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The effect of a water-soluble tris-galactoside terminated cholesterol derivative on the in vivo fate of small unilamellar vesicles in rats

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When the water-soluble cholesterol derivative, *N*-[tris[(β-D-galactopyranosyloxy)methyl]methyl]-N^{α}-[4-(5-cholesten-3β-yloxy)succinyl]glycinamide (tris-gal-chol) (Kempen et al. (1984) J. Medicin. Chem. 27, 1306–1312) is added as an aqueous micellar solution to a dispersion of small unilamellar phospholipid vesicles it rapidly associates with the vesicles, without causing significant leakage of liposome contents. Incorporation of 10 mol% tris-gal-chol in the liposomal membrane caused a substantial increase in the rate and extent of rat liver uptake and a shift in intrahepatic distribution of an intravenously administered dose of liposomes. For neutral liposomes composed of equimolar amounts of cholesterol and sphingomyelin incorporation of tris-gal-chol led to a 7-fold increase in total liver uptake, which was mainly accounted for by an increase in uptake by the Kupffer cells (12-fold) and by only a small increase in uptake by the hepatocytes (1.4-fold). The increased liver uptake is blocked by preinjection of *N*-acetyl-D-galactosamine and not affected by preinjection of *N*-acetyl-D-glucosamine. This indicates that the increased interaction of liposomes as a result of tris-gal-chol incorporation is mediated by galactose-specific recognition sites on both Kupffer cells and hepatocytes. Targeting of liposomes to the asialoglycoprotein receptor of the hepatocytes is thus frustrated by the highly active galactose-specific receptor on Kupffer cells. Comparable results on lactosylceramide incorporation into liposomes were recently reported by us (Spanjer et al. (1984) Biochim. Biophys. Acta 774, 49–55).

Introduction

Mammalian liver cells possess on their plasma membrane a specific receptor for glycoproteins whose oligosaccharide chains have a β-linked terminal galactose or *N*-acetyl-galactosamine [1]. Binding of a glycoprotein to this receptor is followed by internalization through endocytosis. By attaching a terminal galactose to other, larger, particles these can also be recognized by this receptor and subsequently internalized; uptake of galactosylated low density lipoprotein (LDL) by hepatocytes was shown to proceed through the galactose receptor [2]. Similarly, this receptor would be an attractive target for delivery of drug-containing liposomes to the hepatocytes [3–6]. With this goal in mind we designed a molecule with three recognition sites for the galactose receptor allowing optimal receptor interaction [7], and which should readily associate with lipid-contain-

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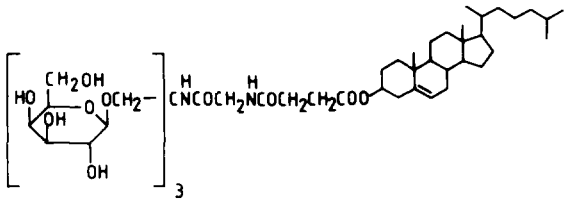

Diagram I. Structure of *N*-[tris[(β-D-galactopyranosyloxy)methyl]methyl]-N^{α}-[4-(5-cholesten-3β-yloxy)succinyl]glycinamide. Short name: tris-gal-chol.

ing particles. The compound with the structure shown in diagram I was prepared by coupling tris(galactosyloxymethyl)aminomethane to cholesterol using glycyl and succinyl as intermediate hydrophilic spacer moieties [8]. The resulting triantennary galactose-terminated cholesterol derivative (tris-gal-chol) was found to form optically clear micellar solutions in water and to associate rapidly with lipid-containing particles like LDL and high density lipoprotein (HDL) [8]. The present paper describes the effect of association of tris-gal-chol with small unilamellar vesicles on blood elimination, liver uptake and intra-hepatic distribution after intravenous injection into rats.

Materials and Methods

Materials

Cholesterol, sphingomyelin, L-α-phosphatidylserine from bovine brain, *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, collagenase type I and fetuin were purchased from Sigma. Fetuin was desialylated as described before [9]. Metrizamide was from Nyegaard & Co, [^{3}H]inulin (904 mCi/mmol) was from Amersham International, Sepharose CL-2B from Pharmacia and Diaflow ultrafiltration membranes PM 10 were obtained from Amicon. Tris-galactosyl-cholesterol (tris-gal-chol) was synthesized as described [8].

Preparation of liposomes

Small unilamellar liposomes composed of lipids as indicated in the legends to the figures and table were made as described before [9,10], with [^{3}H]inulin as a marker for the internal water space. Size determinations were done as described before [9] and the presence of vesicles larger than 50 nm in the total population was estimated according to Barrow and Lentz [11].

Animals

Male Wistar rats were used varying in body weight from 190 to 230 g. Determinations of blood elimination and liver uptake at time intervals exceeding 10 min after liposome administration were performed as described in Ref. 9. Blood elimination studies up to 10 min followed by the determination of total liver uptake and intra-hepatic distribution were done as follows: rats were anesthetized by intraperitoneal injection of 20 mg nembutal. The abdomen was opened and the liposomes were injected in the inferior vena cava at the level of the renal veins. The body temperature of the animals was maintained at 36.5 to 37°C (rectal) by an infra-red heating lamp. At the indicated times 0.2 ml blood was taken from the inferior vena cava at least 2 cm distal of the injection point for the determination of radioactivity as described [9]. At 10 min after injection the vena portae was canulated and a liver perfusion was started with Hanks' buffer with 10 mM Hepes (pH 7.4) at 8°C. After 8 min a liver lobule was tied off and excised for the determination of the total liver uptake by weighing the lobule, determining its radioactivity content and extrapolating this to total liver uptake on the assumption that the liver makes up 3.75% of the total body weight [12]. In order to separate the various cell types the liver perfusion was continued at low temperature (8°C) with inclusion of 0.05% w/v collagenase. After 15 min of perfusion with collagenase the liver was excised, cut into pieces in ice-cold Hanks'-Hepes buffer and the suspension was filtered through a nylon gauze with 90-μm pores. The filtrate was centrifuged for 30 s at $50 \times g$. The pellets were resuspended and this procedure was repeated three times. The final pellet consisted of pure (100%) parenchymal cells as judged by light microscopic examination and the absence of M_2-type pyruvate kinase [13], with a yield of about 200 mg protein (10%). The first two supernatants of the 30 s $50 \times g$ centrifugation, which contained the non-parenchymal cells, were combined and centrifuged again for 30 s at $50 \times g$ in order to remove any residual parenchymal cells. The supernatant was centrifuged for 10 min at $500 \times g$ and the resulting pellet was resuspended in 5 ml Hanks' buffer + Hepes, mixed with 7.3 ml 30% metrizamide and distributed over two Sorvall tubes. On top of the mixture 0.5 ml Hanks' +

Hepes + albumin (4 g/l) was added and the tubes were centrifuged for 15 min at 1400 × *g*. The cells which had floated to the top phase were aspirated in a syringe and washed with Hanks' buffer (two times 5 min at 500 × *g*). As was determined before [14] the non-parenchymal cells thus obtained contained less than 1% parenchymal cells (approx. 7% as protein), 70% Kupffer cells (77% as protein) and 30% endothelial cells (16% as protein). Kupffer and endothelial cells could be discriminated by peroxidase staining and Papanicolaou counterstaining [14] while the presence of parenchymal cells could be detected by analyzing the isoenzymes of pyruvate kinase [13]. As found earlier with other substrates [14,15] no loss of cell bound radioactivity occurred during the low temperature cell isolation procedure leading to a quantitative recovery of the radioactivity associated with total liver in the subsequently isolated cells. A further subdivision of the non-parenchymal cell fraction into an endothelial and a Kupffer cell fraction was performed exactly as described earlier [14]. Tris-gal-chol was added to the liposomes at room temperature 10 min before injection. Asialofetuin (25 mg), *N*-acetyl-D-galactosamine and *N*-acetyl-D-glucosamine (110 mg) were tested for their effect on the liver uptake and were injected as a bolus one min before the injection of liposomes. Liposomes were injected in a dose of 2 μmol total lipid per 100 g body weight.

Subcellular fractionation

Fractionation of total liver was performed by differential centrifugation according to De Duve et al. [16].

Assay of enzyme activities

5'-Nucleotidase activity was determined according to Ref. 17 and acid phosphatase activity according to Ref. 18.

Results

Addition of 10 mol% tris-gal-chol to small unilamellar vesicles composed of cholesterol, sphingomyelin and phosphatidylserine in a molar ratio of 5 : 4 : 1 led to a small variation from the control values for blood elimination as shown in Fig. 1 panel A. Liver uptake values of the negatively charged liposomes with and without tris-gal-chol at four hours after injection were 56.7% and 61.4% of the injected dose, respectively. The influence of tris-gal-chol on the blood elimination of liposomes composed of cholesterol and sphingomyelin only was, however, much more pronounced as is shown in Fig. 1 panel B. There is also a large effect on liver uptake as shown in Fig. 2. Here, at two hours after injection, an almost 7-fold increase in liver uptake is measured: from 7.3% of the injected dose for the control vesicles to 47.8% for the liposomes with tris-gal-chol. Since in an earlier study we found a striking stimulation of

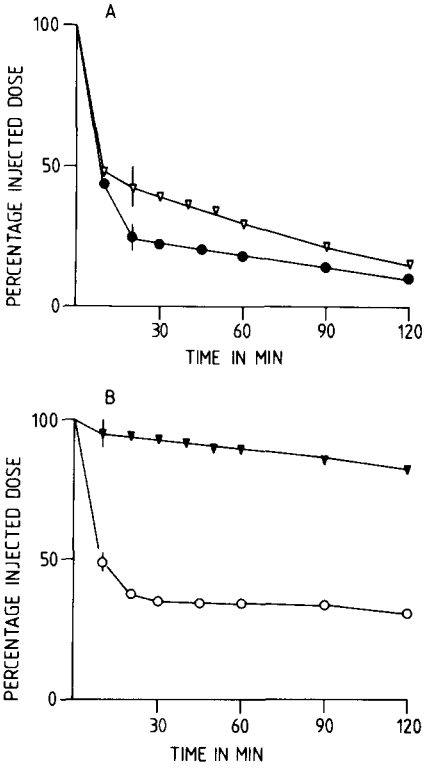

Fig. 1. Elimination of control liposomes and tris-gal-chol liposomes from blood. (Panel A) Rats were injected with negatively charged liposomes composed of cholesterol, sphingomyelin and phosphatidylserine in a 5 : 4 : 1 molar ratio (▽———▽) or with these liposomes after incorporation of 10 mol% tris-gal-chol (●———●). (Panel B) Rats were injected with neutral liposomes composed of cholesterol and sphingomyelin in an equimolar ratio (▼———▼) or with these liposomes after incorporation of 10 mol% tris-gal-chol (○———○). Liposomes were injected in a dose of 2 μmol per 100 g body weight. Blood liposome content was determined as described in Methods.

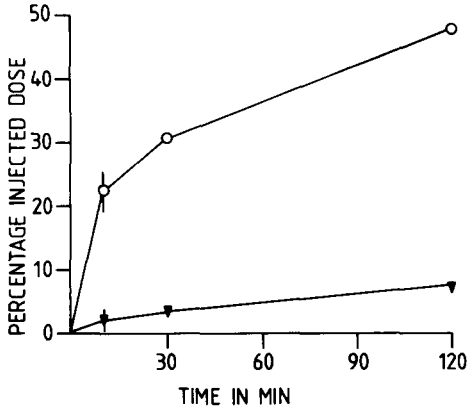

Fig. 2. Liver uptake of control liposomes and tris-gal-chol liposomes. Rats were injected with neutral liposomes composed of cholesterol and sphingomyelin in an equimolar ratio (▼———▼) or with these liposomes after incorporation of 10 mol% tris-gal-chol (○———○). Liposomes were injected in a dose of 2 μmol per 100 g body weight. Total liver uptake was determined as described in Methods.

Kupffer cell uptake of liposomes as a result of lactosylceramide incorporation [10] it was also of interest to determine which cell type in the liver was responsible for the increased uptake of the tris-gal-chol liposomes. Because the effect of tris-gal-chol is particularly pronounced in the first minutes after liposome administration and since inhibition studies with acetylated amino-sugars are restricted to short term incubations due to their rapid excretion, we measured the effects of tris-gal-chol addition shortly after injection (10 min). Fig. 3 shows the blood elimination curves of tris-gal-chol-containing liposomes with and without preinjection of various test compounds. The results show that preinjection of asialofetuin does not influence the blood elimination of tris-gal-chol liposomes. Considerable inhibition was, however, accomplished by preinjection of GalNAc, whereas GlcNAc did not have an appreciable effect. The liver uptake values of the tris-gal-chol loaded liposomes were invertedly related to the blood elimination with the various inhibitors. With the liver uptake values of tris-gal-chol liposomes without preinjection set at 100, the relative liver uptake values after preinjection of asialofetuin, GalNAc and GlcNAc were 122, 34 and 82, respectively. The specific inhibition by GalNAc indicates that galactosyl-residue binding sites play a role in the increase in total liver uptake due to tris-gal-chol incorporation into the liposomes. In order to establish which cell types contribute to the total liver uptake of the liposomes and which cells specifically recognize the exposed galactosyl-residues, we separated the liver into a parenchymal, a Kupffer and an endothelial cell fraction. For control liposomes the uptake at 10 min by hepatocytes, Kupffer cells and endothelial cells was 15, 424 and 97% of the injected dose $\times 10^4$ per mg protein, respectively, whereas incorporation of tris-gal-chol into the liposomes resulted in uptake values of 22, 4877 and 1118% of the injected dose $\times 10^4$ per mg protein, respectively. Thus, both parenchymal cells and non-parenchymal cells contribute to the total liver uptake of both control and tris-gal-chol loaded liposomes. The data for the endothelial cell fraction were not corrected for Kupffer cell contamination and thus the measured uptake of the endothelial cell fraction is likely to reflect the presence of Kupffer cells. The constant ratio of uptake by the Kupffer cell fraction over the uptake by the endothelial cell fraction for control and tris-gal-chol liposomes (4.4) is compatible with this assumption. Since it is known that Kupffer cells have a preference for uptake of large particles over smaller particles including liposomes [19] the observed tris-gal-chol induced increase in Kupffer cell up-

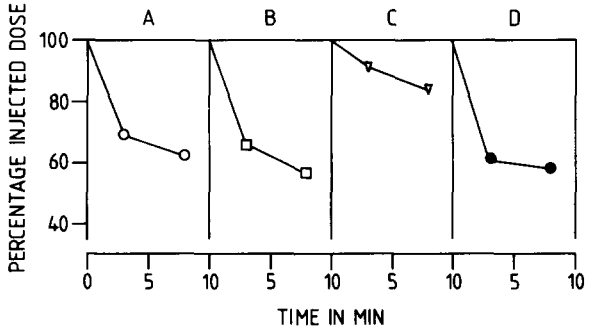

Fig. 3. Elimination of tris-gal-chol liposomes from blood. Effect of preinjection of various compounds. Rats were injected with liposomes composed of cholesterol and sphingomyelin in an equimolar ratio and containing 10 mol% tris-gal-chol in a dose of 2 μmol per 100 g body weight. Liposome elimination from blood was determined (panel A, ○———○) and liposome elimination was also determined after preinjection of asialofetuin (panel B, □———□), *N*-acetyl-D-galactosamine (GalNAc) (panel C, ▽———▽), or *N*-acetyl-D-glucosamine (GlcNAc) (panel D, ●———●).

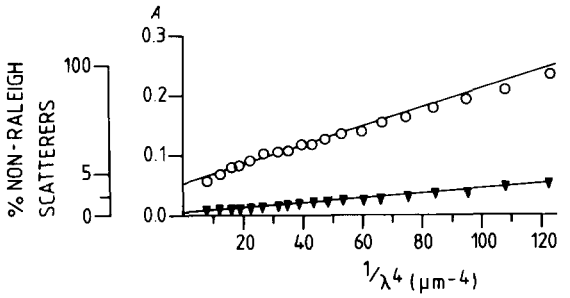

Fig. 4. Determination of non-Raleigh scatterers in liposome preparations with and without tris-gal-chol. The absorbance of small unilamellar vesicles was determined between 300 and 600 nm and plotted against the reciprocal fourth power of the wavelength. Measurements were performed with liposomes composed of cholesterol and sphingomyelin in an equimolar ratio (▼———▼) and with such liposomes after incorporation of 10 mol% tris-gal-chol (○———○). The percentage non-Raleigh scatterers was estimated from the data of Barrow and Lentz [11].

take could conceivably be due to an increase in liposome size as a result of tris-gal-chol incorporation. The effect of tris-gal-chol on liposome size was investigated according to Barrow and Lentz [11]. The absorbances of the liposome preparations were measured between 300 and 600 nm and plotted against the reciprocal fourth power of the wavelength (Fig. 4). The intercept with the ordinate provides an estimate of the proportion of vesicles which do not behave as Raleigh scatterers, i.e. with diameters larger than 50 nm [11]. From the internal volume of the control liposomes a mean diam-

TABLE I

GALACTOSE-SPECIFIC INHIBITION OF TRIS-GAL-CHOL LIPOSOME UPTAKE BY CELL FRACTIONS OF THE LIVER

Rats were injected with liposomes composed of cholesterol and sphingomyelin in an equimolar ratio and containing 10 mol%tris-gal-chol in a dose of 2 μmol per 100 g body weight. The isolation of a parenchymal and a non-parenchymal cell fraction was started 10 min after injection. Preinjections were done 1 min before liposome administration. Liposome uptake in these cell fractions was determined as described in Methods.

Preinjection with	Uptake (%ID × 10^3/mg protein)	
	Parenchymal cell fraction	Non-parenchymal cell fraction
–	2.1	615
GalNAc	1.4	146
GlcNAc	2.4	584

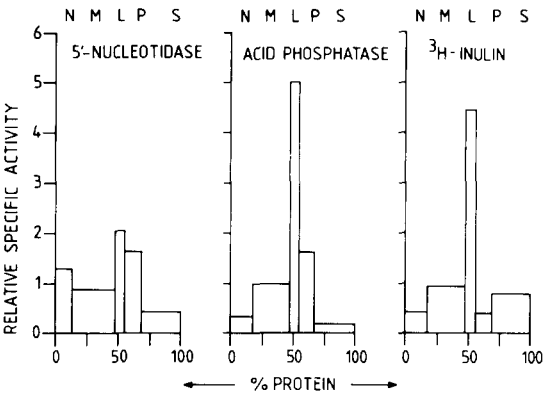

Fig. 5. Subcellular distribution of ^{3}H label in liver following injection of [^{3}H]inulin-containing tris-gal-chol liposomes. Liposomes composed of cholesterol and sphingomyelin in an equimolar ratio with [^{3}H]inulin as a marker of the internal space were allowed to incorporate tris-gal-chol and injected intravenously in rats in a dose of 2 μmol per 100 g body weight. At 1 h after injection the liver was excised, homogenized and subjected to differential centrifugation as described in Methods. Of each fraction 5'-nucleotidase, acid phosphatase, radioactivity and protein were determined. Specific (radio)activity relative to that of the homogenate was plotted against relative total protein content (in percentage of homogenate). N, nuclear fraction; M, mitochondrial fraction; L, lysosomal fraction; P microsomal fraction; S, final supernatant.

eter of 32.4 nm could be calculated [9], while all vesicles were smaller than 50 nm according to the results presented in Fig. 4. Addition of tris-gal-chol to the liposomes resulted in the formation of a small fraction of particles larger than 50 nm; according to the titration values determined by Barrow and Lentz [11] this fraction accounts for less than 4% of the total population. An increase in particle size is thus not likely to be responsible for the observed increase in Kupffer cell uptake. Furthermore, the results presented in Table I provide evidence that the increased liposome uptake is accomplished by mediation of galactose specific binding sites. Table I shows that the increase in liposome uptake due to tris-gal-chol incorporation could in both the parenchymal and the non-parenchymal cell fractions (uptake in the latter being equivalent to the Kupffer cell uptake) be inhibited by preinjection of GalNAc, but not of GlcNAc; this indicates that for both cell types galactose-specific binding sites are involved in the uptake of tris-gal-chol loaded liposomes. Finally, to investigate if the tris-gal-chol liposomes were

internalized or remained merely bound to the plasmamembrane of the liver cells a subcellular distribution study was done. One hour after the injection of tris-gal-chol liposomes the liver was perfused with saline, homogenized in sucrose buffer and subjected to differential centrifugation as described in Methods. As shown in Fig. 5, the liposomal marker [^{3}H]inulin co-distributed with the lysosomal marker enzyme acid phosphatase indicating that the tris-gal-chol liposomes do become internalized by the cells by an endocytic mechanism, which causes them to end up in the lysosomes.

Discussion

The affinity of the hepatocytic galactose receptor strongly depends on the degree of branching of the oligosaccharide chain: triantennary structures are bound with higher affinity than bi- or monoantennary chains [7,20]. Thus it was reasoned that for optimal targeting of lipid particles to hepatocytes via the galactose receptor the construction of an amphipathic molecule with a triantennary galactose structure would be desirable. After synthesis of the tris-gal-chol, the molecule turned out to be water-soluble, i.e. it formed optically clear solutions in water, presumably consisting of micellar aggregates [8]. By adding such a micellar solution to lipidic particles in aqueous dispersion the tris-gal-chol molecule quantitatively associates with liposomes, LDL and HDL [8]. The addition of tris-gal-chol to liposomes prior to intravenous injection had a pronounced effect on blood elimination and liver uptake of uncharged liposomes (Figs. 1 and 2). With negatively charged liposomes the differences were moderate. The inclusion of phosphatidylserine in the liposomal bilayer, which in itself stimulates cellular uptake [19], obscures the additional effect of tris-gal-chol comparable to the observations on the effect of this and other negatively charged lipids on the uptake of opsonized liposomes by mouse macrophages [21]. Fractionation of the liver, after injection of neutral tris-gal-chol liposomes into different cell populations showed that both the hepatocytes and the Kupffer cells were involved in the uptake of liposomes, whereas the endothelial cells did not significantly contribute, in line with previously published data [9,10,22]. Since tris-gal-chol incorporation does not significantly affect the size distribution of the small unilamellar vesicles as shown in Fig. 4, we expect them to have no more difficulties in crossing the endothelial fenestrations, with mean diameters of about 100 nm [23] and thus in reaching the hepatocytes than the control liposomes. We rather tend to ascribe the shift in intrahepatic distribution to differences in receptor characteristics between the hepatocyte and the Kupffer cell. Not only are the hepatocyte receptors randomly distributed on the sinusoidal side of the plasma membrane, while the Kupffer cell receptors are preclustered, there is also a different response to particle size, with the Kupffer cell receptor being more responsive to larger particles [24]. Especially this latter difference may cause tris-gal-chol liposomes or tris-gal-chol LDL to be taken up efficiently by Kupffer cells but the smaller tris-gal-chol HDL by hepatocytes [25].

The lack of inhibition of total liver uptake of tris-gal-chol liposomes by asialofetuin already suggested that the hepatocytes were only minimally involved in the specific uptake of these liposomes.

Asialofetuin, which itself is a well known substrate for the galactose receptor on hepatocytes serves for those cells as a competitive inhibitor of the uptake of other substrates, but it fails to inhibit uptake by the Kupffer cell receptor [25], despite the observation that the asialofetuin does bind to the Kupffer cell receptor [24]. GalNAc, on the other hand, is a potent inhibitor of galactose-specific uptake by both hepatocytes and Kupffer cells [20,24] and for both cell types it inhibites tris-gal-chol mediated uptake (Table I).

In the subcellular distribution experiments the activities of the marker enzyme of the plasmamembrane (5′-nucleotidase) and the lysosomes (acid phosphatase) were determined in order to be able to discriminate between binding and internalization of the tris-gal-chol liposomes. The co-distribution of the liposomal marker [^{3}H]inulin and acid phosphatase indicates an ultimately lysosomal destiny of the liposomes and thus an endocytic route of internalization.

The main purpose for the design of the tris-gal-chol molecule, i.e. to direct lipid particles to liver parenchymal cells is only partially fulfilled: only HDL particles with tris-gal-chol were cleared by

the hepatocytes. On the other hand, larger lipid particles were shown to be specifically directed to Kupffer cells lending support to the presence of a galactose specific receptor on these cells [24], but strongly frustrating the targeting of galactose-residue exposing liposomes to hepatocytes. Also, with liposomes containing lactosylceramide as a ligand for the hepatocyte receptor we previously reported a minor [9] or a major [10] contribution to total liposomal liver uptake by the Kupffer cells. From our present results we conclude that the tris-gal-chol molecule with its triantennary galactose residues is a ligand with a higher affinity for the Kupffer cell galactose receptor than lactosylceramide. This should also be taken into account when attempting to target liposomes to hepatocytes.

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