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Synthesis and transfection activity of novel galactosylated polycationic lipid

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ABSTRACT

In this study, we synthesized a new galactosylated cationic lipid and investigated its biological activity. The structure of lipid combines both spermine residue for DNA compaction and galactose moiety for the improvement of aggregation behavior of lipoplexes. Lipid was low toxic for different mammalian cells, and was able both to compact plasmid DNA and to mediate cellular accumulation of various nucleic acids (ODN, pDNA and siRNA) exhibiting biological activity (transgene expression, gene silencing).

Gene and antisense oligonucleotides based therapies have been developed for the treatment of both acquired and inherited diseases.[1] These approaches are based on the correction of the basis of diseases by therapeutic nucleic acids, namely DNA, oligonucleotides, small interfering RNA etc. Naked nucleic acids degrade easily within the organism, therefore special transport systems were developed to protect and to deliver nucleic acids into the targeted cells. Cationic lipids are widely used as non-viral delivery systems for the improvement of nucleic acid delivery into eukaryotic cells.[2] However, lipids used for the gene delivery often display both the high cytotoxicity and insufficient transfection efficiency. Hence, intensive development and study of the new non-toxic and efficient cationic lipids for the gene delivery are currently in progress.[3,4] Cationic lipids have common structural features such as the presence of positively charged and hydrophobic domains connected by a spacer. Numerous chemical modifications have been made to augment the delivering activity of cationic lipids.[2,5,6]

One promising strategy for the improvement of nucleic acids delivery is the design of cationic agents for the cell specific gene targeting, with the aid of covalently bounded ligands for specific cell receptors. Recently, cationic lipids with incorporated carbohydrate residues were under intensive investigation as targeted systems for the delivery of nucleic acids to hepatocytes, macrophages, and into the nucleus.[7–9] Several groups have reported that carbohydrate fragments increase the colloid stability of nucleic acid–lipid complexes (lipoplexes) in a blood serum and decrease the toxicity of cationic lipids.[10,11] Some polycationic single and double long-alkyl chain galactospermine bolaamphiphile have been synthesized and appeared as a promising non-viral synthetic vector for specific gene delivery.[12,13] Here we describe the new and convenient route to obtain a galactosylated polycationic lipid **1** (Fig. 1) and assess its biological activity in terms of cytotoxicity and transfection activity, in respect to different nucleic acids.

The structure of lipid **1** combines both spermine residue for DNA compaction and galactose moiety for improvement of aggregation behavior of lipoplexes. Galactosyl residue may then serve as a molecular signal for hepatocytes targeting. 1,2-di-*O*-tetradecyl-*rac*-glycerol was used as the hydrophobic anchor for incorporation into a lipid bilayer. It is known that cationic lipids containing tetradecyl substituents possess higher in vitro transfection efficacy

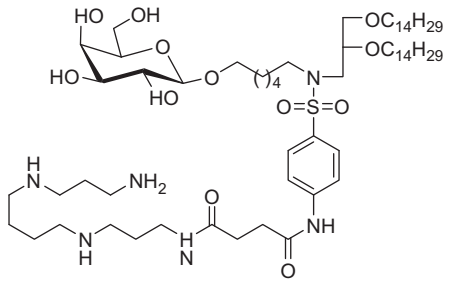

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Figure 1. Galactose-containing polycationic lipid.

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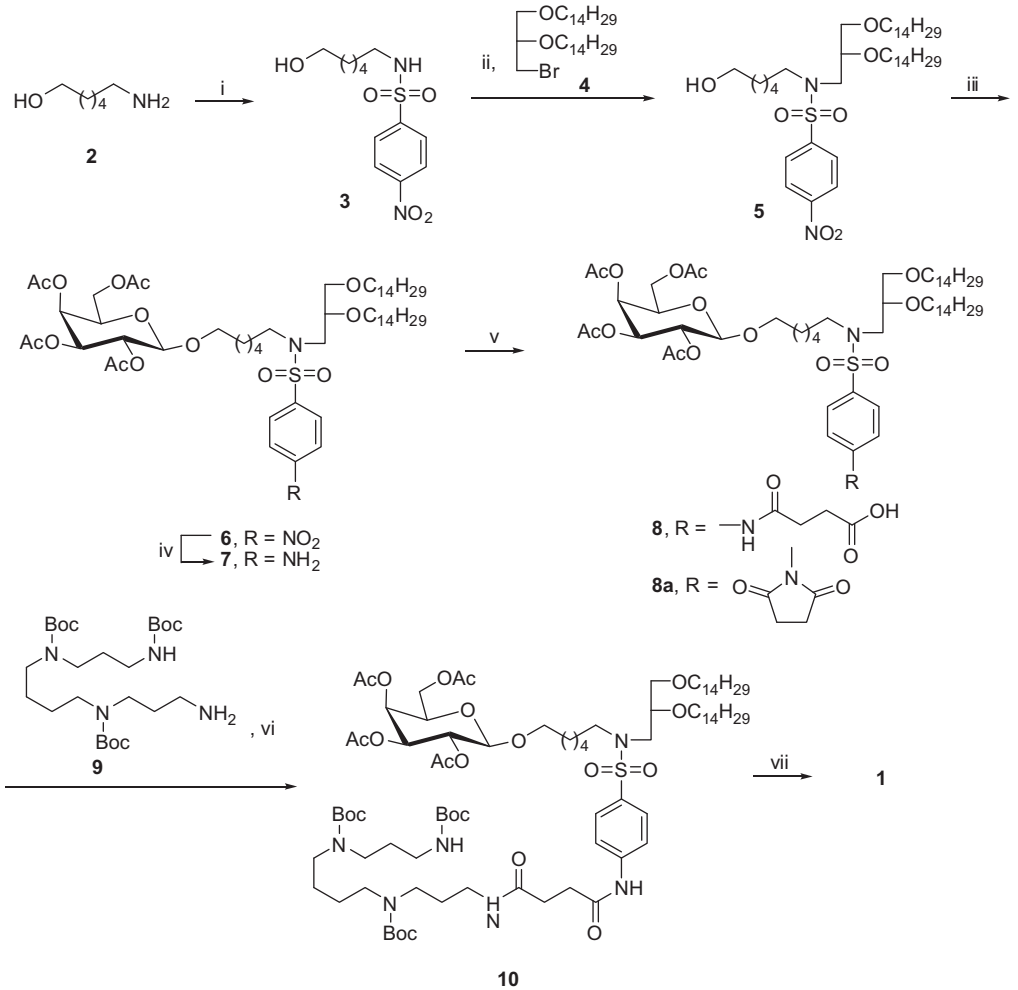
compared to other long-chain hydrocarbon analogues.[14,15] Natural polyamines such as spermine or spermidine are capable of binding and condensing DNA molecules into small, dense particles. Lipophilic derivatives of polyamines condense DNA more efficiently than polyamines themselves.[16] Among the cationic lipids tested lipophilic spermines are the most efficient synthetic gene delivery agents.[17] Therefore, for the creation of new polycationic lipid natural polyamine—spermine have to be covalently attached to the hydrophobic anchor by means of an amide bond. On the other hand it was necessary to link to the same anchor galactosyl residue. The attachment of hydrophobic, cationic, and carbohydrate domains was performed by using a Fukuyama amine synthesis—the reaction of 6-hydroxyhexyl 4-nitrobenzenesulfonamide with 1,2-ditetradecyl-3-bromo-*rac*-glyceride. This convenient approach permits to obtain a bifunctional molecule for the assembly of both spermine and galactose residues into a single structure of polycationic glycolipid.

According to the developed synthetic route the sulfonylation of 6-aminohexanol (**2**) with 2-nitrobenzenesulfonyl chloride in the presence of Et_3N afforded amide **3** in 92% yield (Scheme 1). Bromide **4** was prepared from 1,2-di-*O*-tetradecyl-*rac*-glycerol by treatment with CBr_4 in the presence of Ph_3P as described previously.[18] The alkylation of amide **3** with bromide **4** was carried out with a Fukuyama amine synthesis[19] and gave **5** in 64% yield.

The glycosylation of compound **5** with 2,3,4,6-tetra-*O*-acetyl-α-D-galactosyl bromide under the modified Königs–Knorr method in a Soxlet apparatus using $CdCO_3$ as the promoter gave galactoside **6a**. It is known that the ratio of the anomeric glycosides obtained is strongly dependent upon the reaction conditions. Upon optimising glycosylation conditions the best results were obtained by reflux of compound **5** (1 equiv) with galactosylbromide (3 equiv) in benzene for 1.5 h. Under these conditions β-glycoside **6** was formed in 65% yield accompanied by only 2% of α-galactoside. The preferable formation of β-glycoside **6** was a result of neighboring-group participation of a 2-*O*-acyl functionality in the glycosyl donor.[20] The reduction of nitro group by catalytic hydrogenolysis on Pd/C in the presence of ammonium formate as a hydrogen source afforded amine **7** with 90% yield. Acylation of lipid **7** with succinic anhydride in the presence of *N*,*N*-dimethyl-4-aminopyridine (DMAP) (2 equiv) and *N*-hydroxybenzotriazole (HOBT) at 70 °C for 24 h gave the corresponding succinate **8** in the very low yield (29%). The main product obtained in this reaction was cyclic imide **8a** (70%). In order to minimize the amount of undesirable **8a** we optimized ratios of reagents and temperature conditions and found that the treatment of compound **7** with succinic anhydride in the presence of Et_3N (2 equiv) and catalytic amount of DMAP (0.2 equiv) for 36 h at the room temperature gave succinate **8** in 73% yield.



Scheme 1. Synthesis of polycationic lipid **1**. Reagents and conditions: (i) 2-NO_2-$C_6H_4SO_2Cl$, TEA, CH_2Cl_2, 24 °C, 36 h, 73%; (ii) Cs_2CO_3, TBAI, DMF, 90 °C, 8 h, 65%; (iii) 2,3,4,6-tetra-*O*-acetyl–D-galactopyranosyl bromide, $CdCO_3$, C_6H_6, reflux, 1.5 h, 65%; (iv) $NH_4{\cdot}HCO_2$, Pd/C, MeOH/THF (2:1), 60 °C, 30 min, 90%; (v) succinic anhydride, TEA, DMAP, CH_2Cl_2, 24 °C, 36 h, 73%; (vi) tri-Boc-spermine (**9**), HBTU, DIEA, DMF, 4 °C, 40 min, 85%; (vii) (a) TFA, CH_2Cl_2, 24 °C, 3 h, 86%; (b) 0.04 N MeONa, MeOH, 24 °C, 4 h, 53%.

The key step of the synthesis involved the condensation of protected spermine **9**[21] with succinate **8**. The use of both 1,3-diisopropylcarbodiimide (DIC)/HOBT/DMAP and 1,1′-carbonyldiimidazole as condensing agents was unsuccessful and provided large amounts of by-products (*N*-acyl diisopropylurea in the case of DIC or cyclic imide **8a** for both approaches). The condensation of amino **8** and carboxy **7** derivatives in the presence of 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) at 4 °C for 40 min afforded lipopolyamine **10** in 85% yield. At the final stage, the unblocking of amino groups in the spermine moiety was carried out by treatment of **10** with trifluoroacetic acid. The removal of acetyl groups under Zemplen reaction using 0.04 N sodium metoxide in methanol and subsequent dialysis against water provided the key compound **1** in 46% yield in two stages.

The cytotoxicity of the polycationic lipid **1** was estimated by the MTT[22] test using several cell lines: HeLa (human cervical carcinoma), HEK 293 (human embryo kidney), BHK (baby hamster kidney) and transgenic BHK IR-780 cell lines. The cells were incubated with either the lipid **1** or with the complexes of **1** with plasmid DNA (2 μg/mL) at lipid concentrations ranging from 1 to 90 μM for 24 h in the presence of 10% fetal bovine serum (FBS) in the cell medium or under serum-free conditions. IC_{50} values of **1** (a lipid concentration corresponding to the viability of 50% of the cells after incubation in the presence of lipid for 24 h) for different cells were around 20 μM (18–21 μM) in the absence and around 40 μM (36–42 μM) in the presence of 10% FBS. Note that cytotoxicity of DNA/lipid **1** complexes was lower than that of **1** alone both in the absence and in the presence of serum. For comparison, IC_{50} value for Lipofectamine2000 was 20 μM for HEK 293 cells.

The cationic lipid **1** was used for the delivery of a 25-mer 5′-fluorescein isothiocyanate-labeled oligonucleotide (hereinafter, FITC-ON); pEGFP-C_2 plasmid, encoding enhanced green fluorescent protein (EGFP); and small interfering RNA (siRNA) targeted EGFP gene into the HEK 293, BHK, and transgenic BHK IR-780 cells expressing EGFP. The results of these experiments monitored by a flow cytometry are summarized in Table 1. The data are presented as a percentage of either the fluorescence-positive cells (FITC-ODN, pDNA) or non-fluorescent cells (when silencing of EGFP gene caused by the delivered siRNA was achieved) in a sample and as an average fluorescence of the cells population in a sample. The cell population incubated with nucleic acids in the absence of **1** were used as a negative control: in these samples the percentage of both the fluorescence-positive cells (FITC-ODN, pDNA) and the non-fluorescent cells (siRNA) did not exceed the experimental error. The transfection of the nucleic acids mediated by Lipofectamine 2000 was used as a positive control.

The cationic lipid **1** (10 μM) enhanced significantly the accumulation of FITC-ON (10 μM) in the cells (HEK 293, and BHK) both in the absence and in the presence of serum (Table 1). The accumulation efficiency (number of FITC-positive cells in a population) was similar for HEK 293 and BHK cells. A comparison of **1** with Lipofectamine 2000 shows that **1** stimulates an accumulation of FITC-ON with an efficiency that is close to or exceeds these parameters for Lipofectamine 2000.

Analysis of the cellular delivery of plasmid DNA (2 μg/mL) mediated by **1** (20 μM) was performed 24 h post incubation of HEK 293 cells with pDNA/lipid **1** complexes to achieve high levels of EGFP expression (Table 1). In the absence of serum **1** mediates the accumulation of pEGFP-C_2 in the cells with the efficiency close to that of Lipofectamine. However, in the presence of serum the number of EGFP-positive cells is decreased noticeably, indicating that cellular delivery of pDNA mediated by **1** is strongly affected in the presence of serum.

Small interfering RNAs are widely used for gene silencing. Analysis of the delivery of siRNA (50 nM) targeted EGFP mediated by **1** (10 μM) was performed using transgenic BHK IR-780 cells. siRNA used in the study delivered in the complex with Lipofectamine (positive control) efficiently inhibits expression of EGFP both in the absence and in the presence of serum in the cell medium, while scrambled siRNA (50 nM) under similar conditions causes insignificant changes of the EGFP expression (primary data not shown). Under serum-free conditions the efficiency of siRNA delivery mediated by **1** is distinctly higher than with Lipofectamine both in terms of the number of non-fluorescent cells in population and an average cell fluorescence (Table 1), however in the presence of serum efficiency of siRNA delivery is decreased 2.5 times. The inhibitory effect of serum on the delivery mediated by **1** of both plasmid DNA and siRNA allow us to suppose that additional stabilization of the complexes nucleic acid/**1** possibly is required.

The ability of the lipid to compact nucleic acids (plasmid DNA) was studied using atomic force microscopy (Fig. 2). The topographic AFM images show that particles formed by **1** (20 μM, as in the experiments with the plasmid DNA delivery) with plasmid DNA (5.5 μg/mL) have a predominately spherical shape 50–100 nm in a diameter (Fig. 2B). The ability of the polycationic lipid to compact the large plasmid DNA (Fig. 2A) to the small granules, having the size lower than 100 nm, was in accordance with the effective uptake and delivery of the nucleic acids by the lipid.[23]

Table 1
Analysis of cellular accumulation of various nucleic acids mediated by polycationic lipid 1

Nucleic acid	10% FBS	Control		Lf		Lipid 1	
		% of positive cells in population	RFU	% of positive cells in population	RFU	% of positive cells in population	RFU
FITC-ODN[a]	–	0.2	95	75.4	2714	63.3	4198
	+	0.2	97	72.7	1654	94.6	13,243
pDNA[b]	–	1.1	236	29	7512	31.2	5108
	+	1.8	250	27	8295	2.9	406
siRNA[c]	–	1.2[d]	13988	39.8[d]	7122	49.4[d]	6872
	+	1.3[d]	16522	45.4[d]	6065	19.6[d]	14,246

Flow cytometry data.

[a] An accumulation of the complexes FITC–ODN (5 μM)/**1** (10 μM) in the BHK cells in the absence (–) or in the presence (+) of 10% FBS. The percentage of FITC-positive cells and the average fluorescence of the cells in a population were determined by FACS analysis after 4 h of cell incubation with the FITC–ODN/**1** complexes.

[b] An expression of EGFP-transgene in the HEK 293 cells. The cells were incubated with the complexes composed of plasmid pEGFP-C2 (2 μg/mL) and **1** (20 μM) in the absence (–) or in the presence (+) of 10% FBS. The percentage of EGFP-positive cells and the average fluorescence of a cell population were estimated by FACS analysis after 24 h of cell incubation with the cationic lipid/pEGFP-C_2 complex.

[c] An inhibition of the EGFP expression in the transgenic TGFP expressing BHK IR-780 cells. The cells were incubated with the complexes composed of **1** (10 μM) and siRNA-EGFP (50 nM) in the absence (–) or in the presence (+) of 10% FBS. The levels of EGFP expression in the cells were evaluated using flow cytometry after 72 h of incubation with these complexes.

[d] The percentage of non-fluorescent BHK IR-780 cells, which means siRNA mediated the silencing of the EGFP gene.

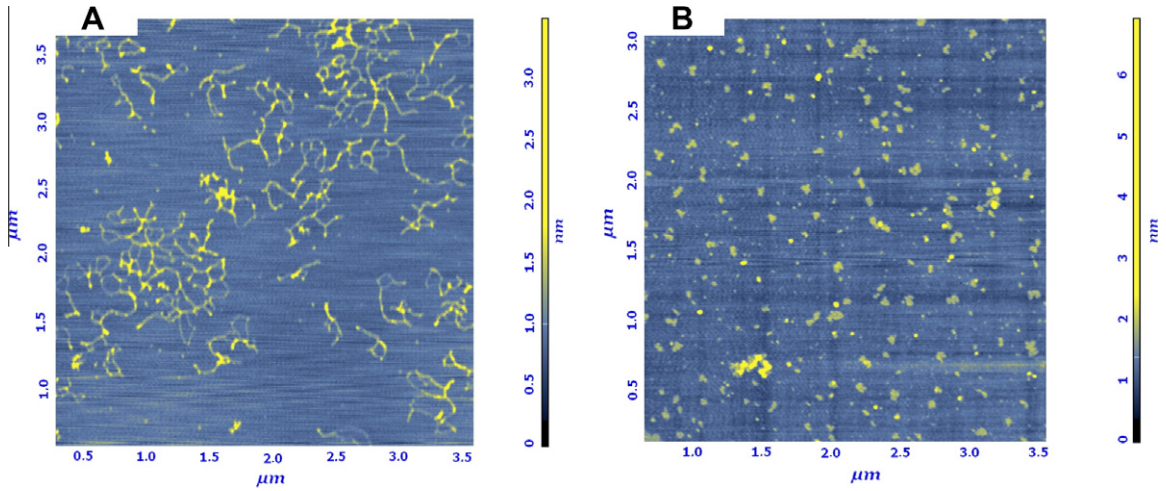


Figure 2. The atomic force microscopy images of the free plasmid DNA (A) and the complexes formed by the plasmid DNA (5.5 μg/mL) and the cationic lipid **1** (20 μM) (B). The AFM analysis was performed in 20 mM Tris–HCl buffer (pH 7.0) containing 5 mM $MgCl_2$. The bar size is 100 nm.

In conclusion, we have elaborated the synthetic route for preparation of the new galactose-containing polycationic lipid, which was shown to be a promising transfection agent in terms of both the low cytotoxicity and the efficient delivery of various nucleic acids in vitro. Lipid **1** stimulates an accumulation of FITC-ON, plasmid DNA and siRNA with an efficiency that is close to or exceeds these parameters for Lipofectamine 2000. The inhibitory effect of serum on the delivery mediated by lipid **1** of both plasmid DNA and siRNA allow us to suppose that additional studies, including the formation of liposomal compositions, nucleic acid-to-carrier ratio, and delivery conditions are required.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.03.056.

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