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# Influence of the galactosyl ligand structure on the interaction of galactosylated liposomes with mouse peritoneal macrophages

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Liposomes bearing at their surface mono- and triantennary galactosyl ligands were prepared and their interaction with the galactose receptor of mouse peritoneal macrophages studied. Triantennary structures were synthesized by coupling derivatives of 1-thio-β-D-galactose to the amino groups of lysyl-lysine dipeptide. Galactosylated liposomes were obtained either by synthesis of neo-galactolipids followed by their incorporation into the vesicles or by neo-galactosylation of preformed liposomes by reaction between thiol-functionalized galactosyl ligands and vesicles bearing maleimido groups. The interaction of the galactosylated liposomes with the macrophage lectin was remarkably sensitive to the topology of the ligands, i.e., a spacer-arm length about 3 nm was necessary and, in contrast to results obtained with the galactose receptor of other cells, the triantennary structure did not provide additional binding. Related to the strategy of drug delivery with targeted liposomes, these results indicate that lectins from different cells might possibly be distinguished by using multiantennary ligands having optimal geometries.

*Keywords:* neogalactolipids, cluster galactosyl glycosides, liposomes, targeting, mouse peritoneal macrophages

*Abbreviations:* Gal, D-galactose; GalNAc, 2-acetamido-2-deoxy-D-galactose; PC, L-α-phosphatidylcholine; PE, L-α-phosphatidylethanolamine; DPPE, dipalmitoyl-L-α-phosphatidylethanolamine; PG, L-α-phosphatidylglycerol; SPDP, *N*-succinimidyl-3-(2-pyridyldithio)propionate; SMPB, succinimidyl-4-(*p*-maleimidophenyl)butyrate; MPB-PE, 4-(*p*-maleimidophenyl)butyryl-PE; Succ-DPPE, *N*-succinyl-DPPE; NHS, *N*-hydroxysuccinimide; DCC, *N*,*N'*-dicyclohexylcarbodiimide; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; NHS-Succ-DPPE, NHS ester of *N*-succinyl-DPPE; REV, vesicles obtained by reversed phase evaporation; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; DMEM, Dulbecco's modified Eagle's medium.

Lectins binding specifically nonreducing terminal β-D-Gal/GalNAc residues are present at the outer surface of mammalian cells such as hepatocytes [1], macrophages, e.g., Kupffer cells [2, 3], resident [4] and elicited [5] peritoneal macrophages, and of certain metastases [6]. The macrophage lectins which interact with glycoproteins and particles such as desialylated erythrocytes are believed to function in opsonin-independent phagocytosis processes. An important feature of the interaction of such receptors with their ligands is the cluster effect, i.e., these lectins show increased affinities for bi-, tri- or tetraantennary galactose structures compared to monogalactosyl ligands [7, 8]. These high affinities are believed to be due to oligomeric arrangements of these lectins in the plane of the cellular membrane [8]. It appears, however, that the galactose-binding lectins are a rather heterogeneous class of receptors, which differ by their structure [9] and their relative affinities for complex ligands [7]. Moreover, optimal recognition requires specific spatial arrangements, for example, for triantennary structures, between the different galactosyl residues [9], which might not be similar for all the lectins.

Such interactions could be used for the targeting of drugs. Indeed, neogalactosylated proteins [10, 11] and vesicles having incorporated galactose residues [12, 13] have been tested with this aim as drug carriers. Recently, we have described a chemical strategy to prepare galactosylated liposomes starting with preformed vesicles, and studied their interaction *in vitro* with mouse resident peritoneal macrophages [14]. These neogalactosylated liposomes were bound by the cells up to 4–5 times better than control vesicles. In order to explore the influence of the structure of the ligand on the binding selectivity of the targeted liposomes, we have now synthesized mono- and triantennary galactosylated structures which were incorporated into liposomes.

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## Materials and methods

Cholesterol (recrystallized in methanol), phosphatidylcholine (egg yolk), phosphatidylethanolamine (egg yolk), dipalmitoylphosphatidylethanolamine, phosphatidylglycerol, 5,5′-dithiobis(2-nitrobenzoic acid), dicyclohexylcarbodiimide, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, ninhydrin, penta-*O*-acetyl-β-D-galactopyranose, methyl β-D-galactopyranoside, SMPB and Dowex 50W resin were purchased from Sigma Chemical Co., St Louis, MO, USA. *N*-Hydroxysuccinimide, succinic anhydride, *p*-nitrophenol, 1,3-diamino-2-propanol and sodium borohydride were obtained from Fluka, Buchs, Switzerland. L-Lysyl-L-lysine.2HCl (Serva, Heidelberg, Germany) was converted into its trifluoroacetate salt after dissolution in $CF_3COOH$ at 35 °C and precipitation into diethyl ether. SPDP was synthesized according to Carlsson *et al.* [15]. 5(6)-Carboxyfluorescein, obtained from Eastman Kodak Co., Rochester, NY, USA, was purified according to Ralston *et al.* [16]. Silica gel for flash chromatography and silica gel precoated TLC plates were from Merck, Darmstadt, Germany. Acidic silica was from Bio-Rad, Richmond, CA, USA. Lectin from *Ricinus communis* (RCA I) was obtained from Boehringer, Mannheim, Germany. Polycarbonate filters were from Nucleopore, Pleasanton, CA, USA. Dulbecco's modified Eagle's medium, and fetal calf serum were obtained from Gibco (Cergy Pontoise, France) and the culture dishes were from Costar (Cambridge, MA, USA). Dimethylformamide and triethylamine were redistilled over ninhydrin before use. All other reagents were of analytical grade.

### *Instrumentation*

NMR spectra were recorded on Brucker WP-200 MHz SY and AM-400 MHz spectrometers. Positive and negative fast atom bombardment (FAB) mass spectra were obtained with a VG-Analytical ZAB-HF instrument. Matrixes used were *m*-nitrobenzylalcohol or thioglycerol containing 1% trifluoroacetic acid. Molecular masses were calculated using monoisotopic masses.

### *Synthesis of the phospholipid derivatives*

MPB-PE was prepared from egg yolk-PE and SMPB according to the method of Martin and Papahadjopoulos [17]. NHS-succ-DPPE was obtained from DPPE by procedures adapted from the literature [18, 19]. In brief, to a solution of DPPE (100 mg, 145 μmol) in 10 ml freshly redistilled pyridine was added succinic anhydride (30 mg, 300 μmol). After 2 h stirring at 55 °C under argon, the mixture was evaporated *in vacuo* to dryness and the excess succinic anhydride was hydrolysed in 10 ml $CHCl_3$:MeOH: 0.58% NaCl in $H_2O$, 1:2:0.8 by vol). After addition of 30 ml $CHCl_3$, the organic phase was then washed with 30 ml aqueous 0.58% NaCl, dried and evaporated. Extracted Succ-DPPE was purified by silicic acid chromatography as described for MPB-PE. TLC indicated a single phosphate-positive and ninhydrin-negative spot with $R_F = 0.2$ in $CHCl_3$:MeOH:$H_2O$, 70:30:5 by vol. The average yield of the conversion was about 90%. For the activation of the carboxylic function of Succ-DPPE, DCC (413 mg, 200 μmol) in 3 ml anhydrous $CH_2Cl_2$ was added dropwise to a solution of Succ-DPPE (158 mg, 200 μmol) and NHS (23 mg, 200 μmol) in 13 ml anhydrous $CH_2Cl_2$:EtOAc, 10:3 by vol. After stirring overnight under argon at room temperature, the precipitate of the corresponding urea was removed by filtration and the filtrate was evaporated under vacuum. The residue was chromatographed on a silica gel column eluted with $CHCl_3$:MeOH:$H_2O$, 70:30:5 by vol). NHS-Succ-DPPE was obtained (yield 60%) as a white solid which gave a single spot on TLC: $R_F = 0.4$ ($CHCl_3$:MeOH:$H_2O$, 70:30:5 by vol).

### *Synthesis of the thiol functionalized β-D-galactosyl residues*

The monogalactosyl derivative *N*-(3-mercaptopropionyl)-*N*′-[3-(1-deoxy-1-thio-β-D-galactopyranosyl)propionyl]-1,3-diamino-2-propanol (**8**) and the trigalactosyl derivative *N*-(3-mercaptopropionyl)-*N*′-[*Nα*-[*Nα*,*Nε*-bis[3-(1-deoxy-1-thio-β-D-galactopyranosyl)propionyl]-L-lysyl]-*Nε*-[3-(1-deoxy-1-thio-β-D-galactopyranosyl)propionyl]-L-lysyl]-1,3-diamino-2-propanol (**13**) were synthesized by procedures outlined in Fig. 1. The known intermediates 2-carboxyethyl 1-deoxy-2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-galactopyranoside (**4**) and its deacetylated homologue 2-carboxyethyl 1-deoxy-1-thio-β-D-galactopyranoside (**5**) were obtained, as described previously [14], from penta-*O*-acetyl-β-D-galactopyranoside, as colourless oils with total respective yields of 56% and 50%.

The analytical data of these compounds are given below:

Compound **4**: $R_F = 0.82$ (AcOEt/AcOH/$H_2O$, 8/2/1 by vol.

$^1$H-NMR (200 MHz, $C^2HCl_3$) δ 1.99 (s, 3H, $-COCH_3$), 2.07 (s, 3H, $-COCH_3$) 2.08 (s, 3H, $-COCH_3$), 2.17 (s, 3H, $-COCH_3$), 2.78 (t, 2H, $-SCH_2CH_2-$), 2.97 (m, 2H, $-SCH_2-$), 3.92 (t, 1H, H-5), 4.15 (m, 2H, H-6A and H-6B), 4.56 (d, 1H, H-1, $J_{1,2} = 9.86$ Hz, β-configuration), 5.05 (dd, 1H, H-3, $J_{2,3} = 10$ Hz, $J_{3,4} = 3.32$ Hz), 5.23 (t, 1H, H-2, $J_{1,2} \approx J_{2,3} = 9.9$ Hz), 5.44 (d, 1H, H-4, $J_{3,4} = 3.29$ Hz).

Compound (**5**): $R_F = 0.15$ (AcOEt:AcOH:$H_2O$, 8:2:1 by vol); $[\alpha]_D^{22} = -9.8°$ ($c = 0.5$, $H_2O$).

$^1$H-NMR (200 MHz, $^2H_2O$) δ 2.42 (t, 2H, $-SCH_2CH_2$), 2.77 (m, 2H, $-SCH_2-$), 3.31–3.46 (m, 5H, H-2, H-3, H-5, H-6A and H-6B); 3.79 (d, 1H, H-4, $J_{3,4} = 3.12$ Hz), 4.31 (d, 1H, H-1, $J_{1,2} = 9.36$ Hz, β-configuration). $FAB^+$ MS: Calculated for $C_9H_{16}O_7S$ $m/z = 268$; found: $m/z = 269$ $(M^\cdot + H)^+$, $m/z = 291$ $(M^\cdot + Na)^+$.

*N*-(2-Hydroxy-3-aminopropyl)-3-(1-deoxy-1-thio-β-D-galactopyranosyl)propionamide (**6**), was obtained in 65% yield, as described previously [14], by coupling 1,3-diamino-2-propanol to compound (**5**) in the presence of EDC in water. Compound **6** was isolated in its chlorhydrate form as a very hygroscopic white solid. It gave a single spot on TLC: $R_F = 0.13$ (AcOEt:AcOH:$H_2O$, 6:4:2 by vol).

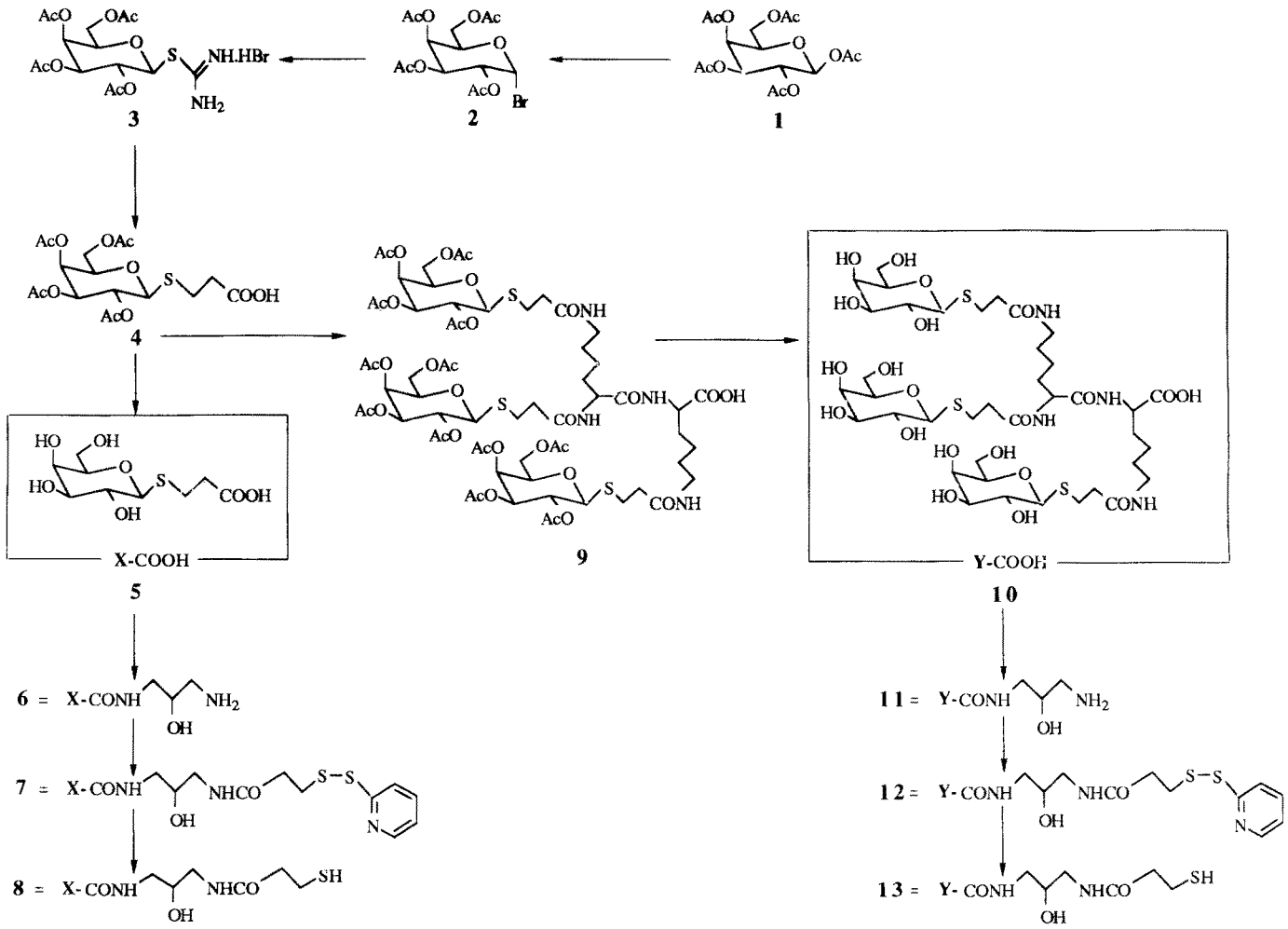


**Figure 1.** Synthesis of the thiol-functionalized mono- and trigalactosyl derivatives.

$[\alpha]_D^{22} = +1.93$ ($c = 0.5$, $H_2O$).

$^1$H-NMR (400 MHz, $^2H_2O$) $\delta$ 2.72 (t, 2H, $-SCH_2CH_2$), 3.04 (m, 2H, $-SCH_2-$), 2.97–3.26 (m, 2H, $-CH_2NH_2$), 3.40 (m, 2H, $-CONHCH_2-$), 3.57–3.63 (m, 5H, H-2, H-3, H-5, H-6A and H-6B); 4.02 (d, 1H, H-4, $J_{3,4} = 4.2$ Hz), 4.06 (m, 1H, $-CH_2CHOHCH_2-$), 4.55 (d, 1H, H-1, $J_{1,2} = 9.8$ Hz, $\beta$-configuration).

FAB$^+$ MS: Calculated for $C_{12}H_{24}O_7N_2S$ $m/z = 340.2$; found: $m/z = 341.2$ $(M^* + H)^+$, $m/z = 363.2$ $(M^* + Na)^+$.

*N*-(2-Pyridyldithiopropionyl)-*N'*-[3-(1-deoxy-1-thio-$\beta$-D-galactopyranosyl)propionyl]-1,3-diamino-2-propanol (**7**).

SPDP (850 mg, 2.66 mmol, in 50 ml ethanol) was added dropwise to a solution of compound **6** (500 mg, 1.33 mmol) in 300 ml of 50 mM sodium phosphate buffer (pH 7.0) containing EDC (1.02 g, 5.32 mmol). The mixture was stirred overnight at room temperature and evaporated *in vacuo* to dryness. The residue was loaded on a column of silica gel and eluted with $CHCl_3$:MeOH:$H_2O$, 70:30:5 by vol. Compound **7** was isolated in 58% yield as an amorphous white solid which gave a single spot on TLC: $R_F = 0.28$ ($CHCl_3$:MeOH:$H_2O$, 70:30:5 by vol).

$^1$H-NMR (200 MHz, $^2H_2O$) $\delta$ 2.71 (m, 4H, $-SCH_2CH_2-$), 3.09 (m, 4H, $-SCH_2-$) 3.25 (m, 4H, $-CONHCH_2CHOHCH_2NHCO-$), 3.51–3.75 (m, 5H, H-2, H-3, H-5, H-6A and H-6B), 3.84 (m, 1H, $-CONHCH_2CHOHCH_2-NHCO-$), 3.98 (d, 1H, H-4, $J_{3,4} = 3.03$ Hz), 4.50 (d, 1H, H-1, $J_{1,2} = 9.27$ Hz, $\beta$-configuration), 7.31 (m, 1H, Pyr H-3); 7.85 (m, 2H, Pyr H-3 and H-4); 8.41 (m, 1H, Pyr H-6).

FAB$^+$ MS: Calculated for $C_{20}H_{31}O_8N_3S_3$ $m/z = 537.1$, found: $m/z = 538.2$ $(M^* + H)^+$, $m/z = 560.1$ $(M^* + Na)^+$.

*N*-(3-Mercaptopropionyl)-*N'*-[3-(1-deoxy-1-thio-$\beta$-D-galactopyranosyl)propionyl]-1,3-diamino-2-propanol (**8**).

Compound **7** (2 mg, 3.7 µmol) was treated with $NaBH_4$ (2 mg, 52 µmol) in 100 µl of water under argon. After 30 min, at room temperature, the excess of reagent was destroyed by decreasing the pH of the reaction to 2 with HCl. As judged by sulfhydryl group determination with 5, 5'-dithiobis(2-nitrobenzoic acid) [20], after neutralization with NaOH the deprotection reaction was essentially complete. Compound **8** was used immediately without any further purification.

*Nα*-[*Nα*, *Nε*-Bis[3-(1-deoxy-1-thio-2,3,4,6-tetra-*O*-acetyl-$\beta$-D-galactopyranosyl)propionyl]-L-lysyl]-*Nε*-[3-(1-deoxy-1-thio-2,3,4,6-tetra-*O*-acetyl-$\beta$-D-galactopyranosyl)

propionyl]-L-lysine (**9**) was synthesized from L-lysyl-L-lysine, trifluoroacetate salt and compound **4** according to Ponpipom *et al.* [21]. Briefly, compound **4** (2.5 g, 5.7 mmol) was activated by esterification with *p*-nitrophenol (800 mg, 5.75 mmol) in 20 ml anhydrous $CH_2Cl_2$ in the presence of DCC (1.25 g, 6 mmol). After 3 h stirring at room temperature under argon, the urea precipitate was removed by filtration and the filtrate was purified on a silica gel column eluted with $CH_2Cl_2$:$Et_2O$, 96:4 by vol. The activated ester was isolated in 51% yield as a pale yellow solid: m.p. 94–96 °C (lit. [21] m.p. 94–96 °C). It was then added to a solution of L-lysyl-L-lysine, trifluoroacetate salt (550 mg, 1.1 mmol) in 13 ml DMF containing $Et_3N$ (650 µl, 4.62 mmol). The mixture was stirred overnight at room temperature under argon and evaporated to dryness. The residue was chromatographed on a silica gel column eluted with $CHCl_3$:MeOH:$H_2O$, 90:10:0.5 by vol. Compound **9** was isolated in 47% yield, as an amorphous white solid, homogeneous on TLC: $R_F = 0.38$ ($CHCl_3$:MeOH:$H_2O$, 90:10:0.5 by vol).

*Nα*-[*Nα*,*Nε*-Bis[3-(1-deoxy-1-thio-*β*-D-galactopyranosyl) propionyl]-L-lysyl]-*Nε*[3-(1-deoxy-1-thio-*β*-D-galacto-pyranosyl)propionyl]-L-lysine (**10**).

Compound **9** (700 mg, 0.46 mmol) was deacetylated in 7 ml of MeOH:$H_2O$:$NEt_3$, 5:4:1 by vol. After 36 h stirring at room temperature, the deprotection reaction was essentially complete and the solvents were evaporated under vacuum to dryness. The residue was purified on a column of silica gel eluted with $CHCl_3$:MeOH:$H_2O$, 60:40:10 by vol and compound **9** was isolated (yield 81%) as a colourless oil (382 mg; 0.37 mmol). TLC ($CHCl_3$:MeOH:$H_2O$, 60:40:10 by vol) indicated a single spot: $R_F = 0.14$ compared to 0.8 for the acetylated homologue.

$[\alpha]_D^{22} = -12.2$ ($c = 0.5$, $H_2O$).

$^1$H-NMR (400 MHz, $^2H_2O$) $\delta$ 2.66 (t, 4H, $-SCH_2CH_2$ COεNH–), 2.75 (t, 2H, $-SCH_2CH_2CO\alpha NH-$), 3.06 (m, 6H, $-SCH_2-$), 3.25 (m, 4H, $-CH_2NHCO-$), 3.59–3.86 (m, 15H, 3 × H-2, 3 × H-5, 3 × H-6A and 3 × H-6B), 4.03 (d, 3H, H-4, $J_{3,4} = 3.25$ Hz), 4.22 (q, 1H, αCH), 4.37 (q, 1H, αCH), 4.55 (d, 2H, H-1, $J_{1,2} = 9.70$ Hz, *β*-configuration), 4.56 (d, 1H, H-1, $J_{1,2} = 9.62$ Hz, *β*-configuration).

FAB$^+$ MS: Calculated for $C_{39}H_{68}O_{21}N_4S_3$ $m/z = 1025$; found: $m/z = 1025.9$ $(M^* + H)^+$, $m/z = 1047.9$ $(M^* + Na)^+$, $m/z = 1069.9$ $(M^* + 2Na - H)^+$.

*N*-(2-Hydroxy-3-aminopropyl)-*Nα*-[*Nα*,*Nε*-bis[3-(1-deoxy-1-thio-*β*-D-galactopyranosyl)propionyl]-L-lysyl]-*Nε*-[3-(1-deoxy-1-thio-*β*-D-galactopyranosyl)propionyl]-L-lysinamide (**11**).

To a solution of **10** (350 mg, 0.34 mmol) and 1,3-diamino-2-propanol (153 mg, 1.7 mmol) in 15 ml water adjusted to pH 5.5 with HCl, was added EDC (192 mg, 1 mmol). After 24 h stirring at room temperature the reaction was essentially complete. Compound **11** was purified by chromatography on a 30 × 2.4 cm Dowex 50W ($H^+$), 100–200 mesh, column eluted with 0.33 N HCl. Fractions containing **11**, which were positive for amine and sugar reagents, were combined and lyophilized. Chlorhydrate of compound **11** was obtained in 62% yield as a very hygroscopic white solid.

$^1$H-NMR (200 MHz, $^2H_2O$) $\delta$ 2.66 (t, 4H, $-SCH_2CH_2$ COεNH–), 2.76 (t, 2H, $-SCH_2CH_2CO\alpha NH-$), 3.06 (m, 6H, $-SCH_2-$), 2.92–3.20 (m, 2H, $-CH_2NH_2$), 3.25 (m, 4H, $-CH_2NHCO-$), 3.41 (m, 2H, $-CONHCH_2-$), 3.58–3.86 (m, 15H, 3 × H-2, 3 × H-3, 3 × H-5, 3 × H-6A and 3 × H-6B), 4.03 (d, 3H, H-4, $J_{3,4} = 3.25$ Hz), 4.06 (m, 1H, $-CONHCH_2$ $CHOHCH_2NH_2$), 4.31 (m, 2H, αCH), 4.54 (d, 2H, H-1, $J_{1,2} = 9.69$ Hz, *β*-configuration), 4.56 (d, 1H, H-1, $J_{1,2} = 9.68$ Hz, *β*-configuration).

FAB$^+$ MS: Calculated for $C_{42}H_{76}O_{21}N_6S_3$ $m/z = 1096.4$; found: $m/z = 1097.2$ $(M^* + H)^+$, $m/z = 1119.4$ $(M^* + Na)^+$.

*N*-(2-Pyridyldithiopropionyl)-*N'*-[*Nα*-[*Nα*,*Nε*-bis[3-(1-deoxy-1-thio-*β*-D-galactopyranosyl)propionyl-L-lysyl]-*Nε*-[3-(1-deoxy-1-thio-*β*-D-galactopyranosyl)-L-lysyl]-1,3-diamino-2-propanol (**12**).

SPDP (32 mg, 100 µmol) in 2 ml ethanol was added dropwise to a solution of compound **11** (56 mg, 50 µmol) in 50 mM sodium phosphate buffer (pH 7.0) containing EDC (38 mg, 200 µmol). The mixture was stirred overnight at room temperature and evaporated to dryness. The residue was loaded on a column of silica gel eluted with $CHCl_3$:MeOH:$H_2O$, 60:40:10 by vol. Compound **12** was isolated (yield 26%) as a colourless syrup (17 mg, 13 µmol). It gave a single spot on TLC: $R_F = 0.26$ ($CHCl_3$:MeOH:$H_2O$, 60:40:10 by vol).

$^1$H-NMR (200 MHz, $^2H_2O$) $\delta$ 2.60 (t, 4H, $-SCH_2CH_2$ CONH–), 2.70 (t, 4H, $-SCH_2CH_2CO\alpha NH-$); 3.0 (m, 8H, $-SCH_2-$), 3.20 (m, 8H, $-CH_2NHCO-$), 3.51–3.76 (m, 15H, 3 × H-2, 3 × H-3, 3 × H-5, 3 × H-6A and 3 × H-6B), 3.84 (m, 1H, $-CONHCH_2CHOHCH_2NHCO-$), 3.98 (d, 3H, H-4, $J_{3,4} = 2.9$ Hz), 4.28 (m, 2H, αCH), 4.49 (d, 2H, H-1, $J_{1,2} = 9.26$ Hz, *β*-configuration), 4.50 (d, 1H, H-1, $J_{1,2} = 9.20$ Hz, *β*-configuration), 7.32 (m, 1H, Pyr H-3), 7.85 (m, 2H, Pyr H-4 and H-5), 8.42 (m, 1H, Pyr H-6).

FAB$^+$ MS: Calculated for $C_{50}H_{83}O_{22}N_7S_5$ $m/z = 1293.4$; found: $m/z = 1294.2$ $(M^* + H)^+$, $m/z = 1316.3$ $(M^* + Na)^+$.

*N*-(3-Mercaptopropionyl)-*N'*-[*Nα*-[*Nα*,*Nε*-bis[3-(1-deoxy-1-thio-*β*-D-galactopyranosyl)propionyl]-L-lysyl]-*Nε*-[3-(1-deoxy-1-thio-*β*-D-galactopyranosyl)propionyl]-L-lysyl]-1,3-diamino-2-propanol (**13**). The thiol function was generated from compound **12**, as described above with **7**, by treating the dithiopyridine moiety with a 14–15-fold molar excess of $NaBH_4$.

*Synthesis of neogalactolipids (Fig. 2)*

*N*-[*N*-(*N*-Succinyl dipalmitoylphosphatidylethanolamine)-2-hydroxy-3-propionamide]-3-(1-deoxy-1-thio-*β*-D-galacto-pyranosyl)propionamide (**14**).

Compound **6** (83 mg, 220 µmol) in 6 ml of anhydrous MeOH containing $NEt_3$ (31 µl, 220 µmol) was mixed with NHS-Succ-DPPE (180 mg, 203 µmol) in 10 ml of anhydrous $CHCl_3$. After 12 h stirring under argon at room temperature the mixture was evaporated *in vacuo* to a residue which was

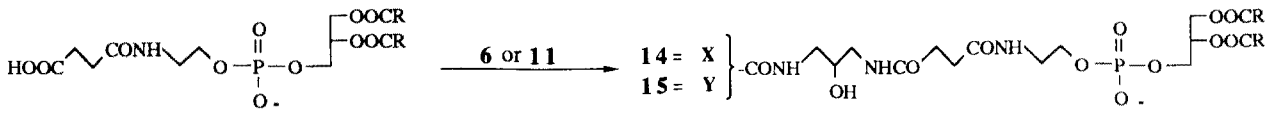


**Figure 2.** Synthesis of neogalactolipids from succinylphosphatidylethanolamine.

purified by flash chromatography on a column of silica gel eluted with $CHCl_3$:MeOH:$H_2O$, 65:25:4 by vol. Fractions containing the neomonogalactolipid, which were positive to phosphate and sugar reagents, were combined and evaporated to dryness. Compound **14** was obtained in 47% yield as a colourless amorphous solid, homogeneous on TLC: $R_F = 0.11$ ($CHCl_3$:MeOH:$H_2O$, 65:25:4 by vol).

FAB$^+$ MS: Calculated for $C_{53}H_{99}O_{17}N_3SP$ $m/z$ = 1113.4; found: $m/z = 1114.5$ $(M^* + H)^+$, $m/z = 1136.5$ $(M^* + Na)^+$, $m/z = 1158.5$ $(M^* + 2Na - H)^+$.

*N* - [*N* - (*N* - Succinyl dipalmitoylphosphatidylethanol - amine)-2-hydroxy-3-propionamide]-*Nα*-[*Nα*,*Nε*-bis[3-(1-deoxy-1-thio-*β*-D-galactopyranosyl)propionyl]-L-lysyl]-*Nε*[3-(1-deoxy-1-thio-*β*-D-galactopyranosyl)propionyl]-L-lysinamide (**15**).

Compound **11** (75 mg, 66 μmol) was mixed with NHS-succ-DPPE (53 mg, 60 μmol) in 10 ml of DMF containing $NEt_3$ (10 μl, 70 μmol). After 5 days stirring under argon at 55 °C the mixture was evaporated *in vacuo* to a residue which was purified by flash chromatography on a silica gel column eluted with CHCl:MeOH:$H_2O$, 60:40:10 by vol. Fractions containing neotrigalactolipid were combined and evaporated to dryness. Compound **15** was obtained in 18% yield as a colourless amorphous solid homogeneous on TLC: $R_F = 0.36$ ($CHCl_3$:MeOH:$H_2O$, 60:40:10 by vol).

FAB$^-$ MS: Calculated for $C_{93}H_{152}O_{31}N_7S_3P$ $m/z$ = 1870; found: 1869 $(M^* - H)^-$.

*Preparation of the galactosylated vesicles*

Large unilamellar vesicles were prepared from 15 μmol lipids (PC, cholesterol, MPB-PE or neogalactolipids at molar ratios indicated in the Results section) by the reversed phase evaporation technique [22], and were extruded through 0.2 μm polycarbonate filters [13]. 5(6)-Carboxyfluorescein (40 mM) to be encapsulated was in 5 mM Hepes buffer (pH 7.4) containing 100 mM NaCl (290 mosmol/kg). For the coupling of ligands to preformed liposomes, freshly prepared vesicles (1 ml) containing MPB-PE were mixed with a threefold molar excess of compound **8** or **13** over MPB-PE content. After 45 min at room temperature under argon, the neogalactosylated liposomes were separated from unencapculated molecules, excess ligands and from reagents by filtration on a 1 × 18 cm Sephadex G-75 column equilibrated and eluted with 5 mM Hepes buffer (pH 7.4) containing 150 mM NaCl. Control liposomes (i.e., non-galactosylated liposomes) contained PG instead of MPB-PE or neogalactolipids. Liposomes were analysed for their phosphorus [24] and galactose [25] contents after prior hydrolysis of the thioether bond in the presence of mercuric acetate [26].

*Interaction between the galactosylated liposomes and RCA-I*

Galactosylated liposomes (final concentration 60 μM) were incubated with RCA-I (40 μg/ml) in a cuvette containing 1 ml (final volume) of 5 mM Hepes buffer (pH 7.4) and 150 mM NaCl. After rapid mixing, aggregation of the vesicles was estimated, at 22 °C, by the time dependent increase in turbidity as measured by the absorbance at 360 nm with a Shimadzu (Model MPS-2000) spectrophotometer equipped with a graphic printer (PR-3). The reversibility of the aggregation was assessed by addition of free methyl *β*-D-galactopyranoside.

*Interaction between the galactosylated liposomes and isolated resident mouse peritoneal macrophages*

Resident peritoneal macrophages, obtained as described [27] from female Balb/c mice (between 6 and 12 weeks old) in DMEM containing 10% FCS, were plated in 1 cm diameter wells of 24-wells plates (Costar) at $1 \times 10^6$ cells per 1 ml (final volume) and maintained for 2 h at 37 °C in a humidified atmosphere of 5% $CO_2$ in air. Nonadherent cells were then eliminated by rinsing the wells three times with DMEM. The adherent macrophages were fed with fresh serumless DMEM and incubated for 2 h at 37 °C with varying amounts of galactosylated or control liposomes containing 40 mM 5(6)-carboxyfluorescein. After the incubation time, the medium was pipetted off and the cells washed six times with phosphate-buffered saline (PBS: 6.48 mM $Na_2HPO_4$, 1.47 mM $KH_2PO_4$, 2.68 mM KCl, 136.75 mM NaCl; pH 7.4). Carboxyfluorescein associated with the cells was measured fluorometrically as described previously [28] after cell digestion in 2 ml PBS containing 1% (v/v) Triton X-100. Standard curves were established under the same conditions with the initial liposome preparations. The results given later in Fig. 5 are those of typical experiments. The points are means of at least duplicates which do not differ by more than 10%.

The stability of the liposome preparations was assessed by their ability to retain encapsulated 5(6)-carboxyfluorescein (self-quenching method [28]). Under our conditions more than 90% of the dye remained encapsulated, for all types of liposomes, at the term of the incubation period.

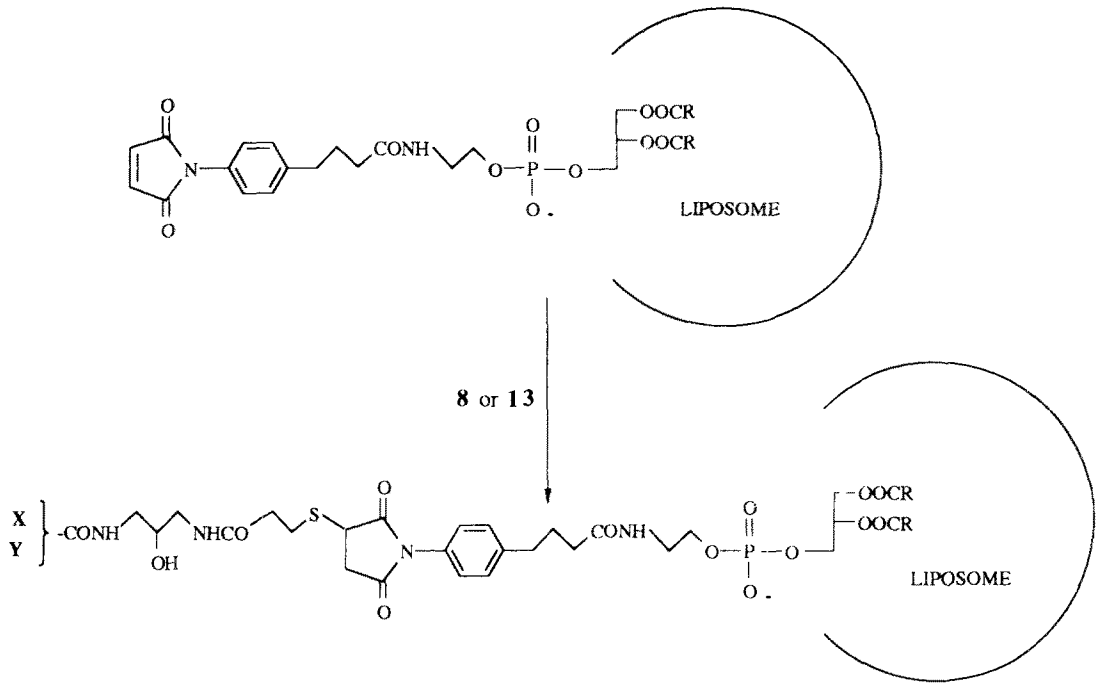


**Figure 3.** Preparation of neogalactosylated liposomes.

## Results and discussion

To explore some structural parameters which are of importance in the interaction of galactosylated liposomes with the galactose receptor expressed at the surface of mouse peritoneal macrophages, we have prepared vesicles bearing several synthetic galactosides. Mono- and trigalactosyl ligands (derivatives of **X** and **Y**, Fig. 1) were synthesized and conjugated to (i) preformed vesicles containing MPB-PE, according to the general technique of Martin and Papahadjopoulos [17], to give neogalactosylated liposomes (Fig. 3) or (ii) a derivative of phosphatidylethanolamine to give neogalactolipids (Fig. 2). These galactolipids could then be used in the preparation of liposomes. We could therefore compare mono- and triantennary galactoside structures which are also differently spaced from the liposome surface.

### *Chemistry*

Synthetic approaches for the preparation of glycosylated liposomes give access to chemically defined and modulable structures, both in the carbohydrate and the lipophilic anchoring moieties, and allow the introduction of spacer arms of variable length, between the liposome surface and the ligands. In this study, derivatives of 1-thio-$\beta$-D-galactopyranose were chosen as building blocks (i.e., compound **5**) because of their greater stability when compared with their *O*-galactosyl homologues. Moreover, in order to provide good accessibility of the ligands, the thiogalactose moieties were conjugated to the vesicles via hydrophilic spacer arms.

*Synthesis of thiol-functionalized galactose ligands and preparation of neogalactosylated liposomes:* The synthesis of a compound analogous to **8** (Fig. 1), starting from pentaacetyl-$\beta$-D-galactopyranose (**1**), has been described in our previous publication [14]. In the present work, however, in order to avoid the side reactions due to succinimidyl *S*-acetylthioacetate [29, 14], we used SPDP for the thiolation step, i.e., **6** → **8**. The intermediate **4** was used to prepare the triantennary galactose ligand (**13**). After activation of its carboxylic acid function with *p*-nitrophenol in the presence of DCC, it was reacted with the amino groups of L-lysyl-L-lysine, to afford the protected trigalactopeptide **9**. Compound **10**, obtained by treating **9** in a mixture of $MeOH:H_2O:NEt_3$, was linked to 1,3-diamino-2-propanol in the presence of a water-soluble carbodiimide. SPDP was then coupled at the extremity of the spacer arm of **11** via its activated acid function, and the terminal thiol group of **13** was generated by reduction of the dithiopyridine moiety with an excess of $NaBH_4$. The thiogalactosyl ligands **8** and **13** were coupled directly to preformed liposomes containing MPB-PE (i.e., a derivative of PE bearing an electrophilic maleimide residue) via their thiol-terminated spacer arm (Fig. 3). This reaction occurred readily, in quantitative yields, in a homogeneous aqueous medium under mild conditions of pH and temperature. Using this approach, the liposomal content (i.e., encapsulated drug or marker) must, however, be unreactive toward thiol groups, since it was found by sugar

analysis that the functionalized galactose **8** penetrates the vesicles.

*Neogalactolipids:* The amino-terminated spacer arm derivatives of monogalactosyl (**6**) and trigalactosyl (**11**) compounds, used as intermediates in the syntheses described above, were linked via an amide bond to succinyl-DPPE after activation of the carboxylic acid function of the phospholipid with NHS in presence of a carbodiimide (Fig. 2). The resulting neogalactolipids **14** and **15** were mixed with the other lipids used for the preparation of liposomes. As shown by sugar analysis, these neogalactolipids were incorporated quantitatively into the liposomes.

*Topological considerations:* Using extended conformations, in the neomonogalactolipid **14**, the distance measured between the sulfur atom at position 1 of the galactose and the phosphorus atom of DPPE was about 2.15 nm compared to 3.35 nm in the MPB-PE conjugate with the monogalactosyl derivative **8**. In the neotrigalactolipid **15**, the distance measured between the sulfur atom at position 1 of the galactose residue linked to the $\alpha NH_2$ of the lysyl-lysine dipeptide and the phosphorus atom of DPPE was 2.87 nm compared to 4.07 nm in the MPB-PE conjugate with trigalactosyl derivative **13**. For the trigalactosylated ligand (**Y**), in the absence of conformational data in solution, an approximation of the distances between the galactosyl residues is given by considering their C-1 sulfur atoms at the apexes of a triangle of sides 1.4, 1.5 and 2.4 nm.

*Interaction of the galactosylated liposomes with lectins:* Interaction between galactosylated liposomes prepared above and lectins (i.e., soluble such as RCA-I and cellular such as the galactose receptor on macrophages) could thus be studied as a function of (i) the structure of the galactosyl ligand (mono- or triantennary) and (ii) their exposure at the surface of the vesicles (length of the ligand-bearing spacer arm).

*Ricin-mediated aggregation of the galactosylated liposomes:* In order to check if the galactosyl ligands were accessible at the surface of the liposomes, prepared according to our methodologies, a classical method consists of testing their agglutination promoted by the lectin from *Ricinus communis* (RCA-I) [30]. This lectin, which is a dimer, has two binding sites selective for terminal β-D-galactosyl residues. By binding and crosslinking the galactosylated vesicles it can provoke their agglutination. In Fig. 4 are given the turbidity (light scattering) increases observed after addition of RCA-I to liposomes containing 15 mol% of β-D-thiogalactosyl residues (mono- and triantennary structures). The conditions used for RCA-I concentration and surface density of galactosyl

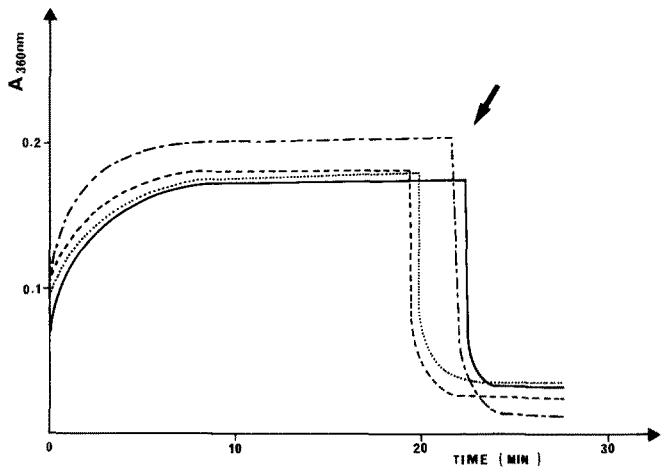


**Figure 4.** Aggregation of the galactosylated liposomes by *Ricinus communis* lectin. Liposomes (60 μM phospholipid) were incubated in 1 ml 5 mM Hepes buffer (pH 7.4), 150 mM NaCl at 22 °C. Time courses of turbidity changes (at 360 nm) were recorded after the addition of the lectin (final concentration: 40 μg/ml). At the arrow, methyl-β-D-galactopyranoside was added (4.5 mM). Liposomes composed of PC/cholesterol/MPB-PE (8.5:5:1.5 molar ratio) conjugated to compound **8** (———) or to **13** (— — — —). (· · · · · ·) Liposomes composed of PC/cholesterol/derivative **14** (8.5:5:1.5) or (– – – – –) liposomes composed of PC/cholesterol/derivative **15** (9.5:5:0.5).

residues were optimal for observing an interaction, indeed threshold and saturation phenomena in galactosylated liposome/lectin interactions were previously found [14]. Figure 4 indicates that all the galactosylated liposomes tested were agglutinated by RCA-I. This effect could be reversed with 4.5 mM free methyl β-D-galactopyranose, providing evidence for the specific nature of the interaction studied. Thus our approach yields vesicles whose mono- and triantennary galactosyl residues are well exposed and are effective and specific ligands of RCA-I.

*Interaction between the galactosylated liposomes and peritoneal macrophages:* Mouse peritoneal macrophages present, at their surface, a well characterized receptor for galactose which presents an increased affinity for clustered ligands, such as triantennary structures [7]. The specific association of neogalactosylated liposomes to this receptor has already been studied in a previous work, suggesting also a cluster effect [14].

In order to check whether an oligomeric ligand, such as the trigalactosyl structure **Y** (Fig. 1), when present at the surface of liposomes, promotes more potent and specific interactions with cellular galactose receptor than a monogalactosyl ligand (e.g., monogalactosyl structure **X**), we studied the interaction of galactosylated large unilamellar vesicles with mouse resident peritoneal macrophages. The liposomes were prepared by the reverse phase evaporation technique from egg PC and cholesterol. Such a composition was chosen to minimize their nonspecific interaction with

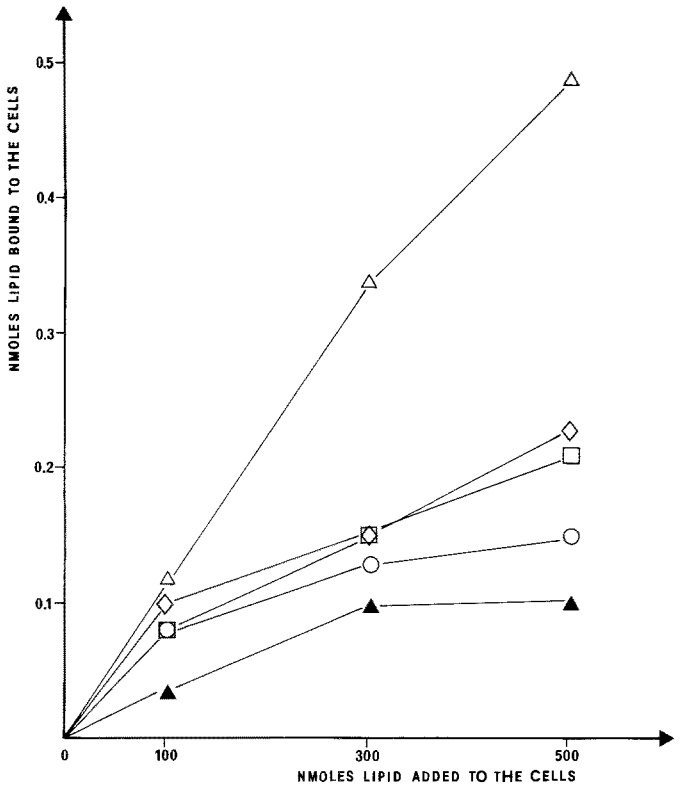


**Figure 5.** Influence of the structure of the galactosyl ligand and of the spacer arm on the association of the galactosylated liposomes with mouse peritoneal macrophages. Resident peritoneal macrophages ($10^6$ cells/well containing 1 ml of DMEM) were incubated 2 h at 37 °C with increasing amounts of liposomes (0–500 nmoles phospholipids) containing 40 mM carboxyfluorescein and composed of △, PC:cholesterol:MPB-PE (8.5:5:1.5) conjugated to **8**; ◇, PC:cholesterol:MPB-PE (9.5:5:0.5) conjugated to **13**; □, PC:cholesterol:derivative **14** (8.5:5:1.5); ▲, PC:cholesterol:derivative **15** (9.5:5:0.5); ○, PC:cholesterol:PG (8.5:5:1.5), control vesicles. Results are given as amounts of liposomes associated to $10^6$ cells.

cells and to increase their stability. From our previous study [14], it was known that a galactose density above 5 mol% at the surface of the liposomes was required to observe a specific association of galactosylated liposomes with peritoneal macrophages, and that serum inhibits liposome/cell association. Thus the liposomes tested here contained at least 15 mol% of thiogalactopyranose and the interaction experiments with cells were carried out in serum-free DMEM. The liposome/cell association was quantified by fluorometric methods using vesicles which had encapsulated high concentrations of 5(6)-carboxyfluorescein.

Figure 5 indicates the amount of liposomes bound to cells as a function of added liposomes for the five types of vesicles tested. The association of the liposomes containing 15 mol% of the monogalactosyl derivative **8** conjugated to MPB-PE was 4–5-fold greater than control. As demonstrated previously [14], this association was due to a specific interaction between the galactosyl residues at the surface of the vesicles and a galactose-specific receptor. Thus, the binding was $Ca^{2+}$-dependent, and at 4 °C was abolished by an excess of free galactose; moreover, the specific binding of this galactosylated liposome containing 5(6)-carboxyfluorescein was inhibited by void vesicles of similar composition (not shown). Phagocytosis of the targeted vesicles was inferred, at 37 °C, by the reduction of the fluorescent marker uptake by the cells in the presence of $NH_4Cl$ [14]. Surprisingly, however, a 2–3-fold lesser interaction was achieved with liposomes containing 5 mol% of the trigalactosyl ligand **Y**, regardless of its mode of association to the vesicles, i.e., **13** conjugated to MPB-PE in preformed liposomes or introduced as the neotrigalactolipid **15**. That this was not due to a threshold phenomenon was evidenced by the fact that increased concentrations of the trigalactosyl ligand **Y** at the surface of the vesicles (from 5 mol% to 10 or 15 mol%, not shown) did not affect this liposome/cell association. More subtle steric factors must probably account for this observation. As discussed in a previous paper [14], the lateral mobility of the phospholipids in liposomal membranes at 37 °C and the conformational flexibility of the MPB-PE derivative with compound **8**, should allow the formation of a favourable arrangement in which monogalactosyl ligands may interact simultaneously, with some affinity, with a clustered arrangement of galactose receptors which form a binding unit at the cell surface. In contrast, in the branched derivative **Y**, the spatial organization of the galactosyl ligands, which is fixed by the chemical structure of **Y**, does not seem to be optimal for its interaction with these macrophage receptor binding units. The trigalactosyl head groups are probably too bulky to compensate this unfavourable arrangement with an optimal clustering of the anchoring lipids in the bilayer of the vesicles.

As indicated in Fig. 5, liposomes containing 15 mol% of the neomonogalactolipid **14** were found to interact with cells less than control vesicles. This lack of specific binding is in sharp contrast to the favourable interaction observed with neogalactosylated liposomes containing **8** (see above). This observation can, in principle, be attributed to the shorter spacer arm of **14**, but unfavourable conformations in solution cannot be overlooked. In this case, where galactose residues seem to be inaccessible for their cellular receptor, a decreased liposome/cell association by hydrophilic coating of the vesicles is reminiscent of the stealth-liposomes [31].

## Conclusion

The interaction of galactosylated liposomes with the galactose receptor of mouse resident peritoneal macrophages showed an important sensitivity to the ligand topology. The following points are of interest. (i) A minimal distance between the vesicle surface and the β-D-galactose residue seems to be required, i.e., compare the liposomes neogalactosylated with compound **8** with those incorporating the

neomonogalactolipid **14**. (ii) A reduced affinity, compared to control vesicles, was observed with liposomes bearing the triantennary ligand (**Y**), in which the galactosyl residues can probably not adopt the adequate topology for optimal binding. Importantly, the steric requirements of the cellular lectin seem to be more stringent than those of a soluble lectin, such as ricin, which bound indiscriminately the four types of galactosylated liposomes used in this study. Related to our work, Kempen *et al.* [32] have synthesized a tris-galactoside-terminated cholesterol. Its incorporation into liposomes led to a rapid clearance from circulation and uptake by liver, mainly by Kupffer cells [33]. The use of a polylysine backbone for the synthesis of branched glycopeptides was proposed by Ponpipom *et al.* [21]. This group, however, mainly used a triantennary mannosyl derivative for their targeting studies [34].

Ligand topology is known to have a paramount influence on its interaction with the galactose receptor at two different levels. (i) The cluster effect, e.g., compared to a single galactosyl residue, triantennary ligands have an increased affinity (up to $10^5$-fold [35]). (ii) The intergalactosyl distances are also important for the binding, e.g., for the galactose-receptor of hepatocytes, the three galactosyl head groups of a triantennary ligand are optimally located at the apexes of a triangle of sides 1.5, 2.2 and 2.5 nm [35]. These considerations are of importance in the design of liposomes targeted towards cellular lectins. It is probable that the topological characteristics of the different receptor binding units at the surface of galactose receptor-bearing cells are different. This could allow the selection of an optimal polyglycosidic ligand (i.e., adequate oligomeric ligand possessing the right intrinsic structure for optimal binding) for each class of cells.

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