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Research paper

Reduced hepatic toxicity, enhanced cellular uptake and altered pharmacokinetics of stavudine loaded galactosylated liposomes

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Abstract

The aim of the present investigation was to reduce the hepatic toxicity, enhance the cellular uptake and alter the pharmacokinetics of stavudine using galactosylated liposomes. β-D-1-Thiogalactopyranoside residues were covalently coupled with dimyristoyl phosphatidylethanolamine, which was then used to form liposomes. The galactosylated liposomal system was assessed for *in vitro* ligand-specific activity. The drug release from liposomes was studied by dialysis method. *Ex vivo* cellular uptake study was performed using liver parenchymal cells harvested from male albino rats. Changes in hematological parameters, hepatic enzymes, hepatomegaly, plasma and tissue distribution of the formulations (free stavudine solution, uncoated liposomal and galactosylated liposomes) were determined using albino rats. Percent cumulative drug release in 24 h was low (34.8 ± 2.6%). Enhanced hepatic cellular d4T uptake (27.96 ± 2.41 pg d4T/million cells) was seen in case of galactosylated liposomal d4T. Galactosylated liposomes maintained a significant level of d4T in tissues rich in galactose specific receptors and had a prolonged residence (11.44 ± 1.25 h) in the body resulting in enhanced half-life of d4T (23.07 ± 1.25 h). This formulation did not show either hematological or hepatic toxicity. Galactosylation of liposomes alter the biodistribution of encapsulated drug thereby delivering the drug to cells bearing galactose specific receptors.

Keywords: Galactosylated liposomes; Stavudine; Hepatic toxicity; *Ricinus communis* lectin; Pharmacokinetics

1. Introduction

Targeted drug delivery involves the design and synthesis of carriers displaying ligands that mediate the binding of a drug/carrier complex to a receptor. Subsequent internalisation of the carrier/drug complex leads to accumulation of the drug in the target cells and exclusion from non-target cells that lack the requisite receptor. Ligand coupled liposomes represents a promising approach for improving the selective targeting of drugs to diseased tissues *in vivo*, leading to reduction in drug toxicity and improved therapeutic outcomes [1,2]. Cells of macrophage lineage represent a key target of human immunodeficiency virus (HIV) in addition to CD4-lymphocytes. Macrophages possess various receptors such as F_c receptors, mannosyl, galactosyl and many other receptors [3]. Asialoglycoprotein receptor specific for galactose is present on parenchymal cells of hepatocytes. Galactosylated liposomes are selectively removed from circulation by Kupffer cells. Kupffer cells carry on the cell surface a lectin-like receptor with specificity for D-galactose residues [4]. These galactose receptor-bearing cells are the reservoirs of HIV-I. The liposome approach has been used successfully in the treatment of specific diseases *in vivo* to enhance drug targeting to the cells [5,6]. Liposomal entrapment is particularly valuable for anti-HIV drugs because liposomes accumulate in the reticuloendothelial tissues in which HIV infection occurs [7]. Uptake of ligand-incorporated liposomes is significantly higher than liposomes without ligands [8–11]. The mechanism of sugar recognition that specific cell types possess

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is well known. The relative hydrophilic glycolipids may reduce immunogenicity; they can be removed from liposomes simply via interaction with plasma lipoproteins or lipids in tissue, resulting in a reduction in cell selectivity after intravenous injection [12,13]. It is, therefore, desirable to develop superior glycosylating agent that can be securely attached to the liposomal membrane, even under *in vivo* conditions.

Stavudine (d4T) is one of the important drugs, belonging to the class of reverse transcriptase inhibitors approved by the FDA for the treatment of AIDS. d4T has greater bioavailability (88–99%). However, long-term administration of stavudine over a period of 6 months results in adverse side effects such as a dose limiting peripheral neuropathy, anemia, hypersensitivity, insomnia and malaise. Lactic acidosis, hepatitis or liver failure has been reported with d4T. A liposomal drug delivery system may be ideal in the case of stavudine, as it may alleviate drug toxicity and also deliver the drug directly to the macrophages in a passive manner. Such a reduction in toxicity has been demonstrated in the case of drugs like Doxorubicin and Amphotericin B [14]. This could increase the time-interval between each drug administration during therapy as well as dose of the drug, consequently improving the quality of life of patients with AIDS.

2. Materials and methods

2.1. Chemicals

Stavudine was received as a gift sample from M/s Hetero Drugs, Hyderabad, India. Egg phosphatidylcholine (PC), cholesterol (CH), dimyristoyl phosphatidylethanolamine (DMPE), β-D-galactose, Sephadex G-50, *Ricinus communis* lectin, Dulbecco's modified Eagle's medium high glucose (DME-HG), L-glutamine, penicillin, streptomycin, heat-inactivated fetal bovine serum, human AB serum, lactate dehydrogenase diagnostic kit, aspartate aminotransferase diagnostic kit, alanine aminotransferase diagnostic kit and Triton X-100 were purchased from Sigma Chemicals Co. (USA). Hanks' balanced salt solution was obtained from Himedia, Mumbai, India. Dimethoxyethane, dicyclohexyl carbodiimide (DCC), 3-thiopropionic acid, boron trifluoroetherate, dichloromethane (DCM) and *N*-hydroxy succinimide (NHS) were procured from Spectrochem, Mumbai, India. Sabouraud dextrose agar was obtained from Merck, Mumbai, India.

2.2. Methods

2.2.1. Synthesis of thiol functionalized β-D-galactosyl residue

Galactosylated phosphatidylethanolamine was synthesized following the method earlier reported by Ashton et al. [15] with little modification in our laboratory.

Galactose was activated to its acetylated derivative containing an activated ester group, the reactive end group for conjugation. Briefly, 19 mL of acetic anhydride was cooled down to a temperature 4 °C and 0.1 mL of 60% perchloric acid was added to it dropwise with stirring. The reaction mixture was then allowed to attain the room temperature. Six grams of galactose was added to this mixture with stirring and the temperature of the mixture was maintained at 35 °C. Reaction mixture was allowed to stand for 24 h at 25 °C. The brown colored solution thus obtained was transferred dropwise to 25 mL distilled water with stirring. The insoluble brown colored sticky galactose pentaacetate got separated. Galactose pentaacetate (mp 85 °C) so obtained was dried in vacuum desiccator.

Two grams of galactose pentaacetate was dissolved in 25 mL dichloromethane. To the resulting solution 3-thiopropionic acid (1.6 mL) and boron trifluoroetherate (0.9 mL) were added. The mixture was stirred overnight at 25 °C, before being diluted with dichloromethane (25 mL) and washed with 1 M HCl (4 × 50 mL). The yellow colored mixture was evaporated and further dried in vacuum desiccator. A solution of thioglycoside (1.20 g) synthesized in dimethoxyethane was treated with DCC (600 mg) and NHS (315 mg) at 0–10 °C for 20 h with stirring. The precipitate was filtered off, the solvent was removed from filtrate under vacuum and the residue obtained was dissolved in 10 mL chloroform, filtered and concentrated. This concentrated reaction mixture was poured in cyclohexane (25 mL) to precipitate the product. The precipitation step was repeated. The solvent was decanted. The viscous oily product so obtained was dried to a white product (*N*-hydroxysuccinimidoyl-3-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosylthio)-propionate).

Dimyristoyl phosphatidylethanolamine (20 mg) was dissolved in 10 mL solution of DCM and chloroform (50:50 v/v). The white product obtained above was added to this solution. The mixture was stirred at 25 °C for 48 h. After dilution with DCM (25 mL) the solution was washed with saturated aqueous sodium carbonate (3 × 50 mL) and dried over sodium sulfate. The solution was concentrated under vacuum to yield white product that was insoluble in water. The deacetylation was carried out by Zemplen reaction followed by treatment with an aqueous NaOH solution [16]. The galactosylated lipid (Gal-DMPE) so formed was confirmed by IR spectrum recorded on FTIR multiscope spectrophotometer (Perkin Elmer, Buckinghamshire, UK) equipped with spectrum v3.02 software, by a conventional KBr pellet method.

2.2.2. Preparation of liposomes

In the present investigation, liposomes were prepared using film hydration method [17]. Briefly, lipids (Egg PC, Ch, Gal-DMPE) in different ratios (Table 1) were dissolved in 5 mL chloroform. The solvent was removed completely in rotary evaporator (York Scientific Ltd., New Delhi, India) under vacuum above the transition temperature of the lipid. The lipid film was further dried under vacuum for 1 h. The thin, uniform lipid film thus produced was hydrated with 1 mL of aqueous phase, i.e. phosphate-buffered saline (PBS, pH 7.4) containing 1 mg/mL d4T for

Table 1
Optimization of lipid ratio on the basis of characterization parameters for liposomes

Formulation code	Molar lipid ratios	Particle size[a] (nm)	% entrapment[a]	Zeta potential[a] (mV)	% entrapment efficiency[a]
PL1	9:0.5:0.5	158.70 ± 2.63	26.31 ± 0.29	+5.62 ± 2.39	9.42 ± 3.20
PL2	8:1:1	145.17 ± 2.36	29.60 ± 1.21	+9.12 ± 0.36	12.0 ± 0.21
PL3	8:1.5:0.5	131.02 ± 2.14	35.62 ± 0.26	+1.26 ± 0.25	15.45 ± 0.69
PL4	7:2:1	122.30 ± 0.29	46.23 ± 0.69	+8.21 ± 0.15	27.93 ± 2.70
PL5	7:2.5:0.5	160.28 ± 5.16	34.03 ± 1.21	+0.20 ± 0.06	24.35 ± 1.98
PL6	6:3:1	164.21 ± 8.25	21.30 ± 1.98	+0.03 ± 0.01	21.76 ± 0.58
PL7	6:3.5:0.5	171.30 ± 4.16	16.72 ± 0.21	−0.28 ± 0.03	20.56 ± 2.63
PL8	5:4:1	189.21 ± 4.52	12.25 ± 0.08	−0.30 ± 0.21	14.83 ± 1.23
PL9	5:4.5:0.5	196.32 ± 7.16	9.41 ± 1.37	−0.32 ± 0.09	13.17 ± 1.98
GL1	9:0.5:0.5	176.43 ± 3.20	26.42 ± 0.27	+7.7 ± 0.25	11.57 ± 0.75
GL2	8:1:1	170.52 ± 2.11	28.39 ± 0.58	+6.1 ± 0.29	14.85 ± 1.33
GL3	8:1.5:0.5	155.28 ± 1.88	31.21 ± 0.14	+5.6 ± 1.25	21.34 ± 2.70
GL4	7:2:1	129.46 ± 0.27	47.06 ± 1.23	+3.2 ± 0.21	29.23 ± 1.27
GL5	7:2.5:0.5	143.29 ± 5.29	36.23 ± 2.13	+0.11 ± 0.05	25.47 ± 1.47
GL6	6:3:1	161.29 ± 4.38	34.12 ± 1.28	−1.31 ± 0.08	22.39 ± 1.93
GL7	6:3.5:0.5	184.26 ± 5.29	19.21 ± 1.29	−3.41 ± 0.16	18.65 ± 2.28
GL8	5:4:1	192.35 ± 4.63	17.49 ± 1.58	−6.69 ± 0.85	16.11 ± 3.41
GL9	5:4.5:0.5	193.21 ± 3.78	15.21 ± 2.05	−10.24 ± 1.25	13.23 ± 2.79

PL1–PL9 are the formulation codes for positively charged liposomes prepared using various lipid ratios (PC:CH:DMPE). GL1–GL9 are the formulation codes for galactosylated liposomes prepared using various lipid ratios (PC:CH:Gal-DMPE).
[a] All values are expressed as means ± SD ($n = 6$).

1 h at room temperature with continuous rotation at 60 rev min^{-1}. The resulting vesicles were allowed to swell for 2 h at room temperature to produce large multilamellar vesicles (LMLVs). A laboratory homogenizer (EmulsiFlex-C5, Avestin, Ottawa, ON, Canada) was used to reduce the particle size of the liposomes. Multiple passes (5 k–10 k psi) were often needed to obtain the desired particle size (~200 nm). Liposomes were then extruded through polycarbonate membranes of 200 nm pore size. Vesicle size was evaluated by dynamic light scattering using Coulter N4 MD Submicron Particle Size Analyzer (Coulter Electronics, Hialeah, FL). Entrapment efficiency was determined after separation of unentrapped drug by Sephadex G-50 minicolumn using centrifugation technique [17,18]. The amount of drug entrapped in the vesicles was then determined by disrupting the vesicles using 0.1% Triton X-100 and the liberated drug was determined using UV spectrophotometer (Shimadzu 1601 UV spectrophotometer, Japan) at 266 nm. As a control, in order to rule out the possibility of any interaction of liposomal membrane with the drug, 1 mL of aqueous dispersion of empty liposomes (without drug) in PBS (pH 7.4) was incubated with 1 mL of d4T (1 mg/mL in d4T) for 2 h followed by passing the dispersion through Sephadex G-50 mini column using centrifugation technique. The vesicles eluted through the column were ruptured by using 0.1% Triton X-100 and were estimated spectrophotometrically at 266 nm for d4T which might have interacted with the liposomal membrane. The zeta potential of the uncoated and galactosylated formulation was measured (Zetasizer 3000 HS, Malvern Instruments Co., UK). Lipid drug interaction was determined using differential scanning calorimetry (Perkin Elmer DSC7, UK). Running conditions were a temperature range of 10–100 °C; scanning range 10 °C/min. Baseline optimization was performed before each run. Developed formulations were characterized *in vitro* before and after surface ligand anchoring.

2.2.3. In vitro ligand agglutination assay

The galactosylated liposomal system was assessed for *in vitro* ligand-specific activity by *R. communis* lectin agglutination assay as reported by Haensler and Schuber [19] with slight modification. One millilitre each of original liposomal formulations (both galactosylated and uncoated) was incubated with increasing concentrations (5, 10, 20, 30, 50 and 60 μg/mL) of *R. communis* agglutinin, in a cuvette containing 1 mL PBS (pH 7.4) at 25 °C. Time dependent (0–60 min) increase in turbidity at 360 nm was monitored spectrophotometrically (Shimadzu 1601 UV spectrophotometer, Japan).

2.2.4. Drug release studies

The drug release from liposomes was studied by dialysis cell membrane method. Two millilitres each of uncoated and galactosylated liposomal formulations was taken in dialysis tube (cellulose dialysis membrane, 2.4 nm porosity, Himedia Labs Ltd., Mumbai, India) against 100 mL of PBS (pH 7.4) and the solution was continuously stirred using magnetic stirrer at 37 ± 1 °C. After appropriate time intervals (1 h), 1 mL of sample was withdrawn and analyzed for drug content. Equal volume of fresh media was added to replace the withdrawn sample. d4T release was measured at 266 nm using UV spectrophotometer (Shimadzu 1601 UV spectrophotometer, Japan). Every experiment was performed in triplicate.

2.2.5. In vivo study

2.2.5.1. Hematological study and estimation of enzymes. Healthy male albino rats (Sprague–Dawley strain) of uniform body weight (100 ± 20 g) with no prior drug treatment were used for the *in vivo* studies. The animals were fed on a commercial pellet diet (Hindustan Lever, Bangalore, India), and water *ad libitum*. The animals were acclimatized to laboratory hygienic conditions for 10 days before starting the experiment. Permission of the Institutional Animals Ethics Committee was obtained for all animal experimentation (Registration Number 379/01/ab/CPCSEA, India). The animals were divided into four groups having six rats in each group. Plain drug solution (f d4T) in PBS (pH 7.4), uncoated liposomal formulation (PL d4T) and d4T loaded galactosylated liposomal formulation (GL d4T) each equivalent to 0.1 mg of d4T were administered for 10 days daily intravenously to the first, second group and third group, respectively. Fourth group was kept as control that was maintained on the same regular diet for 10 days. Hematological parameters, i.e. white blood corpuscles (WBC), red blood corpuscles (RBC), hemoglobin (Hb), platelet count and polymorphonuclear neutrophils (PMN) were determined in an Erma Particle Counter (Erma Inc., Tokyo, Japan).

The blood samples withdrawn 1 day and 10 days post treatment were estimated for lactate dehydrogenase and glutamate oxaloacetate transaminases. The collected blood samples were clotted and washed by vortexing with normal saline and the washings were centrifuged at 2000 rpm for 15 min. Serum was deproteinized by acetonitrile (1 mL/mL of serum).

Reduced nicotinamide adenine dinucleotide (0.1 mL; 2.5 mg $NADH_2$/mL of phosphate buffer), serum (0.1 mL) and 2.7 mL of phosphate buffer (pH 7.6) were mixed and kept for 20 min at 25 °C. To this mixture pyruvate substrate (2.5 mg sodium pyruvate/mL of phosphate buffer) was added, for determination of lactate dehydrogenase. Absorbance was measured at 340 nm at intervals of 1 min for 5 min. Average decrease in absorbance per min was determined [20].

For estimation of aspartate aminotransferase (serum glutamate oxaloacetate transaminases, SGOT), substrate (0.5 mL; 0.3 g of L-aspartic acid and 50 mg α-ketoglutaric acid in 30 mL of phosphate buffer, pH 7.4) for SGOT was added to a tube containing pyruvate standard (0.05 mL). Then serum (0.1 mL) was added to the above mixture and incubated for 60 min at 37 °C. Then, dinitrophenylhydrazine (0.1 mL DNPH) reagent was added to this mixture with stirring. The mixture was allowed to stand at 25 °C for 20 min for color development. Sodium hydroxide (5 mL of 0.4 N) was added to the mixture with continuous stirring. This mixture was left at 25 °C for 5 min. Absorbance was measured at 505 nm using UV spectrophotometer (Shimadzu 1601 UV spectrophotometer, Japan).

For alanine aminotransferase (serum glutamate pyruvate transaminase, SGPT) estimation similar method as mentioned above for SGOT determination was used except that alanine (DL alanine and 20 mg α-ketoglutaric acid in 30 mL of phosphate buffer, pH 7.4) substrate was used and incubation period was 30 min instead of 60 min.

After 1 day and 10 days of treatment, three animals from each group were sacrificed for the determination of hepatomegaly. The liver was removed, washed with cold saline solution, pressed between filter paper pads and weighed.

2.2.5.2. Plasma and tissue distribution study. Albino rats (Sprague–Dawley strain) of either sex of uniform body weight (100 ± 20 g) were used for the determination of tissue distribution of drug. The rats were divided into six groups of eighteen animals each. To the first group plain drug solution (1 mg/kg) in PBS (pH 7.4); to the second and third groups equivalent amount of drug loaded uncoated liposomal formulations and galactose coated liposomal formulation were administered, respectively, through the caudal vein. Fourth group served as control for first group to whom saline PBS (pH 7.4) was administered, fifth group served as control for second group to whom plain (without d4T) uncoated liposomal formulation was administered and sixth group served as control for third group to whom plain (without d4T) galactosylated liposomal formulation was administered. After 15, 30, 60, 120 min, 12 h and 24 h three animals from each group were sacrificed. The organs (lymph nodes, spleen, kidney, liver, and lungs) were excised and homogenized using tissue homogenizer (MAC Micro Tissue Homogenizer, Delhi, India) in a minimal volume of 0.25% Triton X-100 solution. The homogenates were deproteinized with acetonitrile. The homogenates were finally centrifuged, filtered and estimated for the drug content by HPLC.

2.2.5.3. Hepatic drug uptake studies. For hepatic uptake study, liver of those rats sacrificed after 1 h of f d4T, PL d4T and GL d4T administration was excised and the capsular membrane was removed. Liver was homogenized using tissue homogenizer (MAC Micro Tissue Homogenizer, Delhi, India) in a minimal volume of 0.25% Triton X-100 solution. The cells were dispersed in ice-cold Hank's Hepes buffer containing 0.1% BSA by gentle stirring. The dispersed cells were filtered through cotton mesh sieves followed by centrifugation (Remi R8C Laboratory Centrifuge, Mumbai, India) at 50*g* for 2 min. The pellets having parenchymal cells (PC) were washed twice with Hank's-Hepes buffer by centrifuging at 50*g* for 2 min. The supernatant so obtained had non-parenchymal cells (NPC). PC and NPC were resuspended separately in ice-cold Hank's-Hepes buffer (5 mL for PC and 3 mL for NPC). Drug uptake was determined by HPLC method [21]. Reverse-phase HPLC method without any internal standard, having mobile phase of 5% acetonitrile and 95% of 50 μM phosphate buffer (pH 6.75), was used. Sample was passed at a flow-rate of 1.25 mL/min by LC10 AT (Shimadzu, Japan) pump on a 5μ-Luna C18 column (Phenomenex,

USA) with UV detection at 266 nm using photodiode array detector (SPD-M10A, Shimadzu, Japan).

One milliliter of blood samples was collected in 0.9% normal saline at 0.25, 0.5, 1, 2, 3 (up to 12 h and 24 h). The collected blood samples were clotted and washed by vortexing with normal saline and the washings were centrifuged at 2000 rpm for 15 min. Serum was deproteinized by acetonitrile (1 mL/mL of serum). The samples were centrifuged and supernatants were analyzed for drug content against similarly treated blood sample of control rats by HPLC. The pharmacokinetic parameters were determined using non-compartmental analysis. The elimination rate constant (K_{el}) was estimated from the slope of the terminal elimination phase. The half-life in plasma of the terminal elimination phase ($t_{1/2}$) was calculated as $0.693/K_{el}$. The area under the plasma concentration–time curve (AUC) and the area under the first moment of the concentration–time curve (AUMC) were calculated using the trapezoidal rule. The mean residence time and clearance were calculated as AUMC/AUC and dose/AUC, respectively.

2.2.5.4. Histopathological study. For histopathological study, liver of those rats sacrificed after 10 days of f d4T, PL d4T and GL d4T administration was excised. The liver was immediately fixed into fixative solution (3:1, absolute alcohol/chloroform) for 3 h and then transferred to pure absolute alcohol for 1/2 h and further treated with absolute alcohol and xylene for 1 h. After this, wax scrappings were added in this solution until saturation and kept for 24 h for histopathological studies. After 24 h, the paraffin blocks were made by embedding the tissue in hard paraffin, matured at 62 °C. After attaching these blocks to carriers (wood cubes) the sections were cut using the microtome (Model 3880/A88, Beck, London) at 5 μm, the sections were mounted on slide and stained with Ehrlich's hematoxylin and eosin for nucleus and cytoplasm, respectively. Histological changes in liver were examined.

2.2.6. Statistical analysis

Statistical analysis was performed with GraphPad InStat software (version 3.00, GraphPad Software, San Diego, California, USA) using one-way ANOVA followed by Tukey–Kramer multiple comparisons test. Difference with $P < 0.05$ was considered statistically significant.

3. Results

3.1. Preparation and characterization of liposomes

Galactosylated dimyristoyl phosphatidylethanolamine was characterized by IR spectrum. O–H stretch (intense broad band) and strong C–O stretch of carbohydrate were obtained around 3350.2 and 1049.1 cm^{-1}, respectively. A characteristic C–H stretching and bending vibration was observed at 2855.3 cm^{-1} and 1470.8 cm^{-1}, respectively. Strong peaks at 1705.5 cm^{-1} and 1205.4 cm^{-1} confirmed the carbonyl (C═O) and C–O groups of ester present in dimyristoyl phosphatidylethanolamine, respectively. Weak N–H stretch was obtained at 3017.2 cm^{-1}. Linkage of galactose with dimyristoyl phosphatidylethanolamine was assured by amide linkage. The characteristic strong C═O stretch and N–H def of amide bond were obtained at 1690.7 and 1505.4 cm^{-1}, respectively. Weak stretch at 684.0 cm^{-1} confirmed C–S linkage.

Table 1 shows that as the lipid and cholesterol ratio varies, entrapment efficiency and particle size of the vesicle also shows difference. Particle size and entrapment efficiency of formulation GL4 were significantly ($P < 0.05$) lower and higher, respectively, as compared to other formulations. The absence of any drug in the ruptured blank liposomes, which was incubated with an aqueous solution of d4T and subsequently passed through Sephadex G-50 mini column, conclusively proves that there is no interaction between the liposomal membrane and the drug. The higher positive value ($+8.21 \pm 0.15$ mV) of zeta potential in case of uncoated liposomes (composed of PC:CH:DMPE) changes to lesser positive value ($+3.2 \pm 0.21$ mV) in case Gal-DMPE is used instead of PE.

DSC measurements of empty liposomal formulation showed a main phase transition temperature (T_m) at 47.9 ± 0.2 °C, whereas d4T loaded liposomes showed a main phase transition temperature at 47.9 ± 0.1°C. Unaltered T_m indicated that d4T was entrapped in the hydrophilic core of the liposomes.

3.2. In vitro specificity of galactosylated liposomes

Turbidity of galactosylated liposomes increased after addition of ricin in various concentrations to galactosylated liposomes. The rate of agglutination depends on lectin concentration. Fig. 1 shows the turbidity changes after addition of varying concentrations of lectin to galactosylated liposomal formulation. The extent of aggregation increased as ricin concentration was increased from 5 μg/mL to 30 μg/mL and time from 5 min to 45 min. Beyond 30 μg/mL lectin concentration, aggregation decreased indicating saturation of the binding sites. After 45 min, no turbidity change was observed. These results are in good agreement with the results of Haensler and Schuber [19].

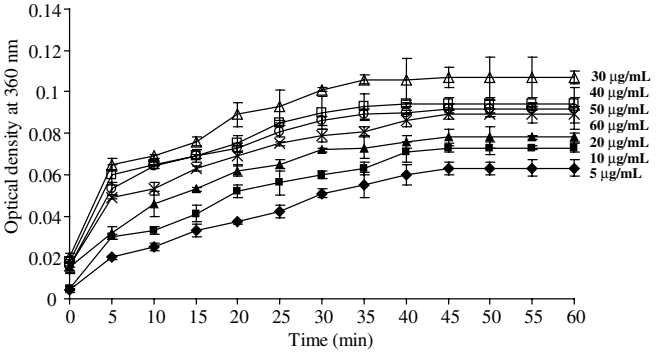

Fig. 1. *Ricinus communis* induced aggregation of galactosylated liposomes. All values are expressed as means ± SD ($n = 3$).

3.3. Drug release studies

Percent cumulative drug release was significantly higher ($P = 0.0011$) with uncoated liposomal formulation as compared to that of galactosylated formulation (Fig. 2). The linear regression coefficient values of % cumulative drug release for the liposomal formulations were very close to one indicating the zero order release profile. Percent cumulative drug release in 24 h was significantly ($P = 0.0011$) low indicating prolonged release carrier potential of galactosylated liposomal vesicular system as compared to uncoated liposomal formulation. In the present study, a blank determination was carried out using free drug and it was observed that the entire quantity of the free drug was able to come out of the cellulose dialysis membrane in less than 2 min, therefore no diffusional resistance through the dialysis membrane was observed.

3.4. Hepatic drug uptake studies

The amount of liposomes accumulated in liver was significantly higher than that for the free drug. Uptake of galactosylated liposomes (Gal L) by PC was significantly ($P < 0.05$) higher than that by NPC (Fig. 3).

3.5. In vivo study

3.5.1. Hematological study and estimation of enzymes

Free d4T solution produced significant decrease in blood cells (leukocyte count, erythrocyte count, Hb content, platelet count, polymorphonuclear neutrophil count), lactate dehydrogenase and significant increase in aspartate aminotransferase, alanine aminotransferase as well as liver weight 1 and 10 days post administration. The same amount of d4T encapsulated in uncoated liposomal system produces insignificant changes in the above-mentioned parameters after 1 day and significant effect on these parameters after 10 days of administration. These changes were insignificant after 1 and 10 days in case of galactosylated liposomes loaded with d4T were administered (Tables 2 and 3).

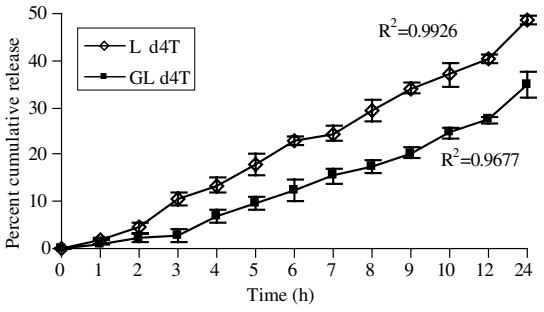

Fig. 2. *In vitro* drug release of d4T from uncoated (◇) and galactosylated (■) liposomal formulation at 37 °C in PBS (pH 7.4) ($n = 3$).

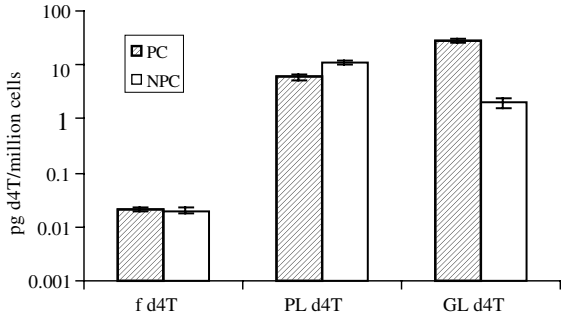

Fig. 3. Hepatic cellular localization of d4T from different formulations (f d4T, PL d4T and GL d4T) following intravenous administration in rats. Drug content was determined 60 min post-injection in parenchymal and non-parenchymal cells. Each value represents means ± SD ($n = 6$).

3.5.2. Plasma and tissue distribution study

To assess the amount of drug reaching lectin receptor rich organs, tissue distribution study was undertaken. Table 4 shows the concentrations of drug measured in plasma and various organs at different times (15, 30, 60, 120 min, 12 h and 24 h) after intravenous injection of f d4T, PL d4T and GL d4T (1 mg/kg), respectively. The incorporation of d4T in liposomal vesicles strongly altered its distribution pattern. After PL d4T injection, plasma levels $\geqslant 0.82 \pm 0.02$ ng/mL were monitored up to 2 h post-injection. In contrast, plasma drug concentration following f d4T injection decreased rapidly (0.01 ± 0.001 ng/mL at 2 h post-injection) and larger drug concentration (9.46 ± 0.1 ng/mL) was found in the kidney at just 15 min after injection. Administration of PL d4T did not result in high renal uptake. In the first 15 min following injection, significantly ($P < 0.0001$) lower d4T levels were observed in the kidney after PL d4T administration as compared with free drug levels.

In case of GL d4T injection although plasma concentration (0.85 ± 0.11 ng/mL) was less at 15 min post-injection yet it retained significantly higher ($P = 0.0036$) concentration even after 12 h as compared to PL d4T. GL d4T maintained a significantly higher level ($P < 0.05$) of the drug in liver, spleen and lungs up to 12 h (more than plasma concentration) compared to f d4T.

3.5.3. Pharmacokinetic study

Plasma level study of different formulations was undertaken to determine the release profile *in vivo*. The plasma concentration–time profiles for f d4T, PL d4T and GL d4T formulations are shown in Fig. 4, which demonstrates a rapid clearance of the free drug from plasma as compared to PL d4T and GL d4T formulations. The concentration–time profile of PL d4T and GL d4T depicted similar sustained release pattern. In case of GL d4T, plasma concentration of d4T was initially less but increased significantly after 9 h ($P < 0.05$) as compared to PL d4T indicating the prolonged sustained release and the retention potential of GL d4T formulation. The pharmacokinetic parameters of f d4T, PL d4T and GL d4T estimated from

Table 2
Hematological parameters after administration of different d4T formulations to albino rats

Group	Leukocyte ($\times 10^3/\mu L$)	Erythrocytes ($\times 10^6/\mu L$)	Hb (g/dL)	Platelet count ($\times 10^3/\mu L$)	Polymorphonuclear neutrophils (%)
Control	8.25 ± 2.1	7.15 ± 1.37	14.89 ± 2.99	681.3 ± 20.3	14.2 ± 2.3
f d4T (days)					
1	5.01 ± 1.46*	5.16 ± 0.13**	5.01 ± 1.46*	560.2 ± 10.3***	8.6 ± 1.3***
10	4.11 ± 1.64***	4.21 ± 1.33***	4.11 ± 1.64***	490.4 ± 11.2***	7.1 ± 2.1***
PL d4T (days)					
1	7.24 ± 1.25^{ns}	6.89 ± 1.21^{ns}	13.89 ± 0.29^{ns}	665 ± 30.2^{ns}	12.9 ± 0.3^{ns}
10	5.12 ± 1.23*	4.13 ± 1.47**	9.21 ± 1.56***	591.9 ± 15.3***	11.2 ± 1.2*
GL d4T (days)					
1	8.12 ± 1.26^{ns}	7.12 ± 1.11^{ns}	14.42 ± 1.29^{ns}	675.3 ± 11.2^{ns}	13.9 ± 1.2^{ns}
10	8.06 ± 1.95^{ns}	7.06 ± 0.59^{ns}	14.03 ± 2.16^{ns}	669.3 ± 10.3^{ns}	13.8 ± 1.1^{ns}

All values are expressed as means ± SD ($n = 6$). ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$, ns – non-significant as compared to control.
PL d4T, d4T loaded positively charged liposomes.
GL d4T, d4T loaded galactosylated liposomes.

Table 3
Serum biochemical parameters and relative liver weight after administration of different d4T formulations to albino rats

Group	Lactate dehydrogenase (IU/L) ($n = 6$)	Aspartate aminotransferase (IU/L) ($n = 6$)	Alanine aminotransferase (IU/L) ($n = 6$)	Liver weight (g)/100 g of body weight ($n = 3$)
Control	180.5 ± 8.3	59.5 ± 2.1	54.6 ± 7.6	3.2 ± 0.1
f d4T (days)				
1	150.3 ± 6.1*	110.3 ± 5.8***	101.6 ± 2.6***	3.6 ± 0.03*
10	62.4 ± 4.7***	450.6 ± 10.3***	389.4 ± 3.8***	5.4 ± 0.05***
PL d4T (days)				
1	176.4 ± 7.2^{ns}	57.4 ± 1.4^{ns}	59.2 ± 3.5^{ns}	3.1 ± 0.05^{ns}
10	74.9 ± 2.7***	200.6 ± 5.9***	196.8 ± 4.5***	4.2 ± 0.02*
GL d4T (days)				
1	175.3 ± 3.5^{ns}	58.2 ± 2.4^{ns}	55.8 ± 2.9^{ns}	3.2 ± 0.2^{ns}
10	172.4 ± 2.6^{ns}	57.1 ± 1.9^{ns}	56.3 ± 3.7^{ns}	3.1 ± 0.06^{ns}

All values are expressed as means ± SD. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$, ns – non-significant as compared to control.
PL d4T: d4T loaded positively charged liposomes.
GL d4T: d4T loaded galactosylated liposomes.

the concentration–time curves in plasma are shown in Table 5. An increase in the elimination half-life of encapsulated drug was observed. The AUC of GL d4T was 17.7 times higher ($P < 0.05$) than that of f d4T formulation. In case of GL d4T, MRT was found to be significantly ($P < 0.05$) higher than that of f d4T and PL d4T, respectively. Thus, the data project that GL d4T has prolonged residence in the body for longer period of time and gets distributed to various organs as observed from the tissue distribution curve. The results indicate that the drug encapsulated in galactosylated liposomes is much more potent in comparison with normal liposome-encapsulated drug or to the free drug. The outcome is in good agreement with the previous studies of Medda et al. [22] for mannosylated liposomes.

3.5.4. Histopathological study

Histopathological studies of liver sections were carried out to ascertain the effect of d4T on the cytoarchitecture of the liver. The liver section of control animals shows the normal appearance of hepatic parenchyma (normal cytoarchitecture of the liver) (Fig. 5A). The liver section of animals treated with f d4T alone showed degenerative changes, bile duct hyperplasia, and coagulative necrosis indicating the damaged cytoarchitecture of the liver (Fig. 5B). The liver section of animals treated with PL d4T showed slightly altered hepatic parenchyma with lobular necrosis, centrilobular necrosis, focal necrosis and caseative necrosis (Fig. 5C). No such type of effect was observed in liver of GL d4T administered rats (Fig. 5D).

4. Discussion

The larger entrapment efficiency of formulation and lesser particle size of formulation GL4 may be ascribed to the optimized ratio of phosphatidylcholine and cholesterol used in this formulation. The reduced entrapment efficiency and particle size of formulation GL1 may be due to less rigid, leaky and fused vesicles. The larger particle size and lesser entrapment efficiency of formulation GL5 to GL9 may be due to the rigidization of vesicles because of larger cholesterol content. The higher positive value of zeta

Table 4
Tissue distribution of free, uncoated liposomes and galactosylated liposomes encapsulated d4T in various tissues as a function of time after a single intravenous dose (1 mg/kg) to rats

Time (h)	Plasma	Liver	Lungs	Spleen	Lymph node	Kidney
f d4T (ng d4T/g or ml of tissue or plasma)						
0.25	0.49 ± 0.09	0.69 ± 0.1	0.79 ± 0.16	0.71 ± 0.11	0.72 ± 0.12	9.46 ± 0.10
0.5	0.42 ± 0.13	0.58 ± 0.06	0.73 ± 0.14	0.67 ± 0.06	0.69 ± 0.01	8.3 ± 0.98
1	0.39 ± 0.07	0.56 ± 0.12	0.59 ± 0.03	0.6 ± 0.01	0.6 ± 0.03	7.42 ± 1.57
2	0.01 ± 0.001	0.03 ± 0.01	0.14 ± 0.04	0.1 ± 0.06	0.06 ± 0.01	4.11 ± 0.52
12	0.001 ± 0.0001	0.02 ± 0.01	0.08 ± 0.01	0.06 ± 0.012	0.04 ± 0.009	0.21 ± 0.01
24	0.001 ± 0.0001	0.01 ± 0.001	0.03 ± 0.02	0.022 ± 0.013	0.021 ± 0.01	0.02 ± 0.001
PL d4T (ng d4T/g or ml of tissue or plasma)						
0.25	1.02 ± 0.16	2.12 ± 0.16	1.29 ± 0.25	1.86 ± 0.21	1.96 ± 0.25	2.87 ± 0.13
0.5	0.91 ± 0.08	3.79 ± 0.21	2.14 ±0.53	2.79 ± 0.39	3.01 ± 0.10	2.11 ± 0.18
1	0.89 ± 0.06	4.13 ± 0.13	3.16 ± 0.11	3.57 ± 0.14	3.99 ± 0.36	1.01 ± 0.10
2	0.82 ± 0.02	6.59 ± 0.21	4.96 ± 0.10	5.24 ± 0.11	6.11 ± 0.21	0.91 ± 0.08
12	0.32 ± 0.07	13.58 ± 0.96	8.01 ± 0.93	11.2 ± 0.84	12.37 ± 0.91	0.16 ± 0.02
24	0.19 ± 0.01	14.11 ± 0.82	10.02 ± 1.01	13.01 ± 0.99	13.99 ± 0.16	0.01 ± 0.001
GL d4T (ng d4T/g or ml of tissue or plasma)						
0.25	0.85 ± 0.11	4.53 ± 0.92	2.16 ± 0.10	2.91 ± 0.01	3.02 ± 0.03	0.96 ± 0.02
0.5	0.85 ± 0.15	5.11 ± 0.11	3.26 ± 0.12	4.01 ± 0.02	4.53 ± 0.11	0.85 ± 0.06
1	0.82 ± 0.06	9.01 ± 0.23	4.43 ± 0.14	5.39 ± 0.16	6.35 ± 0.14	0.79 ± 0.01
2	0.775 ± 0.09	11.28 ± 0.16	5.39 ± 0.56	8.06 ± 0.23	10.01 ± 0.63	0.62 ± 0.12
12	0.59 ± 0.03	18.24 ± 2.68	8.96 ± 1.01	11.25 ± 0.14	13.64 ± 0.91	0.06 ± 0.001
24	0.57 ± 0.02	26.97 ± 3.11	11.11 ± 0.68	13.62 ± 0.15	14.38 ± 0.29	0.01 ± 0.001

All values represent means ± SD ($n = 3$).

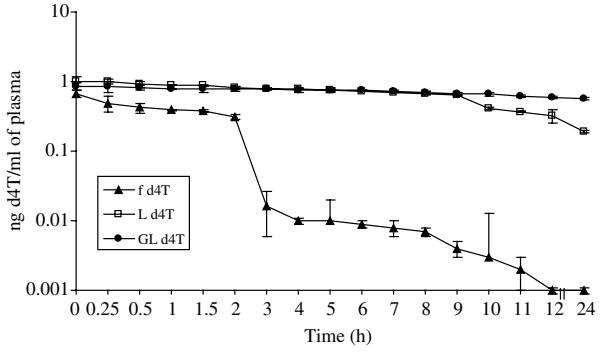

Fig. 4. Concentration–time curves of free d4T (▲), uncoated liposome encapsulated d4T (□), galactosylated liposome encapsulated d4T (●) in plasma following the administration of a single intravenous dose (1 mg/kg) to rats. Values represent means ± SD ($n = 3$).

potential in case of uncoated liposomes may be due to the presence of phosphatidylethanolamine. Galactosylation of dimyristoyl phosphatidylethanolamine decreases its more positive charge to lesser positive charge.

Because of the important roles of protein–carbohydrate interactions in normal human biology, the need for specificity in carbohydrate-based therapeutics is of great importance. *R. communis* agglutinin, a lectin selective for β-D-galactose residues, confirms that galactose present on the surface of liposomes, as a part of lipid (phosphatidylethanolamine) layer, is perfectly available to the lectin.

Liposomes either uncoated or galactosylated have release sustaining potential. Slow release in case of galactosylated liposomal formulation may be attributed to the presence of galactose residues on liposomal surface, which interfere with the drug release.

Larger cellular d4T uptake in case of liposomal formulation (uncoated and galactosylated) may be due to the particulate nature of this carrier. Galactose specific lectin on the parenchymal cells could account for the greater uptake of galactosylated liposomes [11]. Macrophages do also have lectin receptor on their surface. Because of macrophage's role in the pathogenesis of some diseases, targeting to macrophages could be potentially useful in the treatment of disorders such as AIDS. The present results show that galactosylated liposomes are recognized by the asialoglycoprotein receptors in PC. Kuiper et al. [23] have reported that galactose-exposing particles are effectively

Table 5
Pharmacokinetic parameters of free, uncoated liposomal and galactosylated liposomal d4T following the administration of a single intravenous dose (1 mg/kg), in rats ($n = 3$)

Pharmacokinetic parameters	f d4T	PL d4T	GL d4T
$t_{1/2}$ (h)	2.15 ± 0.12	7.98 ± 0.85	23.07 ± 1.25
AUC_{0-24} (ng h/mL)	0.82 ± 0.02	11.30 ± 0.21	14.55 ± 1.52
K_{el} (h^{-1})	0.32 ± 0.02	0.08 ± 0.06	0.03 ± 0.01
MRT (h)	0.90 ± 0.002	8.03 ± 1.19	11.44 ± 1.25
Cl (mL min^{-1} kg^{-1})	20.17 ± 0.02	1.60 ± 0.032	1.14 ± 0.01

The pharmacokinetic parameters were determined using non-compartmental analysis.
Values are expressed as means ± SD ($n = 3$).
$t_{1/2}$-plasma half-life of the terminal elimination phase, AUC – area under the plasma concentration–time curve, K_{el} – elimination rate constant, MRT – mean residence time and Cl – clearance.
PL d4T: d4T loaded positively charged liposomes.
GL d4T: d4T loaded galactosylated liposomes.

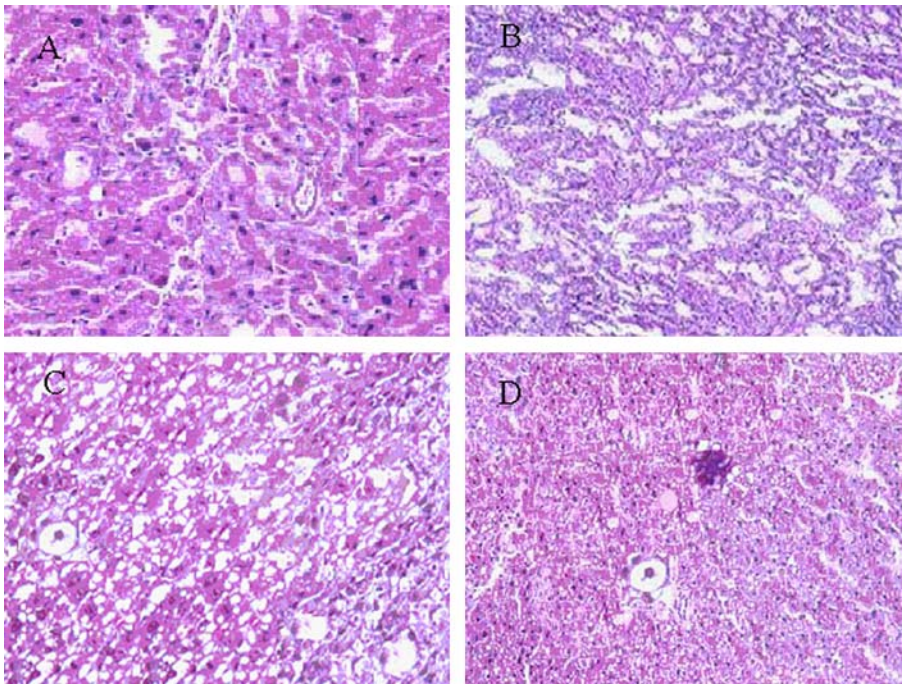

Fig. 5. Photomicrographs of liver section showing structure integrity of hepatic architecture after 10 days of different treatments. (A) control rat, (B) f d4T, (C) PL d4T and (D) GL d4T (1000×).

taken up by isolated Kupffer cells. Sliedregt et al. [12] found that galactosylated liposomes containing a relatively low content of galactolipids were taken up by the asialoglycoprotein receptors on PC, while galactosylated liposomes with a high content of galactolipids (over 50%) were taken up by the galactose particle receptors on NPC. These results support the conclusion that galactosylated liposomes can be taken up by both PC and NPC.

Significant decrease in blood cells such as (leukocyte count, erythrocyte count, Hb content, platelet count, polymorphonuclear neutrophil count) in case of free d4T solution may be due to the direct contact of d4T with blood cells causing anaemia, thrombocytopenia and neutropenia. The same amount of drug encapsulated in uncoated liposomes caused toxicity only after treatment for 30 days. This may be because of the release sustaining potential of liposomes due to which they accumulate in the body. In case d4T encapsulated in galactosylated liposome administration no significant change in blood cells was observed. This may be due to the fact that galactosylated liposomes release their content directly to the target sites (macrophages and hepatocytes) thereby causing no harm to the non-target sites. These results are in good agreement with the earlier reported results [5,24] using azidothymidine.

Estimation of the serum enzymes is a useful quantitative marker of the extent and type of hepatocellular damage. Alanine aminotransferase is a cytosolic enzyme primarily present in the liver. The levels of this enzyme in serum increase due to leakage of this cellular enzyme into plasma if there is any hepatic injury. Serum levels of SGPT can increase due to damage of the tissue produced by d4T. SGOT is a mitochondrial enzyme released from heart, liver, skeletal muscle and kidney. Liver damage is also associated with increased serum level of SGOT. Though the galactosylated liposomes are releasing d4T directly to hepatocytes no toxicity (hepatomegaly i.e. increase in liver weight) was observed. This may be due to the reason that galactosylated liposomes are releasing their content in a sustained manner. As soon as they release d4T it could be converted intracellularly to the triphosphate thereby halting DNA synthesis of retroviruses, including HIV. No excess drug gets deposited in the liver in case of galactosylated liposomes to cause any adverse effect.

Liposomal encapsulation slows down the renal elimination of d4T. PL d4T injected intravenously was rapidly taken up by organs of the reticuloendothelial system (spleen, liver and lungs) whereas free drug was not. Indeed, f d4T was so rapidly eliminated from the organism that no traces of it were found in any tissue 2 h after injection. This may be due to short elimination half-life of free d4T. In case of GL d4T, significant ($P < 0.05$) amount of d4T was found in spleen, liver and lungs because galactose specific receptors are present at these sites, which slowly localized the coated system in these organs [25,26]. The plasma half-life of liposome encapsulated 2′,3′-dideoxyinosine (ddI) was shown to be 46 times higher than those of free drug [27]. The systemic clearance of foscarnet encapsulated in conventional liposomes was found to be 77-fold lower than that of free drug [28]. Stavudine encapsulated in mannosylated liposomes is much more potent in comparison with normal liposome-encapsulated drug or to the free drug [29].

5. Conclusion

Conclusively, galactosylation of dimyristoyl phosphatidylethanolamine decreases its more positive charge to lesser positive charge. Galactosylated liposomes alters the biodistribution of encapsulated drug thereby delivering the drug to cells bearing galactose specific receptors. Many of the HIV-I infected cells have these receptors. Liposomes sustain considerable interest to develop ways to fabricate drug delivery systems that would provide a good release without inducing any systemic reactions into the host. However, in many cases, liposomes injected into the bloodstream are rapidly cleared from the system and only a fraction reaches the target site. Liposomes linked to other substrates such as galactose can be good candidates for certain types of drug release to achieve a localized treatment.

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