNAc 6-H), 4.49 (1H, d, J=3.2 Hz, GalNAc 4-H), 4.82 (1H, dd, J=8.4, 10.6 Hz, GalNAc 2-H), 5.05 (1H, d, J=8.4 Hz, GalNAc 1-H), 8.84 (1H, br t, CONH)

Solvent; pyridine d_5–D_2O 100 : 1 (v/v).

Liposomes were prepared as described in our previous report.[5] The control liposomes contained 80 μmol DPPC, 80 μmol CH, and 8 μmol DCP. Neoglycolipid modified liposomes were prepared using 16 μmol neoglycolipid together with 80 μmol DPPC, 80 μmol CH and 8 μmol DCP. These lipids were dissolved in chloroform/methanol (1/1 v/v). The solvent was removed in a stream of nitrogen with heating. The residue was hydrated with 8 ml of phosphate buffer saline (PBS(−)) containing cold 0.5% (w/v) inulin and 5.92 MBq [^{3}H]inulin as an aqueous phase

marker. The suspensions were vortexed mechanically and then sonicated. The liposomes were extruded through polycarbonate membranes with pore sizes of 200, 100 and 80 nm in order, successively. Non-encapsulated [^{3}H]inulin was removed by ultracentrifugation at 150000 × *g* for 15 h, 2 h and 2 h, successively. Liposome size was determined using a submicron particle analyzer (NICOMP 370, Pacific Scientific, MD, U.S.A.). The mean diameter of the liposomes ranged from 120—150 nm. The liposomes exhibited a multi-lamellar structure on the negative-stain electron micrographs.

***In Vivo* Distribution of Neoglycolipid-Modified Liposomes in Rats** Male Sprague–Dawley rats (180—230 g body weight) were given a single intravenous injection of 2.5 ml/kg of a liposome suspension, containing about 1×10^7 dpm per dose, into the jugular vein under ether anesthesia. At 6 h after injection, the rats were sacrificed under ether anesthesia and the organs were removed and combusted using an automatic sample combustion system (ASC-113, Aloka, Tokyo, Japan). The radioactivity was measured in a liquid scintillation counter (LSC-3600, Aloka).

The intrahepatic distribution of liposomes was evaluated according to the method of Dasgupta *et al.*[9] Rats were given a single intravenous injection of 2.5 ml/kg of liposomes containing about 1×10^7 dpm per dose. Rat liver cells were isolated by the collagenase perfusion method[10] 2 h after injection. The cells were dispersed in ice-cold Ca^{2+}-free Hanks' balanced salt solution (pH 7.4). The parenchymal cells were allowed to settle under gravity by placing the suspension on ice for 1 h. The nonparenchymal cells were isolated as described by Seglen,[11] with the exception that protease was used to destroy the contaminating parenchymal cells. Parenchymal and nonparenchymal cells were combusted using an automatic sample combustion system and the radioactivity measured in a liquid scintillation counter.

Evaluation of the Tissue-Targeting Ability As an index of targeting ability, the increased tissue concentration was calculated using Eq. 1.

$$C_{t,sub} = C_{t,modified} - C_{t,control} \quad (1)$$

where, $C_{t,modified}$ and $C_{t,control}$ are the tissue concentrations (% of injected dose/g tissue) of neoglycolipid modified liposomes and control liposomes, respectively.

RESULTS AND DISCUSSION

Syntheses of Neoglycolipids Syntheses of Glc-t-psa (**2**), Lac-t-psa (**3**): Glc-t-psa (**2**) was prepared from penta-*O*-acetyl-β-D-glucoside (**5**) in the same procedure as Gal-t-psa (**1**).[4] Similarly, Lac-t-psa (**3**) was prepared from octa-O-acetyl-β-D-lactoside (**6**).

Synthesis of GalNAc-t-psa (**4**): Intermediate **8** was prepared from an oxazolidine derivative (**7**)[12] by means of glycosylation using TMSOTf for activation. Compound **8** was converted to GalNAc-t-psa (**4**) following the procedure shown in Chart 2.

***In Vivo* Distribution of Neoglycolipid-Modified Liposomes in Rats** Neoglycolipid-modified liposomes were incubated with rat plasma or PBS for 30 min, ther

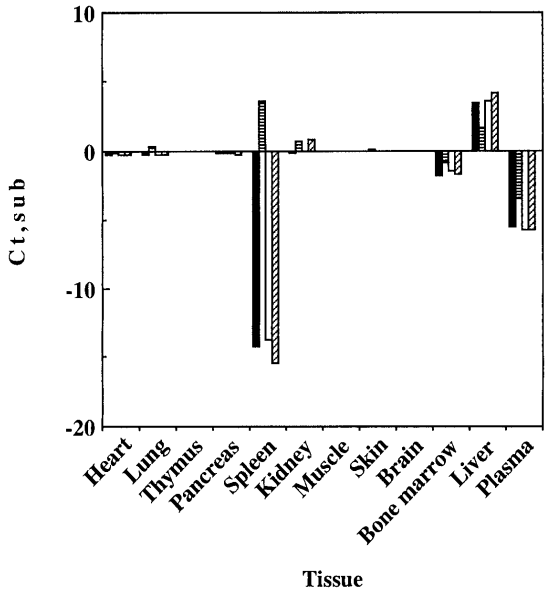

Fig. 2. Substracted Tissue Concentration ($C_{t,sub}$) of Neoglycolipid-Modified Liposomes in Twelve Tissues (6 h)

Each column represents the mean ± S.E. of three animals. ■, Gal-t-psa; ▤, Glc-t-psa; □, GalNAc-t-psa; ▨, Lac-t-psa.

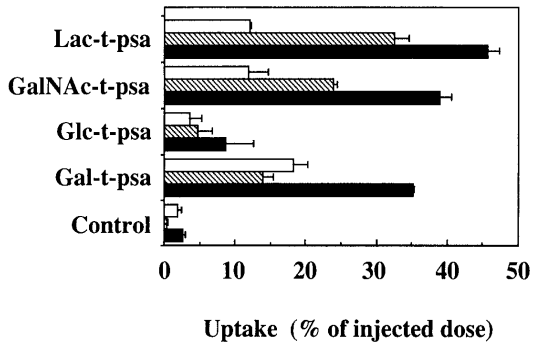

Fig. 3. Intrahepatic Distribution of Neoglycolipid-Modified Liposomes after Intravenous Injection in Rats (2 h)

Each column represents the mean ± S.E. of three animals. □, nonparenchymal; ▨, parenchymal; ■, total.

subjected to gel filtration using a Sephacryl S-400. The gel filtration after the incubation of liposomes with the rat plasma resulted in the elution of [^{3}H]inulin into the void, as after the incubation with PBS. This result shows that the liposomes modified using these neoglycolipids were not destroyed after incubation with rat plasma for 30 min.

Figure 2 shows the subtracted tissue concentrations ($C_{t,sub}$) of several organs. If the $C_{t,sub}$ of a particular tissue is positive, the accumulation of liposomes in the tissue is enhanced by the modification with a neoglycolipid. Six hours after injection of the liposomes, all of the modified liposomes were eliminated from plasma faster than the control liposomes. Liposomes modified with Gal-t-psa (**1**), Lac-t-psa (**3**) and GalNAc-t-psa (**4**) showed significantly higher accumulation in the liver than the control liposomes. Modification with these glycolipids decreased the distribution of liposomes to the spleen, presumably due to significant accumulation in the liver. Glc-t-psa (**2**) modified liposomes accumulated to a greater degree than the control liposomes in both the liver and spleen. Since there have been no reports indicating that glucose on the

liposome surface is recognized specifically by the liver and spleen, this result suggests that Glc-t-psa (**2**) modified liposomes accumulate in the reticuloendothelial system due to their allotrylic property.

The intrahepatic distribution of liposomes modified with Gal-t-psa (**1**), Lac-t-psa (**3**), GalNAc-t-psa (**4**) and Glc-t-psa (**2**) is shown in Fig. 3. Total accumulation in the liver 2 h after injection was similar to that of the *in vivo* examination 6 h after injection (Gal < GalNAc < Lac). The distribution of liposomes modified with GalNAc-t-psa (**4**) and Lac-t-psa (**3**) to the parenchymal cells was 1.7- and 2.3-fold greater, respectively, than that with Gal-t-psa (**1**). In contrast, the distribution of these two liposome preparations to non-parenchymal cells decreased. Connolly *et al.* reported that galactose and lactose were equally recognized by the asialoglycoprotein receptor in the liver *in vitro*.[13] However, the results of the present study indicate that the affinity of the lactosyl residue on the liposome surface is higher than that of the galactosyl residue. The glucose moiety of Lac-t-psa (**3**) may function as a spacer and/or affect the recognition of the terminal galactose residue by the asialoglycoprotein receptor. However, the effect of embedding ligands in the liposomal surface is unclear.

The results of the present study indicate that the modification of liposomes with Lac-t-psa (**3**) is more effective than that with Gal-t-psa (**1**) with respect to selective delivery to liver parenchymal cells.

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