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Cholesterol-containing lactose derived neoglycolipids serve as acceptors for sialyltransferases from rat liver Golgi vesicles

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The cholesterol-containing lactose derived neoglycolipids β-Lactosylcholesterol, Cholesteryl-β-lactosylpropane-1,3-diol, 3-Cholesteryl-1-β-lactosylglycerol, 2-Cholesteryl-1-β-lactosylglycerol, 2,3-Dicholesteryl-1-β-lactosylglycerol, 1-Deoxy-1-cholesterylethanolaminolactitol, 1-Deoxy-1-cholesteryl (*N*-acetyl)-ethanolaminolactitol, 1-Deoxy-1-cholesterylphosphoethanolaminolactitol, and 1-Deoxy-1-cholesterylphospho (*N*-acetyl)-ethanolaminolactitol were synthesized and used as acceptors for sialytransferases from rat liver Golgi vesicles. Relative activities with the neoglycolipids as acceptors varied from 28 to 163% compared to those obtained with the authentic acceptor lactosylceramide. Product identification by thin layer chromatography and fast atom bombardment mass spectrometry showed that the neoglycolipids yielded mono- and disialylated products. The results of competition experiments suggested that lactosylceramide and the neoglycolipids were sialylated by the same enzymes.

Keywords: neoglycolipids, sialyltransferases, rat liver Golgi vesicles, lactosylceramide, GM3, GD3

Introduction

Glycosphingolipids (GSL) are known to be present not only in vertebrates but also in other organisms like molluscs, plants and even microorganisms. Their constituents and structures, however, differ in varying degrees from GSL of mammalian tissues that have been most extensively studied [1]. As components of the plasma-membrane GSL are located with their nonpolar part (ceramide) in the outer leaflet of the membrane. The oligosaccharide moiety is facing the extracellular fluid. Besides their location in plasma membranes, some specific GSL may also be highly concentrated at intracellular membranes, e.g. lactosylceramide at cytoplasmic granular membranes in neutrophils [2]. Many studies concerning the biosynthesis, function and degradation of GSL have been done (for review see [3, 4]).

Recently neoglycolipids (neoGL) have been used as substitutes for natural GSL in investigations on glycosyltransferases. For example 1-deoxy-1-phosphatidylethanolamino-lactitol-type neoGL and even alkylglycosides were shown to serve as acceptors in sialyltransferase assays [5–7]. The latter neoGL were, however, of restricted use, because of their detergent properties.

In the present study we used cholesterol instead of alkylchains as lipid anchors. Lactose-derived neoGL carrying different spacers between cholesterol and the oligosaccharide moiety were synthesized (for structures and abbreviations see Table 1). These neoGL were used as acceptors for sialyltransferases, the resulting products were examined by fast atom bombardment mass spectrometry (FAB MS) and the involved enzymes were identified.

Materials and methods

MATERIALS

Lactose, acetic anhydride, pyridine, trichloroacetonitrile, 1,3-propanediol and 1,8-diazabicyclo[5,4,0]undec-7-ene (DBU) were purchased from Merck (FRG). p-Toluenesulfonic acid, morpholine, 3,4-dihydro-2H-pyrane and o-phosphocolamine were obtained from Fluka (Switzerland). Cholesterol, hydrazinium acetate and sodiumcyanoborohydride were from Aldrich (FRG), 2,3-*O*-isopropylidene-sn-

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Table 1. Names and abbreviations of cholesterol containing neoGl.

Name	*Abbreviation*
β-Lactosylcholesterol	LacChol
Cholesteryl-β-lactosylpropane-1,3-diol	Lac-prop-Chol
3-Cholesteryl-1-β-lactosylglycerol	Lac-Gro-3-Chol
2-Cholesteryl-1-β-lactosylglycerol	Lac-Gro-2-Chol
2,3-Dicholesteryl-1-β-lactosylglycerol	Lac-Gro-$Chol_2$
1-Deoxy-1-cholesterylethanolaminolactitol	Lac-CholEtn
1-Deoxy-1-cholesteryl (*N*-acetyl)-ethanolaminolactitol	Lac-CholEtn (NAc)
1-Deoxy-1-cholesterylphosphoethanolaminolactitol	Lac-CholPEtn
1-Deoxy-1-cholesterylphospho (*N*-acetyl)-ethanolaminolactitol	Lac-CholPEtn (NAc)

glycerol from Bacher (FRG). Cytidine-5′-monophospho-*N*-acetyl[4,5,6,7,8,9–^{14}C] neuraminic acid (CMP-[^{14}C]Neu Ac) was from Amersham-Buchler (FRG) and was used after dilution with the unlabelled sugar nucleotide from Sigma (FRG). Iatrobeads 6 RS 8060 were purchased from Macherey & Nagel (FRG). TLC plates and silica gel 60 were from Merck and Sephadex G-25 was from Pharmacia (Sweden). Scintillation cocktail Picofluor 40 was obtained from Packard (FRG). X-ray film XAR-5 was from Eastman Kodak Company (USA). Rats of the Wistar strain (male, 300–350 g) were procured from Harlan-Winkelmann (FRG). All reagents and solvents were of analytical grade.

SOLUTIONS

Solvent A: chloroform:methanol: 0.2% $CaCl_2$ 60:35:8 v/v/v
Solvent B: chloroform:methanol:water 80:20:2 v/v/v
Solvent C: chloroform:methanol:water 75:25:4 v/v/v
Solvent D: chloroform:methanol:water 65:25:4 v/v/v
Solvent E: chloroform:methanol:water 60:35:8 v/v/v
Reduction reagent: 1% (m/v) sodium cyanoborohydride, 0.1% (v/v) acetic acid in chloroform:methanol 1:1 (v/v).

Phosphomolybdic acid spray reagent: 2.5% (m/v) phosphomolyddic acid, 1% (m/v) cer(IV)sulfate in 6% (v/v) concentrated sulfuric acid.

Orcinol spray reagent: 0.5% (m/v) orcinol, 15% (v/v) concentrated sulfuric acid.

SYNTHESIS OF THE NEOGLYCOLIPIDS

General methods

Lactose was peracetylated as described previously [8]. 1-*O*-Deacetylation, using hydrazinium acetate was performed by the method of Excoffier *et al.* [9]. The trichloroacetimidates of 2,3,6,2′,3′,4′,6′-hepta-*O*-acetyllactose and cholesterol were obtained by reaction with trichloroacetonitrile in the presence of DBU [10]. 1-bromo-2,3,6,2′,3′,4′,6′-hepta-*O*-acetyllactose was made from the peracetylated sugar and hydrogen bromide in acetic acid [11].

The cholesterol anchor was attached to each individual spacer via its trichloroacetimidate [10].

All steps of synthesis were controlled by TLC and the reaction products were characterized by FAB MS. The anomeric purity of the peracetylated lactosides was corroborated by ^{1}H-NMR-Spectroscopy.

The peracetylated neoGL were deacetylated by sodium methanolate treatment as described previously [12]. The resulting neoGL were checked by FAB MS.

β-Lactosyl-cholesterol

2,3,6,2′,3′,4′,6′-Hepta-*O*-acetyllactose trichloroacetimidate was coupled to cholesterol in the presence of catalytic amounts of borontrifluoride as described previously [10].

Lac-prop-Chol

One of the hydroxylgroups of 1,3-propanediol was selectively protected by the tetrahydropyranyl-group [13]. During the coupling reaction with the cholesteroltrichloroacetimidate [10] the protective group was cleaved and 3-cholesteryloxy-propanol was obtained. Finally lactose was attached to the lipid part from 1-bromo-2,3,6,2′,3′,4′,6′-hepta-*O*-acetyllactose using the Königs-Knorr method [14].

Cholesteryl-glyceryl-lactosides

2,3,6,2′,3′,4′,6′-Hepta-*O*-acetyllactose was coupled to 2,3-isopropylidene-glycerol via the imidate method [10]. Subsequently the isopropylidene group was removed by stirring in 60% acetic acid for 4 h at room temperature. Cholesterol was attached as described above. The reaction yielded three acetylated products: 3-cholesteryl-1-lactosyl-glycerol, 2-cholesteryl-1-lactosyl-glycerol, and 2,3-dicholesteryl-1-lactosyl-glycerol. They were separated by column chromatography using silica gel 60 using toluene:acetone 4:1 (v/v) as eluent.

Cholesterylethanolamino- and -phosphoethanolaminolactitols

The amino group of the spacer-molecule was protected by the fluorenylmethoxycarbonyl-group (Fmoc) [15] and cholesterol was coupled as described above. The Fmoc-group was removed by stirring in morpholine for 4 h at

room temperature. Attachment of lactose by reductive amination and *N*-acetylation were performed as described previously [5].

PREPARATION OF GOLGI VESICLES

Golgi derived vesicles were isolated from rat liver by the method of Sandberg *et al.* [16]. A detailed description of the procedure has been given previously [17, 18]. Golgi specific glycosyltransferases were enriched 50–60-fold and contamination with other cellular membranes was below 5% [17].

SIALYLTRANSFERASE-ASSAYS

In a total volume of 50 μl, the assay solution contained the acceptor (LacCer or neoGL) in concentrations varying from 10 to 200 μM, 0.15 to 0.23% (m/v) Triton CF-54, 150 mM sodium cacodylate/hydrochloric acid (pH 6.6), 10 mM magnesium chloride, 10 mM mercabtoethanol, 1 mM CMP-[^{14}C]NeuAc (5000–10000 cpm nmol^{-1}) and 50 μg Golgi protein or 1 mU commercially available α-2,6-sialyltransferase from rat liver. Performance of the assays as well as separation of the products by Sephadex G-25 gel chromatography were executed as described previously [17].

Radioactivity was determined in a liquid scintillation counter. Appropriate blanks without exogenous acceptor were run for each determination. All experiments were performed at least in duplicate, and mean values are presented in the figures and tables. Unless otherwise stated the standard deviations were below 5%.

PRODUCT-IDENTIFICATION

The eluates of the Sephadex G-25 gel chromatography (see previous section) were dried under a stream of nitrogen, redissolved in 200 μl chloroform:methanol (1:1 v/v) and applied to silica gel 60 TLC plates. The chromatograms were developed in solvent A. Radioactivity was detected with a TLC linear analyser (Berthold, FRG) and by autoradiography. For FAB MS analysis, the Sephadex G-25 eluates of 10 assays (15 for the poorer acceptors) were combined and the sialylated products were purified on a 10 × 0.5 cm Iatrobeads 6 RS 8060 column using a stepwise gradient of solvents B to E as eluent. Fractions of 2 ml were collected and aliquots were examined for radioactivity. The fractions containing radiolabelled material were combined, dried under a stream of nitrogen and the residues were analysed by FAB MS.

MASS SPECTROMETRY

FAB MS was carried out with a VG Analytical ZAB-HF reverse geometry mass spectrometer fitted with an ION-Tech atom gun as described previously [19]. Either negative or positive ions were detected. The spectra were recorded and evaluated on a SAM II/68 K computer (KWS, Ettlingen, FRG) using the DP10 program of AMD (Harpstedt, FRG). Processing and plotting of the data were performed on the central computer system of the University of Bonn.

Results

Sialylation of the neoglycolipids

The neoGL were used as acceptors in sialyltransferase assays as described in Materials and methods. The resulting reaction rates were compared with those obtained with the genuine acceptor lactosylceramide (LacCer), which was set to 100%. The results are shown in Table 2. Obviously all the neoglycolipids tested were sialylated. The relative activities varied between 28 and 163%. The worst acceptors were the non-acetylated neoglycolipids carrying an ethanolamino- or phosphoethanolamino-spacer (see Discussion).

Product-Identification by TLC and FAB-MS

The neoGl were incubated with Golgi protein and CMP[^{14}C]NeuAc as described in Material and methods and the reaction products were separated by TLC. The results are shown in Fig. 1. Except for Lac-CholEtn all neoGl were converted to two main products. In an analogy to the natural acceptor LacCer, that is sialylated to GM3 and GD3, the cholesterol-derived neoGL were converted to monosialylated (further-migrating) and disialylated (slower-migrating) products. The formation of GM3 and GD3 and of their corresponding neoGL analogues is, most probably, due to the successive action of GM3 synthase (SAT I) and GD3 synthase (SAT II). Both enzymes are known to be present in Golgi preparations of rat liver [20].

To support this assumption the time-dependent product formation from Lac-Gro-3-Chol and Lac-CholPEtn (NAc) was monitored. The result is shown in Fig. 2. The presumed GD3 analogues indeed increased after a lag phase of at least 10 min, suggesting that they were derived from the respective monosialylated precursors.

For a final identification the reaction products of 10 (or in cases of low sialyltransferase activities the products of 15) assays were combined, purified as described in Materials and methods, and subsequently analysed by FAB MS. Only in the case of Lac-CholEtn if no FAB MS data have been obtained because of the extremely low sialylation rates. The monosialylated products from all other neoGL could be identified by FAB MS in positive and/or negative ion mode. The data are summarized in Table 2. From Lac-CholPEtn(NAc) even the lower migrating product could be analysed by FAB MS. The negative ion mode spectrum showed molecular ions at *m/*

z 1480 ($[M - 2H + Na]^-$), *m/z* 1502 ($[M - 3H + 2Na]^-$), and *m/z* 1524 ($[M - 4H + 3Na]^-$). They confirm the expected molecular weight of 1459 (nominal mass) for $NeuAc_2$Lac-CholPEtn(NAc). These results unambiguously proved that the products formed from the neoGL were the respective mono- and disialylated derivatives.

Table 2. Relative sialyltransferase activities with lactose-derived cholesterol neoGl as acceptors and *m/z* values of the molecular ions obtained from FAB-MS of the monosialylated products.

Acceptor	*Rel. Act.* (% ± SD)	*Monosialylated product*		
		Molecular weight	*m/z* $(M - H)^-$	*m/z* $(M + H)^+$
LacCer	100	–	–	–
LacChol	138, 5 ± 11, 9[a]	1001	1000	1024[d]
Lac-prop-Chol	143, 5 ± 7, 7[a]	1059	–	1082[d]
Lac-Gro-3-Chol	163, 7 ± 16, 9[a]	1075	1074	1120[e]
Lac-Gro-2-Chol	138, 3 ± 23, 3[a]	1075	1074	1120[e]
Lac-Gro-$Chol_2$	113, 9 ± 18, 4[a]	1443	1464[c]	–
Lac-CholEtn	28, 8 ± 7, 6[a]	1044	ND	ND
Lac-CholEtn(NAc)	115, 6 ± 4, 4[a]	1088	1087	1111[d]
Lac-CholPEtn	60, 6 ± 18, 7[b]	1126	1125	1127
Lac-CholPEtn(NAc)	151, 4 ± 8, 4[a]	1168	1167	1213[e]

The acceptors (200 μM) as indicated above were incubated with CMP-[^{14}C] NeuAc and Golgi vesicles as described in Materials and methods. The resulting reaction rates were calculated relative to those obtained with LacCer (set to 100%). (The LacCer sialylation rates varied from 2.29 to 4.65 nmol mg^{-1} h, depending on the Golgi preparation used.)
The *m/z* values were obtained from negative ion ($(M - H)^-$) or positive ion ($(M + H)^+$) FAB mass spectra of the sialylation products prepared as depicted in the product identification section.
[a] $n = 5$.
[b] $n = 4$.
[c] $(M - 2H + Na)^-$.
[d] $(M + Na)^+$.
[e] $(M - H + 2Na)^+$.

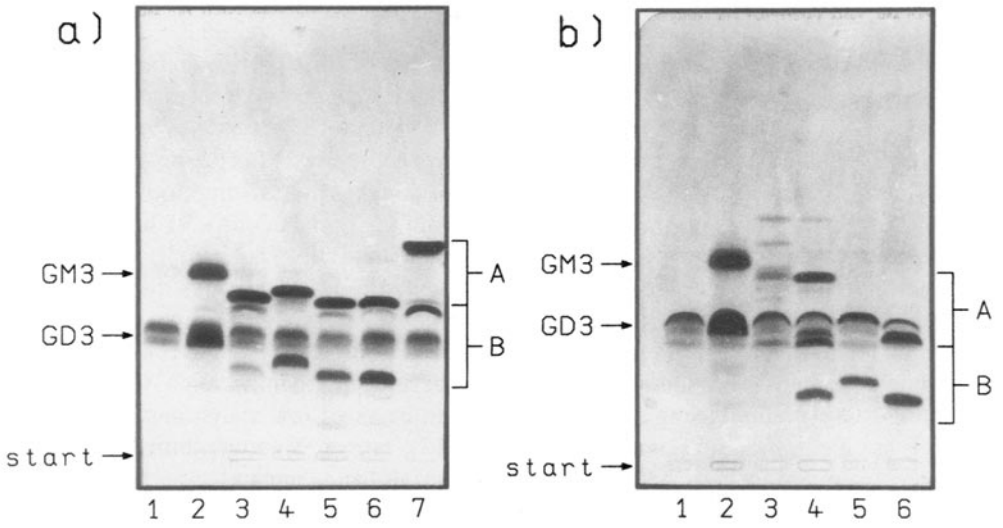

Figure 1. TLC separation of the sialylation products obtained with LacCer and cholesterol-type neoGL as acceptors. The acceptors (100 μM) as indicated below were incubated for two hours with Golgi protein and CMP-[^{14}C] NeuAc as described in Materials and methods. The radioactive products were separated by TLC (solvent A) and visualized by autoradiography. Acceptors: (a) Lane 1, none; lane 2, LacCer; lane 3, LacChol; lane 4, Lac-prop-Chol; lane 5, Lac-Gro-3-Chol; lane 6, Lac-Gro-2-Chol; lane 7, Lac-Gro$Chol_2$. (b) Lane 1, none; lane 2, LacCer; lane 3, Lac-CholEtn; lane 4, Lac-CholEtn(NAc); lane 5, Lac-CholPEtn; lane 6, Lac-CholPEtn(NAc). The presumed products are: (A) GM3 and monosialylated neoGl; (B) GD3 and disialylated neoGl.

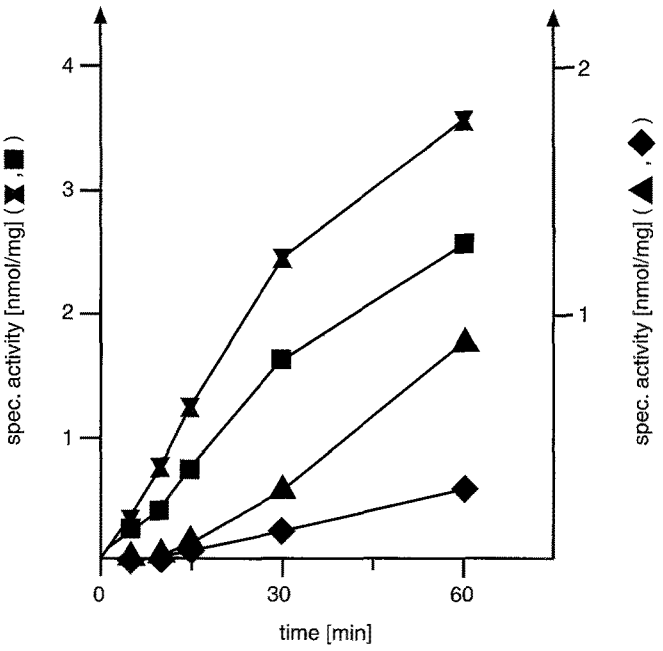

Figure 2. Time-dependent formation of the mono- and disialylated products obtained from Lac-Gro-3-Chol and LacChol-PEtn(NAc). The respective neoGl (100 μM) were incubated with Golgi protein and CMP-[^{14}C] NeuAc for the times indicated as described in Materials and methods. The reaction products were separated on TLC (solvent A) and quantified using a linear analyser. (▼) NeuAcLac-Gro-3-Chol; (■) NeuAcLac-CholPEtn(Nac); (◆) $NeuAc_2$Lac-Gro-3-Chol; (▲) $NeuAc_2$Lac-CholPEtn(NAc).

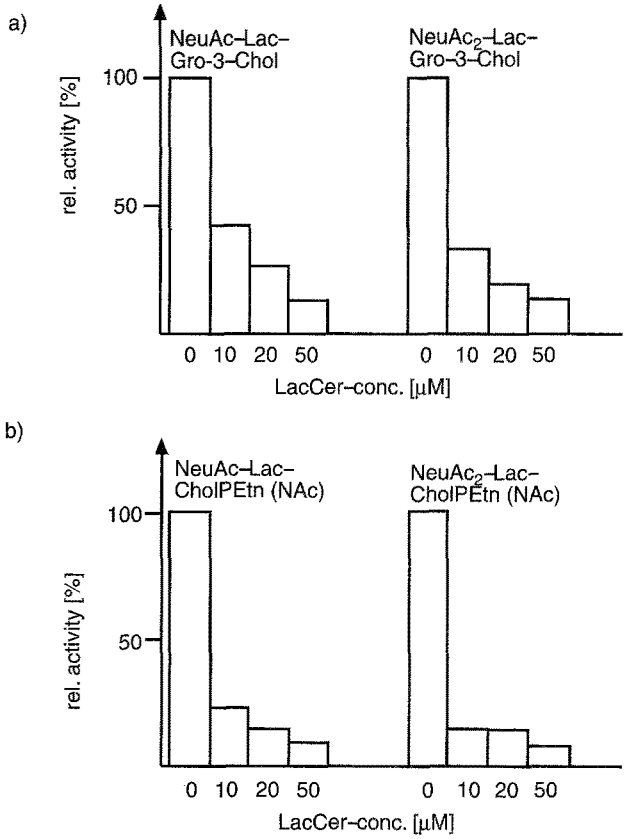

Figure 3. Competition between the neoGl Lac-Gro-3-chol (a) and Lac-CholPEtn(NAc) (b) and LacCer in the sialyltransferase assay. The neoGL acceptors (50 μM) were incubated with CMP-[^{14}C]NeuAc and Golgi vesicles in the presence of increasing LacCer concentrations as indicated. The reaction products were separated on TLC (solvent A) and quantified using a linear analyser. The transferase activities obtained with the respective acceptors in the absence of LacCer were set to 100%.

Competition experiments

In order to demonstrate that the neoGL and LacCer are sialylated by the same enzyme(s), competition experiments with Lac-Gro-3-Chol and Lac-CholPEtn(NAc) have been carried out. The assays contained a constant neoGL concentration and increasing LacCer-concentrations. As Fig. 3 shows the formation of the monosialylated as well as of the disialylated neoGL-derived products was strongly inhibited in the presence of LacCer. This result indicates that the neoGL and LacCer are primarily utilized by the same sialyltransferase (GM3 synthase). A decrease of the neoGL-derived monosialylated products, i.e. the potential acceptors for GD3 synthase, caused by competition of LacCer finally leads to an inhibition of $NeuAc_2$-neoGL formation.

Discussion

In the present study we synthesized LacCer analogues carrying cholesterol instead of ceramide as a lipid anchor and used them as acceptors for sialyltransferases from rat liver Golgi vesicles. Besides β-lactosylcholesterol a series of spacer-containing neoglycolipids were produced to avoid putative steric effects due to the bulky cholesterol residue. A comparison of the relative reaction rates (see Table 2) did not, however, reveal significant differences between those obtained with β-lactosylcholesterol and the spacer-containing derivatives. With the exception of Lac-CholEtn and Lac-CholPEtn all neoGl tested gave slightly higher sialylation rates than the authentic acceptor lactosylceramide. The non-acetylated ethanolamine-spaced neoGl were the poorest acceptors, most probably due to the positive charge of the free amino group. Abolishing the positive charge by *N*-acetylation restored the sialylation rates. The same effect had been observed in previous studies dealing with neoGL of the 1-deoxy-1-phosphatidylethanolamino-lactitol-type and lysogangliosides, respectively [5, 21].

The three following facts exhibit the similarities in acceptor behaviour of the lactose-containing cholesterol-type neoGL and LacCer.

(1) Like LacCer the neoGL were converted to monosialylated (GM3 analogues) and disialylated products (GD3 analogues) by successive action of SAT I and SAT II. For SAT II (GD3 synthase) the type of spacer seemed to have an influence on the affinity to the acceptor. From LacChol only a small amount (< 1%) of the $NeuAc_2$-derivative was obtained. Lac-prop-Chol and the glycerol-spaced neoGL yielded 5–7.5% disialylated product, whereas Lac-CholEtn(NAc), Lac-CholPEtn and Lac-CholPEtn(NAc) furnished about 22% of the respective disialo-derivatives. The same amount of GD3 was obtained from the genuine precursor LacCer.

(2) Competition experiments performed exemplarily with Lac-Gro-3-Chol and Lac-CholPEtn(NAc) as acceptors and LacCer as inhibitor indicated that the neoGl and LacCer are glycosylated by the same sialyltransferases.

(3) In contrast to alkyllactosides [7] the cholesterol-type neoGl (like LacCer) were not utilized by commercially available α-2-6-sialyltransferase from rat liver (data not shown).

The above findings show that cholesterol-containing neoGL could serve as excellent substitutes for natural glycosphingolipids in glycosyltransferase assays. Instead of lactose other oligosaccharides can easily be coupled to the cholesterol-derived lipid anchors yielding neoGL that could be used for investigations on a variety of glycosyltransferases *in vitro*.

Moreover the option of introducing radioactive labels (by *N*-[^{14}C]acetylation of the ethanolamine derivatives [6] or by [^{3}H]-hydrogenation of the cholesterol double bond [22] in general) offers the use of cholesterol-containing neoGL as probes for explorations of glycosphingolipid metabolism *in situ* (e.g. in cell culture).

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