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Hepatocellular targeted α -tocopherol based pH sensitive galactosylated lipids: design, synthesis and transfection studies†

Venkanna Muripiti,^a Brijesh Lohchania,^b
Srujan Kumar Marepally^b and Srilakshmi V. Patri *^a

Receptor mediated gene delivery to the liver offers advantages in treating genetic disorders such as hemophilia and hereditary tyrosinemia type I (HTI). Prior findings demonstrated that tethering the D-galactose head group to cationic lipids directs genes to the liver via asialoglycoprotein receptors (ASGPRs). In our continued efforts to develop safer and efficient lipofectins, we demonstrated that cationic lipids bearing α -tocopherol, an antioxidant, as a hydrophobic domain could deliver genes efficiently with high safety profiles in multiple cell lines. Towards developing ASGPR targeted pH sensitive cationic lipids, we have designed a galactosylated cationic lipid (Toc-Gal) with α -tocopherol as the hydrophobic core covalently connected with a pH responsive triazole moiety and a non-targeting control lipid (Toc-OH) without the galactose head group. In this study, we present the design and synthesis of a pH sensitive galactosylated cationic lipid (Toc-Gal), its comparative transfection biology, cellular uptake studies, serum stability and cytotoxicity profiles in both ASGPR positive and negative liver cells, i.e. HepG2 and SK-Hep-1, respectively.

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1. Introduction

Cationic lipid based delivery systems hold great promise among the non-viral transfection vectors for nucleic acid delivery applications.^{1,2} These delivery systems are considered as the safest alternatives to their viral counter parts owing to their easy preparation, non-immunogenic nature and efficiency in forming stable, injectable complexes even with large-size DNA (up to 10 000 bp).^{3–5} Typical non-viral cationic lipid delivery systems used for nucleic acid delivery contain a polar head group with a positive charge and a hydrophobic domain of steroidal origin (cholesterol or similar skeletons) or aliphatic long chains separated with/without a spacer/linker.^{6,7} Cationic liposomes can deliver therapeutic payload to specific body cells by tethering receptor specific ligands in a target-guided manner with enhanced efficacies.^{8,9} Liver targeted gene therapy can make a great impact on the treatment of genetic disorders such as hemophilia and hereditary tyrosinemia type I (HTI).^{10–12} Prior findings

demonstrated that galactosylated cationic lipids could effectively deliver therapeutic payload to the liver through asialoglycoprotein receptors (ASGPRs).^{13,14} However, cationic liposomes exhibit necroptosis due to the high density of positive charges at the surface of the liposomes which depolarize the negatively charged cell surface, triggering the up regulation of intracellular reactive oxygen species (ROS) which in turn leads to the necroptosis mechanism. To address these issues, we developed cationic lipids with α -tocopherol as the hydrophobic core. α -Tocopherol, a form of vitamin-E, is a membrane antioxidant, and helps in preventing lipid peroxidation as compared to cholesterol.¹⁵ Other analogues, such as vitamin-E-TPGSs, are used extensively as solubilizers in nanoparticle formulations and in clinical use.^{16,17} In our continued efforts to develop safer and efficient transfection reagents, α -tocopherol derivatized cationic lipids were found to be effective and safe in multiple cultured cell lines.^{18,19} More importantly, α -tocopherol based lipids exhibited improved transfections in the presence of serum.²⁰ The rationale for using triazole as a linker between the hydrophilic moiety and the hydrophobic core is that triazole imparts pH sensitivity and stability and improves DNA binding properties.²¹ The triazole linker facilitates the escape of lipoplexes from endosomes in a pH responsive manner.^{22,23} V. V. Kumar *et al.* demonstrated that

^a National Institute of Technology, Warangal-506004, Telangana, India.E-mail: patrisrilakshmi@gmail.com^b Centre for Stem Cell Research, Vellore, Tamil Nadu, India

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Chemical structures of cationic lipids

Taking cues from our previous findings, in the present study, we developed an α -tocopherol based ASGPR targeted liposomal delivery system by conjugating the galactose ligand to tocopherol through a triazole linker, **Toc-Gal**, for efficient delivery of nucleic acid into hepatocytes (Chart 1). In addition, we synthesized a control non-targeting lipid, Toc-OH, similar to the Toc-Gal lipid except for the conjugation of the ASGPR targeting ligand (D-galactose). We characterized biophysical properties such as the size and zeta potential and performed DNA binding studies with liposomes of Toc-Gal and Toc-OH lipids using electrophoresis and circular dichroism. Cell viabilities, transfection efficacies and ASGPR specific targeting properties were evaluated in ASGPR positive and negative cell lines (HepG2 and SK-HEP-1) using reporter gene assays and

Chart 1

Scheme 1 A: Synthesis of galactosylated lipid (Toc-Gal). Reagents: i) 50% NaOH solution, tetrabutylammonium hydrogen sulphate; ii) NaN₃, 1:1 THF/H₂O, 12 h reflux; iii) β-D-galactose tetraacetate *o*-propargyl glycoside, CuSO₄·5H₂O, sodium ascorbic acid, 1:1 THF/H₂O, 18 h, r.t.; iv) dry MeOH, NaOMe, 12 h, r.t.; v) 6 N HCl, diethyl ether, 0.5 h, r.t. B: Synthesis of control lipid (Toc-OH). Reagents: i) CuSO₄·5H₂O, sodium ascorbate, 1:1 THF/H₂O, 24 h, r.t.; ii) dry acetonitrile, 6 N HCl, 24 h, reflux.

receptor saturation studies. In addition, serum compatibility studies were also performed in representative HepG2 cells.

2. Results and discussion

2.1 Chemistry

Synthetic routes adopted for preparing the α -tocopherol based cationic glycolipid, Toc-Gal, and its control lipid, Toc-OH, are shown schematically in Scheme 1A and B, respectively. The precursor intermediate α -tocopheryl-1,2-azido alcohol (2A, Scheme 1A) for the synthesis of both lipids, Toc-Gal and Toc-OH, was prepared conventionally in 2 steps. Briefly, *o*-alkylation of α -tocopherol with epichlorohydrin in the presence of 50% sodium hydroxide and tetrabutylammonium hydrogen sulfate provided the epoxide linked α -tocopherol, 1A, which upon epoxide opening with sodium azide afforded intermediate 2A (Scheme 1A). Intermediate 2A upon treatment with β -D-galactose tetraacetate-*o*-propargyl glycoside, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, and sodium ascorbic acid provided intermediate 3A (Scheme 1A). Intermediate 3A upon *o*-acetyl deprotection using sodium methoxide in the presence of dry methanol yielded intermediate 4A (Scheme 1A). Protonation/quaternization of intermediate 4A with 6 N hydrochloric acid afforded the title glyco-lipid, Toc-Gal (Scheme 1A). The control lipid, Toc-OH, was synthesized by reacting intermediate 2A (from Scheme 1A) with propargyl alcohol in the presence of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ and sodium ascorbate, which yielded α -tocopherol-triazole intermediate 1B (Scheme 1B). The resulting triazole ether intermediate 1B (Scheme 1B) was quaternized/protonated with 6 N hydrochloric acid to afford the target compound Toc-OH (Scheme 1B). The structures of all the synthetic intermediates as shown in Scheme 1A and B were confirmed by ^1H NMR and ESI-MS. The structures of the target lipids, Toc-Gal & Toc-OH, were confirmed by ^1H NMR, ^{13}C NMR, ESI-MS and HRMS. Severe line broadening (particularly in the range δ 3–5 ppm) was observed in the ^1H NMR spectra of Toc-Gal presumably due to the presence of multiple exchangeable hydroxyl protons; thus, Toc-Gal was characterized by the molecular ion peaks in the ESI-MS and HRMS spectra. The purities of the target lipids, Toc-Gal & Toc-OH, were confirmed by reverse phase HPLC analysis using methanol as the mobile phase. The purity of the target lipids was found to be more than 95%.

2.2 Liposomal formulations

Initially, we used Toc-Gal and Toc-OH with cholesterol as the co-lipid at a 1:1 molar ratio (1 mM concentration w.r.t. Toc-Gal/Toc-OH) to prepare liposomes, which didn't yield uniform vesicle formations resulting in precipitation. Subsequently, we used the conventional co-lipids DOPC (1,2-dioleoyl-*sn*-glycerol-phosphocholine) and DOPE (1,2-dioleoyl-*sn*-glycerol-phosphoethanolamine) to prepare liposomes with Toc-Gal & Toc-OH in the above indicated ratio and concentration. We observed the formation of a uniform liposomal solution with DOPE:Toc-Gal/Toc-OH only. However, the liposomal formulation prepared with DOPE as the co-lipid at a 1:1

DOPE:Toc-Gal/Toc-OH ratio exhibited poor DNA binding efficiencies (discussed in the following section). Hence, we had to opt for the next formulation using DOPE with Toc-Gal/Toc-OH at a 2:1 ratio and performed all the experiments with the above formulation.

2.3 Physico-chemical characterization of liposomes and lipoplexes

2.3.1 DNA binding & heparin displacement study. To begin with, we performed a conventional agarose gel electrophoresis assay to evaluate the relative DNA binding efficiencies of the above prepared liposomal formulations at varying lipid:DNA charge ratios. Initially, we used liposomal formulations of Toc-Gal and Toc-OH with DOPE at an equimolar ratio, *i.e.* 1:1 DOPE: Toc-Gal/Toc-OH. The liposomal preparation involving Toc-Gal at a 1:1 molar ratio showed optimal binding at 8:1 and 4:1 lipid:DNA charge ratios. The binding efficiencies were drastically reduced at a 2:1 charge ratio, while there was poor binding of DNA at a 1:1 charge ratio (Fig. S1, ESI†). However, the liposomal preparation of Toc-OH showed little/no binding across the lipid:DNA charge ratios of 8:1–1:1 (Fig. S1, ESI†). The reason for compromised DNA binding could be due to the aromatic planar ring of tocopherol which might be limiting the binding efficiencies. To overcome this limitation, we used liposomal formulations with DOPE and Toc-Gal/Toc-OH at a 2:1 ratio for DNA binding studies. Results from this gel electrophoresis experiment demonstrated excellent DNA binding interactions for Toc-Gal across 10:1 to 2:1 lipid:DNA charge ratios with slight reduction of binding at a 1:1 charge ratio (Fig. 1). However, Toc-OH exhibited high binding at 10:1 and 8:1 charge ratios only. We observed optimal DNA binding at a 4:1 charge ratio while at 2:1 & 1:1 charge ratios poor DNA binding was observed (Fig. 1A). Further, a heparin displacement experiment was performed to confirm the DNA binding data obtained with the above liposomal formulations. The liposomal formulation with Toc-Gal resisted displacement of DNA with

Fig. 1 Electrophoretic gel patterns for lipoplex-associated DNA in agarose gel retardation assay (A) and heparin displacement assay (B). The lipid/DNA charge ratios are indicated at the top of each lane.

heparin across lipid:DNA charge ratios of 10:1–1:1 (Fig. 1B). However, the Toc-OH formulation could survive DNA displacement at 10:1 and 8:1 charge ratios. DNA from Toc-OH lipoplexes was gradually displaced as the lipid:DNA charge ratio decreased from 4:1 to 1:1 (Fig. 1B). Results from the above experiments suggest that both Toc-Gal and Toc-OH showed similar DNA binding efficiencies at higher lipid:DNA charge ratios, *i.e.* 10:1 and 8:1.

2.3.2 Circular dichroism (CD). Circular dichroism (CD) is useful for investigating the structure of pDNA and the conformational changes produced by ligand or cationic lipid binding.^{26–28} Generally, the CD phenomenon of pDNA occurs due to the electronic transitions of nucleobases. The interactions between these nucleobases and cationic lipids give intense CD, which is very sensitive to the total conformation of these biomolecules. The correlations between a few characteristic CD spectral features and the structure of various DNA–liposome complexes (B-DNA, A-DNA, C-DNA, *etc.*) might be useful to get information about the mechanistic pathway for gene delivery. In this paper, we report the characterization of DNA–liposome complexes (Toc-Gal and Toc-OH) by circular dichroism (CD) at a charge ratio of 10:1, which is shown in Fig. 2. The CD spectra were recorded from 200 nm to 500 nm to find the changes in the conformation of pDNA arising due to its interaction with the cationic liposome. The CD spectrum of the pDNA present in the canonical B form shows a positive band at 225 nm, a negative signal at 240 nm, and a crossover point near 305 nm, which changed to 250 nm, 218 nm and 260 nm, respectively, for the cationic liposome Toc-Gal, whereas the CD spectrum of the cationic liposome Toc-OH exhibits a positive band at 210 nm and a negative band at 212 nm. The pDNA showed significant conformational changes when it interacted with liposomes of Toc-Gal and

Toc-OH. This result showed that the difference in transfection activity may be due to the difference in the conformational changes of DNA by the addition of Toc-Gal and Toc-OH. It was indicated that α -tocopherol containing cationic lipids formed a stable complex suspension in water.

2.3.3 Liposome sizes and zeta potentials (ζ). Following the DNA binding studies, we have evaluated the physicochemical properties such as the hydrodynamic diameter and zeta potential of the liposomes and lipoplexes of Toc-Gal and Toc-OH using the Dynamic Laser Scattering (DLS) method. Initially, the liposomes of Toc-Gal and Toc-OH showed hydrodynamic sizes of 500 nm and 367 nm, respectively, while their potentials were comparable with +7.12 mV for Toc-Gal and +6.3 mV for Toc-OH (Fig. 3). Next, size data from the lipoplexes showed significant variation for Toc-Gal and Toc-OH across the lipid:DNA charge ratios of 1:1 to 10:1 (Fig. 3A). The size of the lipoplexes prepared from liposomes of Toc-OH was 600 nm at a 1:1 charge ratio and was further found to be within the range of 324–484 nm across 2:1 to 10:1 charge ratios (Fig. 3B). Interestingly, we observed an incremental pattern for the lipoplexes of Toc-Gal, which showed sizes of 855 nm and 985 nm at 1:1–2:1 charge ratios. However, we found a slight decrease in size at the 4:1 charge ratio (953 nm) when compared with the 2:1 charge ratio. At higher charge ratios of 8:1 and 10:1, the sizes of the lipoplexes were found to be 1068 nm and 1154 nm, respectively (Fig. 3A). Unlike the variation observed in hydrodynamic sizes of the lipoplexes of Toc-Gal and Toc-OH, global surface potentials were found to be comparable to each other across 1:1–10:1 lipid:DNA charge ratios (Fig. 3A). Zeta potential data for lipoplexes at 1:1 and 2:1 charge ratios indicated a less cationic nature, showing negative values of –15.8 mV and –11.5 mV for Toc-Gal and Toc-OH, respectively (Fig. 3B). However, the global surface charges were found to be increasing for the lipoplexes of Toc-Gal and Toc-OH across 4:1–10:1 charge ratios (3.9 mV, 9 mV & 16 mV for Toc-Gal; 4.5 mV, 8 mV & 14 mV for Toc-OH) (Fig. 3B). Results from the DLS experiment reveal that the hydrodynamic sizes of liposomes and the global surface charges of both liposomes and lipoplexes of Toc-Gal and Toc-OH didn't show significant variation. However, there was an increase in the hydrodynamic diameters of the lipoplexes prepared using the liposomes of Toc-Gal as the lipid:DNA charge ratio increased. It can be argued that the observed increment in the sizes of the Toc-Gal lipoplexes could be due to the higher hydration at the surface of the liposomes since Toc-Gal contains a galactose head-group, which has 3 additional hydroxyl groups when compared with Toc-OH, which may lead to a higher degree of hydration. Hence, the observed size increase with the increase in lipid:DNA charge ratio could be due to the additional hydroxyl groups present in the head-group of Toc-Gal.²

Fig. 2 Circular dichroism (CD) spectra of lipid–pDNA complexes. Liposomes were prepared at a 2:1 molar ratio of colipid DOPE and cationic lipids, *i.e.* (Toc-Gal and Toc-OH). Complexes were prepared at a charge ratio of 10:1 and CD was recorded at a pDNA concentration of 50 $\mu\text{g mL}^{-1}$. The CD spectra were compared to the profile of pDNA alone. Spectral profiles of liposomes with MilliQ water alone were subtracted as blank.

2.4 Cell viability

The cell viabilities, using cationic liposomes of Toc-Gal & Toc-OH in complex with pDNA (pCMV-SPORT- β -

Fig. 3 Graphical representation of hydrodynamic diameters (A) and zeta potentials (ζ , mV) (B) of the lipoplex-associated DNA in plain DMEM.

galactosidase), were assessed by the MTT assay in 2 cell lines, HepG2 and SK-HEP-1. Results from the study showed that the lipoplexes of both Toc-Gal and Toc-OH exhibited minimal cytotoxicity across the 1:1–10:1 lipid:DNA charge ratios in both cell lines. The cell viability was found to be more than 90% up to the 8:1 lipid:DNA charge ratio for the 2 lipids and ~85% viable cells were found at the 10:1 charge ratio (Fig. 4). It is evident from the cell viability data that the variation/enhancement in transfection efficacies of Toc-Gal and Toc-OH lipids cannot be attributed to their cytotoxic effects.

2.5 Transfection biology and cellular expression study of α 5GFP

The relative *in vitro* transfection efficacies of Toc-Gal and Toc-OH were initially evaluated using pEGFP (a plasmid DNA encoding green fluorescent protein) in ASGPR positive and negative cells, HepG2 & SK-HEP-1, at lipid:DNA charge ratios of 10:1 & 8:1 (optimal charge ratios as revealed by DNA binding studies). Transfection efficacies obtained in HepG2 cells treated with the lipoplexes of Toc-Gal were found to express higher percentages of the green fluorescent protein than the control lipid, Toc-OH, at the 8:1 charge ratio followed by the 10:1 charge ratio (Fig. 5A). Further, SK-HEP-

1 cells revealed similar transfection profiles for both Toc-Gal and Toc-OH at 10:1 & 8:1 charge ratios (Fig. 5B). Interestingly, the transfection activities obtained for Toc-Gal in HepG2 cells were found to be 2–3 fold higher when compared with the transfection activities in SK-HEP-1 cells. However, no such variation was observed for Toc-OH transfection activity (Fig. 5B).

Microscopy image data obtained from the cellular uptake and expression study revealed the highest GFP expression for Toc-Gal at the 8:1 charge ratio. Surprisingly, Toc-Gal at the 10:1 charge ratio showed ~20% less fluorescence as compared to Toc-Gal at the 8:1 charge ratio. Further, GFP expression in Toc-OH treated HepG2 cells was found to be ~60% less when compared with Toc-Gal at their respective lipid:DNA charge ratios (Fig. 5C). In addition, GFP expression for Toc-Gal was found to be 2-fold higher than Lipofectamine 2000 in the HepG2 cell line (Fig. 5). The enhanced transfection activity for Toc-Gal in HepG2 cells could be due to the presence of ASGPRs on the cell surface of HepG2 cells which might increase the endocytosis through receptor mediated lipoplex internalization resulting in enhanced protein expression. Since SK-HEP-1 cells do not express ASGPRs on their cell surfaces, reduced transfection activities for the lipoplexes of Toc-Gal might be observed when compared with HepG2

Fig. 4 MTT assay based percent cell viabilities in HepG2 (A) and SK Hep-1 cells (B). Cells were treated with lipoplexes of lipids (Toc-Gal & Toc-OH) and pDNA (pCMV-SPORT- β -gal) with lipid:DNA charge ratios of 1:1–10:1.

Fig. 5 *In vitro* transfection studies for the liver targeted lipid, Toc-Gal, and the control lipid, Toc-OH, in HepG2 (A) and SK Hep-1 (B) cells. % of GFP positive cells quantified by FACS. Representative epifluorescence microscopy images of HepG2 cells transfected with lipoplexes of Toc-Gal & Toc-OH with eGFP encoded plasmid DNA at lipid/DNA charge ratios of 8:1 & 10:1 (C).

cells. This is supported by the transfection data obtained in HepG2 & SK-HEP-1 cells for the lipoplexes of Toc-OH (which do not have the ASGPR targeting ligand) which showed comparable activity in both cell lines. Plasmid DNA expression results suggested that receptor mediated endocytosis of Toc-Gal had better transfection efficiencies.

2.6 Study of ASGPR specificity of Toc-Gal

High affinity ASGPRs are over-expressed in hepatocytes (liver cells).²⁹ D-Galactose is one of the well-known ligands for ASGPRs as such ligands play a key role in targeted delivery of nucleic acids.¹³ Toc-Gal was designed for targeting ASGPRs expressed in hepatocytes along with a negative control lipid, Toc-OH (without a galactose head-group), to establish the receptor specificity of Toc-Gal in ASGPR +ve HepG2 cells. To determine the ASGPR specificity of the newly synthesized lipid Toc-Gal and its control lipid Toc-OH, we performed cellular uptake studies in ASGPR positive cells (HepG2) with a concentration dependent ligand saturation experiment. Asialofetuin, a naturally occurring ligand for ASGPRs, was used as the saturating ligand at 0–100 μ M and liposomes

were labeled with rhodamine (Rh). The uptake efficiency for Toc-Gal without ligand saturation was found to be the highest (35% Rh positive cells) and the activity significantly diminished dose-dependently in HepG2 cells pre-treated with asialofetuin, a natural ligand for ASGPRs (Fig. 6A). Upon receptor saturation with 25 μ M asialofetuin, the transfection activity was reduced by 20% for Toc-Gal (showing 28% Rh positive cells, normalized value) and ~70% reduction was observed with 100 μ M asialofetuin (Fig. 6). However, the control lipid, Toc-OH, in HepG2 cells did not show significant variation in the transfection activity in both normal (no receptor saturation) and asialofetuin pre-treated (receptor saturation) cells (Fig. 6). These data were further supported by the microscopy images obtained using HepG2 with/without ligand pre-treatment for Toc-Gal and Toc-OH transfections. In the microscopy images of Toc-Gal lipoplex treatment, the number of Rh positive cells was found to be remarkably less when cells were pre-incubated with asialofetuin than for untreated cells while the number of Rh positive cells didn't change with Toc-OH lipoplex treated cells (Fig. 6). Together, these findings confirmed that the cellular uptake of Toc-Gal is likely to be mediated *via* the ASGPRs present in the cell surface of HepG2 cells.

2.7 Serum stability

In general, transfection activities of liposomes (prepared using different cationic lipids) are evaluated mostly in complete medium, *i.e.* medium containing 10% (v/v) serum or in serum-free medium.^{24,30–32} It is believed that the transfection activity of cationic lipids decreases drastically due to the interaction of negatively charged serum proteins with liposomes containing a cationic charge, ultimately hampering the efficient interaction with the cell surface and reducing the internalization of lipoplexes.^{33,34} The clinical success of *in vitro* transfection efficient cationic lipids also depends on the serum stability/serum compatibility. Hence, it is necessary to evaluate the compatibility of such transfection efficient lipids with varying serum concentrations. To evaluate the serum compatibility of Toc-Gal and Toc-OH lipids, we have performed transfection studies in representative HepG2 cells with increasing amounts of added serum (from 10–90%) at an 8:1 lipid:DNA charge ratio. As shown in Fig. 6, the transfection efficacies of Toc-Gal increased as the percentage of added serum increased (up to 50% added serum). For Toc-Gal, we observed ~30% enhancement in the gene transfer activity at 50% added serum (Fig. 6). However, the transfection activity of Toc-Gal was found to be decreasing at high concentrations of added serum (60–90% added serum) (Fig. 7). In contrast, the transfection activity of the Toc-OH lipid showed poor serum compatibility with increasing amount of added serum. Further, we observed that the gene transfer efficacy of Toc-OH was drastically reduced with 40–90% of added serum (Fig. 7). The enhanced serum compatible transfection activities of the Toc-Gal lipid when compared with the Toc-OH lipid could be due to the multiple hydroxyl

Fig. 6 Transfection properties of Toc-Gal and the control lipid Toc-OH after ASGPR saturation with asialofetuin at varying concentrations in HepG2 cells (A). Representative epifluorescence microscopy images of HepG2 cells transfected with lipoplexes of rhodamine labelled Toc-Gal & Toc-OH liposomes with and without ASGPR saturation (B).

Fig. 7 Transfection efficacies of Toc-Gal & Toc-OH in the presence of increasing concentration of added serum. The *in vitro* transfection efficiencies of lipid:DNA complexes prepared using the pCMV-β-gal-SPORT reporter gene at a lipid:DNA charge ratio of 8:1 were evaluated in the presence of increasing concentration of added serum in representative HepG2 cells.

functionalities of the galactose moiety present in the polar head-group region which might be shielding the surface charge of the lipid:DNA complexes. Overall, such enhanced transfection efficacy of the Toc-Gal lipid with increasing amount of serum (up to 50% added serum) shows the serum stability of the lipoplexes prepared using Toc-Gal liposomes which are likely to find use in transfecting cells in systemic settings.

3. Conclusions

In summary, we demonstrated that cationic galactosylated lipids linked with a triazole moiety to an anti-oxidant tocopherol hydrophobic tail efficiently delivered genes into hepatocellular carcinoma cells (HepG2) through ASGPRs. The novel cationic lipids were found to be safe in both HepG2 liver cells and SK-HEP-1 liver endothelial cells. Pre saturation of ASGPRs with the synthetic ligand asialofetuin retarded the cellular uptake of Toc-Gal lipoplexes, whereas the uptake of Toc-OH lipoplexes was unchanged. Further, Toc-Gal was

found to be serum compatible with enhanced transfection activity (up to 50% added serum). These findings collectively suggested that Toc-Gal could be used for liver specific gene delivery without damaging the liver cells.

4. Materials and methods

4.1 General procedures and reagents

Mass spectral data were acquired by using a commercial LCQ ion trap mass spectrometer (ThermoFinnigan, San Jose, CA, USA) equipped with an ESI source or a micromassQuattro LC triple quadrupole mass spectrometer for ESI analysis. ^1H NMR spectra were recorded on an AV 400 MHz NMR spectrometer. Unless otherwise stated, all reagents were purchased from local commercial suppliers and were used without further purification. Column chromatography was performed with silica gel (Acme Synthetic Chemicals, India, 60–120 mesh). Cell culture medium, fetal bovine serum, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and HepG2 (human hepatocarcinoma cells) cell line were procured from the National Centre for Cell Sciences (NCCS), Pune, India and SK-HEP-1 cells from ATCC. Cells were grown at 37 °C in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS in a humidified atmosphere containing 5% CO_2 and 95% air.

4.2 Synthesis

i. Synthesis of liver targeted lipid 1 (Toc-Gal), Scheme 1A

a. Synthesis of α -tocopheryl glycidyl ether (1A). A mixture of α -tocopherol (8.2 g, 20 mmol), epichlorohydrin (5.6 g, 60.5 mmol), sodium hydroxide pellets (2.4 g, 60 mmol), water (1 mL, 56 mmol) and tetrabutylammonium hydrogen sulfate (0.322 g, 1 mmol) was stirred for 24 h at 40 °C. The reaction mixture was filtered and the filtrate obtained was extracted with dichloromethane (3×20 mL). The combined organic layers were dried with anhydrous magnesium sulfate. The solvent and excess epichlorohydrin were distilled under reduced pressure and the obtained residue was purified by

silica gel column chromatography (v/v 40:1, pet ether/ethyl acetate) to give intermediate 1A as oil (7.8 g, 82% yield).

^1H NMR (400 MHz, CDCl_3): δ/ppm = 0.85 (s, 12H), 1.05–1.61 (m, 24H), 1.80 (m, 2H), 2.08 (s, 3H), 2.14 (s, 3H), 2.18 (s, 3H), 2.57 (t, 6.7 Hz, 2H), 2.71 (m, 1H), 2.88 (t, 4.6 Hz, 1H), 3.35 (m, 1H), 3.65 (m, 1H), 3.90 (m, 1H).

ESI-MS m/z : calculated 486 (for $\text{C}_{32}\text{H}_{54}\text{O}_3$); found 487 $[\text{M}^+]$.

b. Synthesis of α -tocopherol-1,2-azido alcohol (2A). To a mixture of tocopherol epoxide (0.486 g, 1 mmol) and $\text{CeCl}_3 \cdot 7\text{H}_2\text{O}$ (0.18609 g, 0.5 mmol) in an acetonitrile & water mixture (9:1, 10 mL) was added NaN_3 (0.0715 g, 1.1 mmol) at reflux temperature for 12 h. The reaction progress was monitored by TLC. After completion of the reaction, the reaction mixture was diluted with water (20 mL) and extracted with ethyl acetate (3 $\times$ 20 mL). The combined organic layers were washed with brine, dried over anhydrous sodium sulfate and evaporated to dryness under reduced pressure. The crude product was purified by column chromatography with 60–120 mesh silica gel using hexane in ethyl acetate as the eluent (10:90, v/v) to give intermediate 2A as a yellow liquid (0.4 g, 88% yield).

^1H NMR (400 MHz, CDCl_3): δ/ppm = 0.83–0.87 (m, 12H), 1.12–1.37 (m, 26H), 2.08 (s, 3H), 2.12 (s, 3H), 2.17 (s, 3H), 2.58 (m, 2H), 2.3 (m, 2H), 3.8 (m, 1H), 4.2 (m, 2H).

ESI-MS m/z : calculated 529.243 (for $\text{C}_{32}\text{H}_{55}\text{O}_3\text{N}_3$); found 552 $[\text{M} + 23]$.

c. Synthesis of the liver targeted lipid intermediate (3A). A mixture of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (15 mg, 0.06 mmol), sodium ascorbate (30 mg, 0.12 mmol), α -tocopherol-2-azido-alcohol (347 mg, 0.65 mmol) and β -D-galactose tetraacetate *o*-propargyl glycoside (1.1 mmol) in $\text{THF}/\text{H}_2\text{O}$ (7 mL, 1:1) was heated for 1 h under microwave irradiation (100 $^\circ\text{C}$). The product was precipitated by the addition of methanol (20 mL). The precipitate was washed with methanol and dried over reduced pressure. The crude product mixture was purified by column chromatography with CHCl_3 /methanol. The product was obtained as a yellow liquid in 89% yield.

^1H NMR (400 MHz, CDCl_3): δ/ppm = 0.83–0.87 (m, 12H), 1.12–1.37 (m, 26H), 2.08 (s, 3H), 2.12 (s, 3H), 2.17 (s, 15H), 2.58 (m, 2H), 3.24 (s, broad OH, 1H), 3.34 (s, broad 4H), 3.39 (m, 2H), 3.4 (m, 1s), 3.7 (m, 2H), 3.8 (m, 1H), 3.81 (m, 1H), 3.84 (d, 1H), 3.94 (d, 1H), 4.95 (d, 1H), 8.01 (s, 1H).

ESI-MS m/z : calculated 915.546 (for $\text{C}_{49}\text{H}_{77}\text{O}_{13}\text{N}_3$); found 916 $[\text{M}^+]$.

d. Synthesis of the liver targeted lipid (Toc-Gal) (4A). In a 25 mL round bottom flask, 0.5 mL of 0.5 N sodium methoxide (freshly prepared by dissolving 0.13 g of sodium in dry methanol) was added to a solution of intermediate 3A (30 mg, 0.035 mmol, prepared above in step h) in dry methanol (1 mL). The reaction mixture was stirred at room temperature for 1 h, neutralized with Amberlite IR120 (H^+), and filtered; the filtrate was dried over anhydrous sodium sulphate and concentrated on a rotary evaporator. Column chromatographic purification of the residue (using 60–120 mesh size silica gel and 9–10% methanol in chloroform as the eluent) was followed by the quaternization of the

compound with anhydrous HCl. The product was obtained as a yellow liquid in 92% yield.

^1H NMR (400 MHz, CDCl_3): δ/ppm = 0.83–0.87 (m, 12H), 1.12–1.37 (m, 26H), 2.08 (s, 3H), 2.12 (s, 3H), 2.17 (s, 3H), 2.58 (m, 2H), 3.24 (s, broad OH, 1H), 3.34 (s, broad 4H), 3.39 (m, 2H), 3.4 (m, 1s), 3.7 (m, 2H), 3.8 (m, 1H), 3.81 (m, 1H), 3.84 (d, 1H), 3.94 (d, 1H), 4.95 (d, 1H), 8.01 (s, 1H).

^{13}C NMR (400 MHz, CDCl_3): δ/ppm = 155.67, 148.00, 147.86, 131.14, 128.91, 127.73, 127.68, 125.78, 125.71, 122.98, 117.61, 74.84, 73.75, 73.69, 69.75, 59.19, 52.68, 50.61, 44.66, 40.07, 39.40, 37.59, 37.49, 37.43, 37.32, 32.81, 32.71, 31.28, 28.01, 24.85, 24.47, 23.90, 22.77, 22.68, 21.06, 20.67, 19.79, 19.72, 12.76, 12.66, 11.89, 11.81.

ESI-MS m/z : calculated 748.51 (for $\text{C}_{41}\text{H}_{70}\text{O}_9\text{N}_3$); found 748 $[\text{M}^+]$.

ii. Synthesis of control lipid 2 (Toc-OH), Scheme 1B. A mixture of intermediate 2A (0.347 g, 0.6 mmol) (Scheme 1A), $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (0.015 g, 94 μmol), sodium ascorbate (0.030 g, 0.15 mmol), and propargyl alcohol (0.0109 g, 0.7 mmol) in $\text{THF}/\text{H}_2\text{O}$ (7 mL, 1:1) was heated for 1 h under microwave irradiation (100 $^\circ\text{C}$). The product was precipitated by the addition of methanol (20 mL). The precipitate was collected, washed with methanol and dried under reduced pressure. The crude product was subjected to column chromatography purification with a CHCl_3 /MeOH solvent system to obtain pure intermediate 1B as a yellow compound, followed by quaternization with anhydrous HCl, affording the title compound lipid 2. The product was obtained as a yellow liquid in 92% yield (0.092 g, 78% yield).

^1H NMR (400 MHz, CDCl_3): δ/ppm = 0.83–0.87 (m, 12H), 1.12–1.37 (m, 26H), 2.08 (s, 3H), 2.12 (s, 3H), 2.17 (s, 3H), 2.58 (m, 2H), 3.42 (s, OH broad), 3.64 (s, OH broad), 3.7 (m, 2H), 3.82 (m, 1H), 4.21 (m, 2H), 4.3 (s, 2H), 7.7 (s, 1H).

ESI-MS m/z : calculated 586.46 (for $\text{C}_{35}\text{H}_{60}\text{O}_4\text{N}_3$); found 586 $[\text{M}^+]$.

4.3 Preparation of liposomes and formulations

1 mM Toc-gal and Toc-OH with DOPE were dissolved in 100 μL of chloroform in glass vials. The solvent was removed with the help of a thin flow of moisture-free nitrogen gas and the glass vial was left to dry under high vacuum for 3 h. Then 1 mL of deionized sterile water is added to the vacuum dried lipid film and the mixture was allowed to swell overnight. The vials were then vortexed for 2–3 minutes at room temperature to produce unstill multi lamellar vesicles (MLVs). After the formation of the MLVs, they were sonicated initially in a water bath followed by probe sonication with the help of a Branson 450 sonifier at 100% duty cycle and 25 W output power to produce small unilamellar vesicles (SUVs). eGFP plasmid was amplified in the DH5 α -strain of *Escherichia coli*, and the purity of the plasmid was checked using the A_{260}/A_{280} ratio (around 1.9).

4.4 Zeta potential (ξ) and size measurements

The hydrodynamic diameters and surface potentials of Toc-Gal and Toc-OH liposomal formulations and their

corresponding lipoplexes at varying lipid:DNA charge ratios were measured by the dynamic light scattering method using a Zeta sizer 3000HS_A (Malvern UK). The sizes of the liposomes and lipoplexes were evaluated in serum-free medium using the following parameters: a sample refractive index of 1.59 and a viscosity of 0.89. The zeta sizer instrument was calibrated by using a 200 nm $\pm$ 5 nm polystyrene polymer (Duke Scientific Corps. Palo Alto, CA) with auto-mode calculation of hydrodynamic diameters. The surface potentials of the liposomes and lipoplexes were measured with the indicated parameters (viscosity, 0.89 cP; dielectric constant, 79; temperature, 25 °C; $F(Ka)$, 1.50 (Smoluchowski); maximum voltage of the current, V). The system was calibrated by using a DTS0050 standard from Malvern, UK. Measurements were done 10 times with zero field correction and the potentials were calculated by using the Smoluchowski approximation.

4.5 DNA-binding assay

The DNA binding ability of Toc-Gal and Toc-OH liposomal formulations at varying lipid:DNA charge ratios was evaluated by the agarose gel retardation assay.^{31–35} 1% agarose gel (pre-stained with ethidium bromide) was used for the assay. Briefly, lipoplexes were prepared using pCMV- β -gal (0.30 μ g) with varying amounts of liposomes in a total volume of 20 μ L in HEPES buffer, pH 7.40, and incubated at room temperature for 20–25 minutes. 4 μ L of 6 $\times$ loading buffer (0.25% bromophenol blue in 40% (w/v) sucrose in H₂O) was added to each of the samples and the resulting solution (24 μ L) was loaded onto each well of the agarose gel. The samples were electrophoresed at 80 V for 45 minutes and the DNA bands were visualized in the gel documentation unit.

4.6 Heparin displacement assay

Heparin was used to study the anionic displacement of DNA within the lipoplexes. The lipid:DNA complexes were prepared as described in the above section (DNA binding assay) and incubated for 20 minutes. Following incubation, 0.1 μ g of sodium salt of heparin was added and further incubated for another 30 minutes. The samples were electrophoresed in an agarose gel (1.5%) for heparin displacement analysis and DNA bands were visualized as mentioned in the above section.

4.7 Circular dichroism

Circular dichroism (CD) spectra of plasmid DNA and pDNA-liposome complexes were obtained using an Aviv 62A spectrometer (Lakewood, NJ). Samples were scanned in the wavelength range of 200–500 nm at 25 °C using a 0.5 mL quartz cuvette, with a path length of 1 cm. Each sample was scanned four times with an integration time of 5 s, and the values were averaged. Scanning was done at 1 nm steps, and the slit width was 1 nm. Liposome-pDNA complexes were prepared at a 10:1 $\pm$ charge ratio at 50 μ g mL⁻¹ pDNA concentration. Sample values were subtracted from those obtained for MilliQ water (for pDNA).

4.8 Toxicity assay

Cytotoxicities of Toc-Gal and Toc-OH liposomal formulations at varying lipid:DNA charge ratios were evaluated using the conventional (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) based colorimetric cell viability assay as reported previously.¹³ The assay was performed in 2 different cell lines (HepG2 and SK-HEP-1 cells) under similar conditions to those used for transfection experiments. After 48 h of lipoplex incubation, MTT was added directly to the 96-well plates and further incubated for 3–4 h. Next, the medium was removed without disturbing the formazan crystals following the addition of DMSO. The well plates were kept for gentle shaking for 15–20 min and the absorbance values were recorded using a microplate reader. Results were expressed as percent viability = $[A_{540}(\text{treated cells}) - \text{background}] / A_{540}(\text{untreated cells}) - \text{background}] \times 100$.

4.9 Transfection biology and cellular expression study of α 5GFP

The transfections were performed using α 5GFP plasmid in HeG2 and SK-Hep-1 cells. Cell lines were seeded onto 24 well plates maintaining the density of 50 000 cells per well a day before the transfection. Then, 0.9 μ g of α 5GFP plasmid DNA encoding green fluorescent protein was complexed with the liposomes of lipids Toc-Gal and Toc-OH at charge ratios (lipid-DNA complexes) of 10:1 & 8:1 in plain DMEM medium (total volume made up to 100 μ L) for 30 min. Just prior to transfection, cells plated in the 24-well plate were washed with PBS (2 $\times$ 100 μ L) followed by addition of lipoplexes. The 400 μ L medium was added after 4 h incubation of the cells. After 48 h, the complete medium was removed from each well, and the cells were washed with PBS (2 $\times$ 200 μ L). Finally, 200 μ L of PBS was added to each well and the cells were visualized under an inverted fluorescence microscope to observe the expression of the green fluorescent protein.

The cells were detached by adding 1 $\times$ trypsin-EDTA (100 μ L per well) and resuspended into PBS (400 μ L per well). The cells expressing α 5GFP were quantified using a FACS Calibur flow cytometer (Becton-Dickinson) equipped with an argon ion laser at 488 nm for excitation and detection at 530 nm. 10 000 cells were analyzed for each sample using the software Cell Quest. Non-transfected cells served as live cell control for gate settings which in turn provided the cutoff threshold for the quantification of the fluorescent cell population.

4.10 Study of ASGPR specificity of Toc-Gal

To study the receptor specificity of Toc-Gal towards the ASGPR, cellular uptake experiments were carried out. HepG2 cells were seeded at a density of 1 00 000 per well in a 24-well plate (Corning Inc., Corning, NY) in 500 μ L of DMEM with 10% FBS 12–18 h before the uptake experiment. Care was taken to maintain 60–70% confluence at the time of uptake. For the preparation of Rhodamine-PE labelled liposomes, 0.5 mM liposomes (Toc-Gal & Toc-OH) were prepared as described in the above section 'Preparation of liposomes and

formulations' with an additional step of adding 0.005 mM rhodamine-PE (*i.e.*, 1% w.r.t. the total formulation content, Avanti-Polar Lipids, USA) to the liposomal solution before evaporating the chloroform. Glass vials containing labelled liposomes were wrapped with aluminium foils to avoid exposure. Rhodamine-PE labelled liposomes of Toc-Gal and Toc-OH were complexed with pCMV-SPORT- β -gal (0.3 μ g per well) at a lipid/DNA charge ratio of 8:1 (total volume of 100 μ L DMEM) for 15–20 min. Prior to the transfection (30 min before transfection), cells were pretreated with asialofetuin in varying concentrations from 0 μ M to 100 μ M. The lipoplexes were then added to the cells and incubated for 3–4 h. After 4 h of incubation, cells were washed with PBS (2100 μ L) and fixed with 3.8% paraformaldehyde in PBS at room temperature for 10 min. The red fluorescent cells were detected under an inverted fluorescence microscope (Nikon, Japan). For FACS analysis, the same transfection procedure is followed, except, after 4 h, the medium was removed, and the cells were washed with PBS (2 $\times$ 200 mL) and trypsinized with a 0.1% Trypsin/EDTA solution. 500 mL of the complete medium was added to the trypsinized cell suspension, the suspension was centrifuged for 3 min at 2000 rpm and the complete medium was removed. PBS (500 mL) was added to the cells and the cell suspension was once again centrifuged for 3 min at 2000 rpm. The supernatant was removed and the cell pellets were resuspended in 100 μ L in PBS for FACS analysis.

4.11 Serum compatibility study

Serum compatibility studies were carried out in representative HepG2 cells at a 8:1 lipid:DNA charge ratio. Briefly, cells were seeded at a density of 15 000 cells per well in a 96-well plate for 18–24 h before the transfection. Plasmid DNA (0.3 μ g) was complexed with 7.2 nmol of individual liposomes (at the 8:1 lipid:DNA charge ratio) in serum-free DMEM (volume of the lipoplex was maintained within 10 μ L) for 20 minutes. Lipoplexes prepared in serum-free medium were made up to 100 μ L by the addition of necessary volumes of serum-free DMEM and FBS (increasing amount of added serum, 10–90%) so that the final serum concentration ranged from 10–90% (for example, to make up 10% added serum with 100 μ L total volume, 10 μ L of FBS and 80 μ L of DMEM were added to 10 μ L of the lipoplex solution prepared in serum-free DMEM). The lipid:DNA complexes with varying amounts of serum (10–90%) were added to the cells and incubated. After 3–4 h of incubation, DMEM was removed, and DMEM with 10% FBS *i.e.* complete medium, was added to the cells. The reporter gene activity was estimated between 36 and 48 h following the same protocol as described in the Transfection biology section of Materials and methods.

Conflicts of interest

The authors declare no competing interest.

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