JPP Journal of Pharmacy and Pharmacology

JPP 2006, 58: 605–616
© 2006 The Authors
Received October 7, 2005
Accepted February 10, 2006
DOI 10.1211/jpp.58.5.0005
ISSN 0022-3573

Stavudine-loaded mannosylated liposomes: in-vitro anti-HIV-I activity, tissue distribution and pharmacokinetics

Minakshi Garg, Abhay Asthana, Hrushikesh B. Agashe, Govind Prasad Agrawal and Narendra Kumar Jain

Abstract

Cells of the mononuclear phagocyte system (MPS) are important hosts for human immunodeficiency virus (HIV). Lectin receptors, which act as molecular targets for sugar molecules, are found on the surface of these cells of the MPS. Stavudine-loaded mannosylated liposomal formulations were developed for targeting to HIV-infected cells. The mannose-binding protein concanavalin A was employed as model system for the determination of in-vitro ligand-binding capacity. Antiretroviral activity was determined using MT-2 cell line. Haematological changes, tissue distribution and pharmacokinetic studies of free, liposomal and mannosylated liposomal drug were performed following a bolus intravenous injection in Sprague-Dawley rats. The entrapment efficiency of mannosylated liposomes was found to be $47.2 \pm 1.57\%$. Protein–carbohydrate interaction has been utilized for the effective delivery of mannosylated formulations. Cellular drug uptake was maximal when mannosylated liposomes were used. MT2 cells treated continuously with uncoated liposomal formulation had p24 levels 8–12 times lower than the level of free drug solution. Further, the mannosylated liposomes have shown p24 levels that were 14–20 and 1.4–2.3 times lower than the level of free drug and uncoated liposomal formulation treatment, respectively. Similar results were observed when infected MT2 cells were treated overnight. Stavudine, either given plain or incorporated in liposomes, led to development of anaemia and leucocytopenia while mannosylated liposomes overcame these drawbacks. These systems maintained a significant level of stavudine in the liver, spleen and lungs up to 12 h and had greater systemic clearance as compared with free drug or the uncoated liposomal formulation. Mannosylated liposomes have shown potential for the site-specific and ligand-directed delivery systems with desired therapeutics and better pharmacological activity.

Introduction

The therapeutic efficacy of antivirals is often hampered by their poor ability to penetrate and target infected cells. Rapid drug clearance and inherent toxicity of parent compounds or their metabolites also constitute some of the major drawbacks, which may slow down the development and use of many antiviral agents (Nassander et al 1990; Lasic 1993; Bakker-Woudenberg 1995; Wasan & Lopez-Berenstein 1995). Receptor-mediated drug targeting is a promising approach to obtain site-specific drug delivery (Kawakami et al 2000). The use of liposomes as a drug carrier represents a convenient strategy to improve the delivery of drugs within infected cells, thereby increasing their efficacy and reducing their toxicity. The localization of liposomes in organs rich in monocytic cells, such as liver, spleen, lungs and kidney, may provide a mechanism for improving the therapeutic index of antiviral agents such as stavudine (d4T) (Poste et al 1982; Herman et al 1983; Lopez-Berestein et al 1984, 1987; Fidler et al 1985; Mehta et al 1987; Treat et al 1989; Rahman et al 1995). One particular method exploits the mechanism of sugar recognition that specific cell types possess. The relatively hydrophilic glycolipids may reduce immunogenicity. However, they can be removed from liposomes simply via interaction with plasma lipoproteins or lipids in

Pharmaceutics Research Laboratory, Department of Pharmaceutical Sciences, Dr Hari Singh Gour University, Sagar 470003, India

Minakshi Garg, Abhay Asthana, Hrushikesh B. Agashe, Govind Prasad Agrawal, Narendra Kumar Jain

Correspondence: N. K. Jain, Pharmaceutics Research Laboratory, Department of Pharmaceutical Sciences, Dr Hari Singh Gour University, Sagar 470003, India. E-mail: jnarendr@yahoo.co.in

tissue resulting in a reduction in cell selectivity after intravenous injection (Slidregt et al 1999). Therefore, it is essential to develop a superior glycosylating agent that can be securely attached to the liposomal membrane, even under in-vivo conditions.

Protein–carbohydrate interactions mediate critical biological recognition processes, such as those involved in cell signalling, organogenesis, fertilization and inflammation. Additionally, carbohydrate–protein interactions facilitate the initial attachment of pathogens to host cells (Weatherman et al 1996). Cells of the mononuclear phagocyte system (MPS) are important hosts for HIV and play a key role in the pathogenesis of AIDS by providing long-term reservoirs for the virus (Roy & Wainberg 1988; Meltzer et al 1990; Levy 1993). As liposomes are preferentially taken up by cells such as macrophages, monocytes and lymphocytes (MPS), their use as carriers of antiviral agents represents an interesting approach (Senior 1987; Gregoriadis 1988; Lasic 1992; Desomeaux et al 1994; Makabi-Panzu et al 1994). Lectin receptors are found on the surface of the cells of the MPS (Auger & Ross 1992). Coating the liposomal formulation with sugar (e.g. mannose, lactose, fucose and galactose) would efficiently target the liposomes to the site of action (Muller & Schuber 1989).

Stavudine (d4T) has become a widely-prescribed antiretroviral agent due to its tolerability and twice-daily dosing schedule. The plasma half-life of d4T is relatively short in man (0.9–1.2 h); however, the putative active metabolite has a longer intracellular half-life of 3.5 h (Williams & Stancel 1990). Entrapment of d4T in liposomes can positively alter the pharmacokinetic profile of this antiviral agent by increasing its half-life in the systemic circulation. A liposomal drug delivery system may be an alternative in the case of stavudine as it can alleviate toxicity associated with the drug and can deliver the drug to the infected cells via passive uptake mechanism. Such a reduction in toxicity has been demonstrated with drugs like doxorubicin and amphotericin B (Lopez-Berestein et al 1985; Szoka et al 1987; Mayer et al 1990; Kanter et al 1993). This could increase the time-interval between each drug administration during therapy, consequently improving the quality of life of patients suffering from AIDS. This investigation was undertaken to develop a macrophage-targeted d4T-loaded mannosylated liposomal formulation. Tissue distribution and pharmacokinetic profiles of free, liposomal and mannosylated liposomal drug were studied in-vivo following a bolus intravenous injection in rats.

Materials and Methods

Materials

Stavudine was received as a gift sample from M/s Hetero Drugs (Hyderabad, India). Egg phosphatidylcholine (PC), cholesterol (CH), stearylamine (SA), D-mannose, Sephadex G-50, penicillin, streptomycin, gentamicin, concanavalin A (Con A), RPMI 1640 culture medium and Triton X-100 were purchased from Sigma Chemicals Co. (USA). Palmitoyl chloride was procured from Fluka (Switzerland). All other chemicals used were purchased from Merck (India).

Synthesis of O-palmitoylmannose

O-palmitoylmannose (OPM) was synthesized by esterification of mannose by the reaction of palmitoyl chloride in dimethylformamide (DMF) under anhydrous catalytic conditions following the method of Sunamoto et al (1985) and adopted with minor modification in our laboratory.

Briefly, 2.0 g mannose was dissolved in 100 mL dry DMF at 70°C. To the resulting solution, 2 mL dry pyridine and 0.2 g palmitoyl chloride dissolved in 0.5 mL dry DMF were added. The mixture was stirred using a magnetic stirrer (Expo India Ltd, Mumbai, India) for 3 h at 60°C followed by 2 h at room temperature. This mixture was then slowly poured into 100 mL absolute ethanol with stirring. The precipitate so formed was collected and washed thrice with 120 mL absolute ethanol and 80 mL dry diethyl ether. The white solid material obtained was dried in vacuum at 50°C for 2 h. The FTIR spectrum was recorded on FTIR multiscope spectrophotometer (Perkin Elmer, Buckinghamshire, UK) equipped with spectrum v3.02 software.

Preparation and development of liposomal formulation

Liposomes were prepared by reverse-phase evaporation (Szoka & Papahadjopoulos 1978). Egg PC, CH and SA were taken in different molar ratios (Table 1) and dissolved in 5 mL diethylether, to which 2 mL of aqueous phase (i.e. phosphate-buffered saline, PBS pH 7.4) containing 2 mg d4T was added. The mixture was sonicated (Titanium probe ultrasonicator; Imeco Ultrasonics, India) for 10 min (with an interval of 1 min after 5 min). A thick emulsion was formed, which was then vortexed (Superfit, India) to remove any residual ether. To this emulsion, 3 mL PBS (pH 7.4) was added to hydrate the vesicles.

Table 1 Composition and characterization of different liposomal formulations

Formulation code	Molar lipid ratios (PC:CH:SA)	Particle size (nm)[a]	% Entrapment efficiency[a]
L1	9:1:0	145 ± 2.36	32.4 ± 0.02
L2	7:2:1	120 ± 1.52	49.60 ± 1.23
L3	6:3:1	169.3 ± 9.6	26.4 ± 1.01
L4	5:4:1	192.3 ± 6.5	14.36 ± 1.22

[a]Values are expressed as mean ± s.d., n = 6. PC, egg phosphatidylcholine; CH, cholesterol; SA, stearylamine; L1, L2, L3 and L4 are the formulation codes of liposomal formulations prepared with different molar ratios of lipids and cholesterol.

Formulations were evaluated for their shape and morphology by phase contrast microscopy (Leica DMLB, Germany). Liposomes were then extruded through polycarbonate membranes (Millipore, USA) of 200 nm pore size. Vesicle size was evaluated by dynamic light scattering using a Coulter N4 MD Submicron Particle Size Analyzer (Coulter Electronics, Hialeah, FL). Entrapment efficiency was determined after separation of un-entrapped drug by a Sephadex G-50 minicolumn using centrifugation technique (Fry et al 1978; New 1990). 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 in a UV spectrophotometer (Shimadzu 1601) at 266 nm. Developed formulations were characterized in-vitro before and after surface ligand anchoring.

Coating of liposomes

For coating, 2 mL of uncoated liposomal formulation was incubated with OPM solution (in PBS, pH 7.4) and was then stirred gently at room temperature. After completion of coating, the excessive unbound OPM was removed by passing the resultant suspension through a Sephadex G-50 column at 2000 rev min^{-1} for 10 min. The two process variables (total lipid to OPM weight ratio and incubation time) were optimized by measuring the change in zeta-potential of the dispersion using 0.1 M KCl buffer in demineralized water at 25°C (Zetasizer 3000 HS; Malvern Instruments Co., UK). OPM (negatively charged) becomes coated onto the positively charged liposomes. This change in zeta-potential value can be used to optimize the process variables. For optimization, lipid and OPM in different ratios were taken to develop the formulations that were incubated for 24 h at 25°C. To optimize the incubation time the formulations with optimum lipid-to-OPM ratio were incubated for different time periods at 25°C and zeta-potential was measured. Both uncoated and OPM-coated liposomes were extruded four times through two stacked 0.2-μm polycarbonate membranes.

In-vitro ligand agglutination assay

Mannosylated liposomes were assessed for in-vitro ligand-specific activity by mannose-binding Con A as reported by Copland et al (2003) with slight modification. A 100-μL sample of the original liposomal dispersion (both plain and OPM-coated) was diluted 10 times with PBS (pH 7.4) and 1 mL of varying concentrations of Con A (100–700 μg mL^{-1} in PBS containing 1 mM $MnCl_2$ and 1 mM $CaCl_2$, pH 7.4) was added to it at 25°C. Time-dependent increase in turbidity at 550 nm was monitored spectrophotometrically (Shimadzu 1601 UV spectrophotometer; Japan) for 4 h. Every experiment was performed in triplicate.

Cellular drug uptake studies

Cultured MT2 cells were used to determine the extent of cellular drug uptake from liposomal formulations. Cells were grown in RPMI 1640 medium containing 10% fetal calf serum (FCS), penicillin (50 IU mL^{-1}), gentamicin (80 μg mL^{-1}) and streptomycin (50 μg mL^{-1}). About 100 μL of the cell suspension, corresponding to a seeding density of 1×10^6 cells/mL, was transferred to 48-well culture plates. Ten microlitres of mannosylated liposomal d4T, liposomal d4T and free d4T solution (1 mg mL^{-1}) was then added to each well of I, II and III columns, respectively. The plates were incubated in a controlled environment at a temperature of 37 ± 1°C for a period of 48 h. During incubation, the cell suspension from each well was transferred to polycarbonate filters (pore size 0.45 μm) at 0, 1, 4, 8, 20 and 48 h. The wells of the cell culture plates were rinsed with 1 mL PBS (pH 7.4) and the washings subsequently transferred to the polycarbonate filters. The cells were separated from the medium in the form of a pellet by centrifuging the filters at 4000 rev min^{-1} for 15 min. About 0.5 mL of Triton X-100 was added to the pellet to rupture the cells and the mixture was incubated at room temperature for 5–6 h (Katragadda et al 2000). Drug uptake was determined by HPLC method.

Cell viability study

Culture of HIV-1 developed in T lymphocyte cell line was used for carrying out cytotoxicity (Hao et al 2005) and antiretroviral activity studies. Cells were cultured in RPMI 1640 supplemented with 10% FCS, penicillin (50 IU mL^{-1}), gentamicin (80 μg mL^{-1}) and streptomycin (50 μg mL^{-1}). Flat-bottom tissue culture plates (Corning Incorporated, Corning NY, USA) with 72 wells were used. One-hundred microlitres of 10% RPMI was added in all the 72 wells of the flat-bottom tissue culture plates. To the first row of column II, III, IV, V and VI, 200 μL of 10 mM free d4T solution, unloaded plain liposomal formulation, unloaded mannose-coated liposomal formulation, liposomal d4T (10 mM drug) and mannosylated liposomal d4T (10 mM drug) were added, respectively. From the first row of each column, 100 μL of formulation or 5 mM of d4T was transferred to the second row, then 100 μL from the second row to the third row, and likewise serial dilutions were made until the last row (each well contained 5000, 2500, 1250, 625, 312.5, 156.2, 78.1, 39.0, 19.5, 9.75, 4.87 and 2.43 μM of d4T). MT2 cell suspension (100 μL; 0.01×10^6 cells/100 μL) was added in each well and mixed thoroughly. The plate was incubated at 37°C for 5 days in a CO_2 incubator. On the fifth day post-infection, cell toxicity was determined by the [4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay (Bedoya et al 2001; Salvatori et al 2001). The cytotoxicity was expressed as the cytotoxic dose 50 (CD50) that induced a 50% reduction of absorbance at 540 nm (Absorbance % = Absorbance of formulation/ Absorbance of cell control).

Anti-HIV assay

MT2 cell suspension (1 mL, 0.1×10^6 cells/mL) was taken in 200 μL of RPMI medium in a 24-well plate. To each

well, 100 μL of HIV-I virus (5 ng p24/mL) was added followed by incubation for 2 h at 37°C in 95% air–5% CO_2 atmosphere. The residual virus was then removed by washing the cell suspension three times with 2% RPMI medium. p24 harvested from the supernatant (200 μL) of the last wash was considered 0 PID (post infection day). A subtoxic concentration (determined from cytotoxicity assay) of d4T was added to each well of 96-well plates and maintained as a positive and negative control for every assay. The plate was then incubated at 37°C in 95% air–5% CO_2 atmosphere for 12 days. On the 5th, 7th, 9th and 12th days, supernatant was harvested for p24 antigen by ELISA determination in harvested culture supernatants (Konopka et al 1990).

For overnight treatment, medium was removed on the next day after infection day; the wells were washed five times with 2% RPMI medium and replaced with fresh medium in the wells. MT2 cells were treated overnight with plain drug solution (f d4T), d4T-loaded mannose-coated (ML d4T) and -uncoated liposomal formulation (L d4T). Then the cells were cultured as usual. For continuous treatment, medium was not replaced, and fresh dilutions of treatments were added with each medium replacement. With our standard curve, p24 was accurately detected down to 0.01 ng mL^{-1}. The results were expressed as the mean ± s.d. of triplicate determinations and were compared with untreated controls unless specified otherwise.

In-vivo study

Haematological study

Healthy male albino rats (Sprague–Dawley strain), 100 ± 20 g, previously untreated with drugs, were used. The rats were allowed free access to a commercial pellet diet (Hindustan Lever, Bangalore, India) and water. The rats 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 rats were divided into four groups of six each. f d4T, ML d4T and L d4T, each equivalent to 200 μM of d4T, were administered intravenously to the first, second and third group, respectively, daily for 30 days. The fourth group was kept as a control, which was maintained on the same regular diet for 7 days. Haematological parameters (i.e. white blood corpuscles (WBC), red blood corpuscles (RBC), haemoglobin (Hb), haematocrit (HCT) and mean corpuscular haemoglobin (MCH)) were determined in an Erma Particle Counter (Erma Inc, Tokyo, Japan) after 30 days.

Plasma and tissue distribution study

Albino rats, 100 ± 20 g, were used to study the tissue distribution of the drug. The rats were divided into six groups of fifteen. To the first group, plain drug solution (0.1 mg kg^{-1}) was administered and to the second and third group an equivalent amount of drug-loaded uncoated liposomal formulation and mannosylated liposomal formulation were administered, respectively, through the caudal vein. The fourth group served as control for the first group to which saline (0.9% NaCl) was administered, the fifth group served as control for the second group to which plain (without drug) uncoated liposomal formulation was administered and the sixth group served as control for the third group to which plain (without drug) mannosylated liposomal formulation was administered. After 15, 30, 60, 120 min and 12 h, three rats from each group were sacrificed. The organs (lymph nodes, spleen, kidney, liver and lungs) were excised and homogenized using a tissue homogenizer (MAC Micro Tissue Homogenizer; Delhi, India) in a minimal volume of 0.25% Triton-X100 solution. The homogenates were de-proteinized with acetonitrile, centrifuged, filtered and estimated for the drug contents by HPLC (Stephen et al 1999). Reverse-phase HPLC method without any internal standard, having a mobile phase of 5% acetonitrile and 95% 50 μM phosphate buffer (pH 6.75) was used. The retention time for stavudine was 6 min. The flow-rate was 1.25 mL min^{-1} by LC10 AT pump on a 5 μm-Luna C18 column (Phenomenex, USA) with UV detection at 266 nm using photo diode array detector (SPD-M10A).

A 1-mL blood sample was withdrawn and collected in 0.9% normal saline at each time point (i.e. 0.25, 0.5, 1, 1.5, 2, up to 12 h). The collected blood samples were clotted and washed by vortexing with normal saline and the washings were centrifuged at 2000 rev min^{-1} for 15 min. Serum was de-proteinized by acetonitrile (1 mL/mL of serum). The samples were centrifuged and supernatants were analysed by HPLC for drug content against a similarly treated blood sample from the control group. 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 (t½) 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 systemic clearance, mean residence time (MRT) and the steady-state volume of distribution (Vd_{ss}) were calculated as dose/AUC, 1/K_{el} and [dose × AUMC]/$[AUC]^2$, respectively.

Statistical analysis

Statistical analysis was performed with GraphPad Prism software (version 4.03, USA) using one-way analysis of variance followed by Tukey–Kramer multiple comparison test. Kruskal–Wallis test followed by Dunn's multiple comparison test was used for the statistical analysis of the results in cases where three replicates were used. $P < 0.05$ was considered statistically significant.

Results

Synthesis and characterization of O-palmitoylmannose

Mannose used in this study was chemically modified by esterification with palmitoyl chloride. OPM was characterized by IR spectrum. Extremely intense O-H stretch of carbohydrate around 3405.2 cm^{-1} was obtained. A characteristic C-H stretching (strong) and bending vibration (medium) was observed at 2832.8 cm^{-1} and 1400 cm^{-1}, respectively. Strong peaks at 1687.1 cm^{-1} and 1750.2 cm^{-1} confirmed the carbonyl group (C = O). C-O stretch was observed at 1115.8 cm^{-1} and 1241.3 cm^{-1}.

Preparation, characterization and coating of liposomes

The reverse-phase evaporation method for preparation of liposomes is reported to encapsulate large hydrophilic molecules with high entrapment efficiency. It is clear from Table 1 that as the lipid ratio used in the preparation of liposomes varies, encapsulation efficiency and particle size shows significant difference ($P < 0.001$). Formulation L2 was found to have the highest entrapment efficiency (49.6 ± 1.23%) and lowest particle size (120 ± 1.52 nm). Therefore, formulation L2 was chosen for mannose coating of the liposomes.

Liposomes initially had positive zeta-potential due to the presence of stearylamine. This positive zeta-potential decreases with addition of OPM (anionic) and it approaches a minimum value at a 10:5 lipid-to-ligand weight ratio (Figure 1A). For optimization of incubation time the optimized formulation (10:5 lipid-to-ligand weight ratio) was incubated for different time periods (0, 15, 30, 60, 120, 180, 240, 300, 360 and 420 min) and the change in zeta-potential was recorded (Figure 1B). There was a decrease in the initial zeta-potential value, with it reaching a minimum after 4 h of incubation and thereafter no significant change was observed in the zeta-potential value.

Entrapment efficiency after coating was 47.20 ± 3.25%, which was insignificantly different ($P = 0.1216$) to the uncoated formulation, indicating no loss of drug after OPM coating. After extrusion through the polycarbonate membranes significant ($P < 0.0001$) increase in vesicle size was observed for OPM-coated liposomes (140 ± 2.25 nm) compared with uncoated liposomes (120 ± 1.52 nm).

In-vitro ligand binding specificity

The development of novel multivalent carbohydrate ligands for the modulation of biological processes continues to be an attractive target for research. Following exposure to Con A an increase in the optical density (turbidity) at 550 nm was noticed with mannosylated liposomes. The extent of aggregation increased as we increased the Con A concentration from 100 to 500 $\mu g\,mL^{-1}$ and the duration of exposure from 5 to 150 min. It was observed that beyond 500 $\mu g\,mL^{-1}$ Con A concentration, turbidity displayed insignificant ($P > 0.05$) increase. After 150 min no significant change in turbidity was observed. The results indicated that even after chemical modification and anchoring onto liposomal surface, mannose displayed binding specificity towards Con A.

Cellular drug uptake study

The liposomes were separated from the cells by centrifugation. The basis for using filters to estimate encapsulation is that liposomes that have a particle size of approximately 0.2 mm readily pass through the filter during centrifugation, whereas the macrophages that are bigger than the liposomes are retained on the filter along with the stavudine they have taken up. Figure 2 shows that drug uptake by macrophages was higher

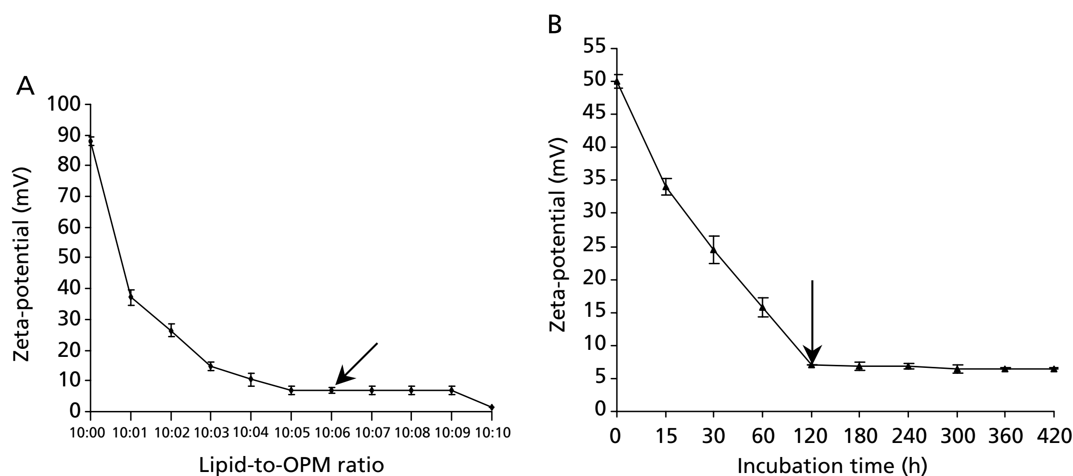

Figure 1 Formulations with different ratios of lipid and O-palmitoylmannose (OPM) were prepared. A. Optimization of total lipid-to-OPM weight ratio (zeta-potential values after time period of 24 h at 25°C). B. Incubation time optimization for coating of OPM at 25°C. Values are expressed as mean ± s.d., n = 6.

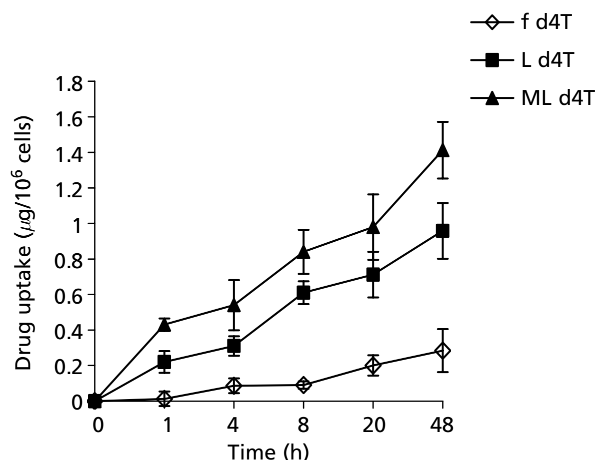

Figure 2 Cellular uptake of stavudine (mg/million cells) as a function of time from different formulations (free d4T (◇), uncoated liposomal d4T (■) and mannosylated liposomal d4T (▲)). Data are expressed as mean ± s.d., n = 3.

when liposomes were used. A 5-fold enhancement (maximal uptake) was observed when mannosylated liposomes were used, whereas in the case of uncoated liposomes the increase was 3 fold compared with the free drug. The extent of drug uptake from liposomal formulations is an important parameter for estimating the dosage of the formulation. It is highly desirable that drug uptake from liposomal formulations be maximal so that a smaller dosage of the formulation would suffice to achieve an optimal therapeutic effect. Moreover, small doses are convenient to the patient in terms of parenteral administration.

Cell viability study

The cytotoxicity of the vesicular formulation was determined by MTT assay. No significant toxicity ($P > 0.05$) was observed up to a concentration of 1250 μM of d4T entrapped within different vesicular formulations as compared with control. It was observed that the encapsulation of drug in liposomes decreased the cytotoxicity of the drug (subtoxic concentration of d4T in free, uncoated liposomal and mannosylated liposomal formulation were 312.5, 1250 and 2500 μM, respectively). To avoid any cytotoxic effects of formulations, an anti-HIV assay was carried out using 312.5 μM of d4T.

Having determined that the subtoxic concentration of d4T was 312.5 μM of free drug solution, we examined a dose range of 625–78.1 μM. When MT2 cells were continuously treated with d4T or liposome-encapsulated d4T for 12 days following infection, there was a dose-dependent inhibition of p24 production compared with control. At day 7, the differences between the p24 values obtained at the various doses were all statistically significant (Figure 3A) (e.g. comparison of the values for 312.5 and 156.25 μM free d4T, and that for 625 and 312.5 μM d4T yielded $P < 0.05$). The p24 values obtained throughout the experiment are shown in figure 3B, C and D for treatments with ML d4T, L d4T and f d4T, respectively. The maximum dosing in the range 156.25–625 μM in all cases kept virus production at a lowest level, whereas the minimum dose (78.1 μM) of f d4T solution could not prevent virus production from increasing over time.

Treatment with ML d4T produced lower p24 levels compared with L d4T and f d4T in the concentration range studied (Figure 3). An empty mannosylated and uncoated liposomal control, at the same concentration of lipid as that of ML d4T and L d4T, was included in every experiment for each type of liposome used in this study, and the results were similar in every experiment. The p24 level in the empty liposomes control (mannosylated and uncoated) was found to be similar to the untreated control.

Continuous treatment versus overnight treatment of infected MT-2 cell line with ML d4T, L d4T and f d4T

T lymphocytes continuously treated with ML d4T and L d4T (312.5 μM d4T) reduced p24 levels to approximately 1/2000 to 1/1000 as against the controls (Figure 3E). MT2 cells treated with L d4T had p24 levels at or below 0.1 $ng\,mL^{-1}$, about 8–12 times lower than the level after f d4T treatment. In the case of ML d4T, p24 levels were below 0.1 $ng\,mL^{-1}$, about 14–20 times and 1.4–2.3 times less than the level of f d4T and L d4T treatment, respectively. The p24 levels of the treated MT2 cells remained relatively steady for 14 days ($P > 0.05$ for ML d4T, L d4T and f d4T treatment), while the control p24 levels increased significantly ($P < 0.05$) from the 5th to the 14th day post infection. After overnight treatment of infected MT2 cell lines (Figure 3F), the levels of p24 increased throughout the course of experiment (14 days), although the levels in the treated wells increased slowly as compared with that of controls. The difference between untreated controls and treated wells increased over time but was less than that with continuous treatment. ML d4T was the most effective of the three treatments, keeping p24 levels at about less than 20% of positive control on day 14 post infection. Significant difference was found in the level of p24 in the case of ML d4T ($P < 0.05$ vs control, Ld4T and f d4T). Formulation f d4T showed no significant effect until day 14 ($P > 0.05$ vs positive control). p24 levels were not kept as low by the single treatment as by continuous treatment; the longer-lasting effect of mannosylated liposome-encapsulated d4T compared with free d4T (to 14 days after a single treatment) suggests that mannosylated liposomes may provide a local reservoir of the antiviral drug in lymphocytes.

In-vivo study

Haematological study

The blood parameters undergoing major changes were RBC counts, WBC counts, Hb content and HCT value. Significant lowering in WBC, RBC, Hb and HCT in rats treated with plain d4T solution and uncoated liposomal formulation was observed (Table 2) as compared with values in control rats. No significant difference was

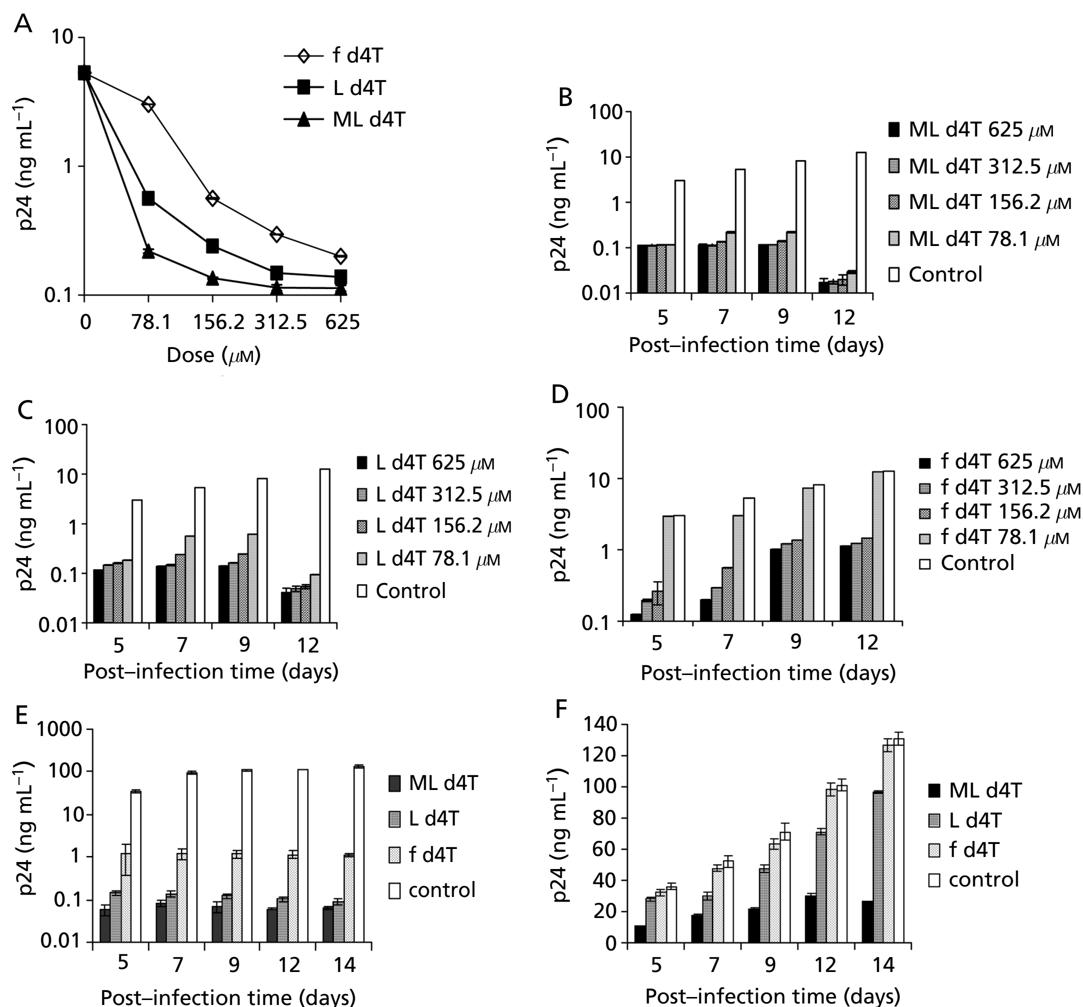

Figure 3 Viral p24 production by infected MT2 cell line treated continuously with free, uncoated liposome- and mannosylated liposome-encapsulated d4T at different doses. MT2 cells were infected with HIV at 5 ng p24/mL on the 7th day after plating, then treated continuously with 625, 312.5, 156.2, 78.1 μM f d4T, ML d4T and L d4T. A. Dose–response of different formulations at 7 days post infection. The zero value is the untreated control. B, C and D. Response with ML d4T, L d4T and f d4T, respectively, on days 5–12 at different doses. E. Ten days after plating with HIV, T lymphocytes were treated continuously with d4T (ML d4T, L d4T and f d4T) at 312.5 μM. F. Ten days after plating with HIV, T lymphocytes were treated overnight with d4T (ML d4T, L d4T and f d4T) at 312.5 μM. Then the cells were washed and cultured. Values are expressed as mean ± s.d., n = 3.

observed in MCH values of any group of rats as compared with the control. The observations clearly showed that d4T either given plain or incorporated in liposomes produced anaemia and leucocytopenia. However, d4T entrapped in mannosylated liposomes overcame these drawbacks as they released their content (d4T) directly to the target site.

Plasma and tissue distribution study

The tissue distribution study was undertaken to assess the amount of drug reaching lectin-receptor-rich organs. Figures 4A, B and C shows the concentration of drug measured in plasma and various organs at different times (15, 30, 60, 120 min and 12 h) after intravenous injection of f d4T, L d4T and ML d4T ($0.1\,mg\,kg^{-1}$), respectively. The incorporation of d4T in liposomal vesicles strongly altered its distribution pattern. After L d4T injection, plasma levels ≥ $346.38 \pm 12.36\,nmol\,L^{-1}$ were monitored up to 2 h post injection. In contrast, plasma drug concentration following f d4T injection decreased rapidly ($7.76 \pm 3.65\,nmol\,L^{-1}$ at 2 h post injection) and a larger drug concentration ($440.01 \pm 20.11\,nmol\,L^{-1}$) was found in the kidney at just 15 min after injection. Administration of L d4T did not result in high renal uptake. In the first 15 min following injection, much lower d4T levels were observed in the kidney for the L d4T formulation than with free drug ($P < 0.05$).

In the case of the ML d4T injection, the plasma concentration ($416.7 \pm 12.25\,nmol\,L^{-1}$) was less at 15 min post injection, although it retained significant ($P < 0.05$) concentration even after 12 h as compared with Ld4T. ML d4T maintained a significantly higher level ($P < 0.05$) of

Table 2 Haematological parameters of control and treated rats

Group	WBC ($\times 10^3/\mu$L)	RBC ($\times 10^6/\mu$L)	Hb (g dL^{-1})	HCT (%)	MCH (pg)
Control	8.25 ± 2.1	7.15 ± 1.37	14.89 ± 2.99	41.69 ± 5.51	20.83 ± 1.35
f d4T solution	4.76 ± 1.74	3.21 ± 1.33	6.27 ± 1.25	29.91 ± 3.17	19.53 ± 1.43
	$P = 0.0106$	$P = 0.0005$	$P < 0.0001$	$P = 0.0011$	$P =$ ns
L d4T	5.11 ± 1.23	4.11 ± 1.45	8.20 ± 1.58	34.18 ± 1.25	19.95 ± 2.17
	$P = 0.0102$	$P = 0.0039$	$P = 0.0007$	$P = 0.0086$	$P =$ ns
ML d4T	8.01 ± 1.54	6.51 ± 1.02	13.43 ± 1.57	39.28 ± 5.64	20.62 ± 1.56
	$P =$ ns	$P =$ ns	$P =$ ns	$P =$ ns	$P =$ ns

Values are expressed as mean ± s.d., n = 6. Each formulation administered intravenously was equivalent to 200 μM of d4T. WBC, white blood corpuscles; RBC, red blood corpuscles; Hb, haemoglobin; HCT, haematocrit; MCH, mean corpuscular haemoglobin.

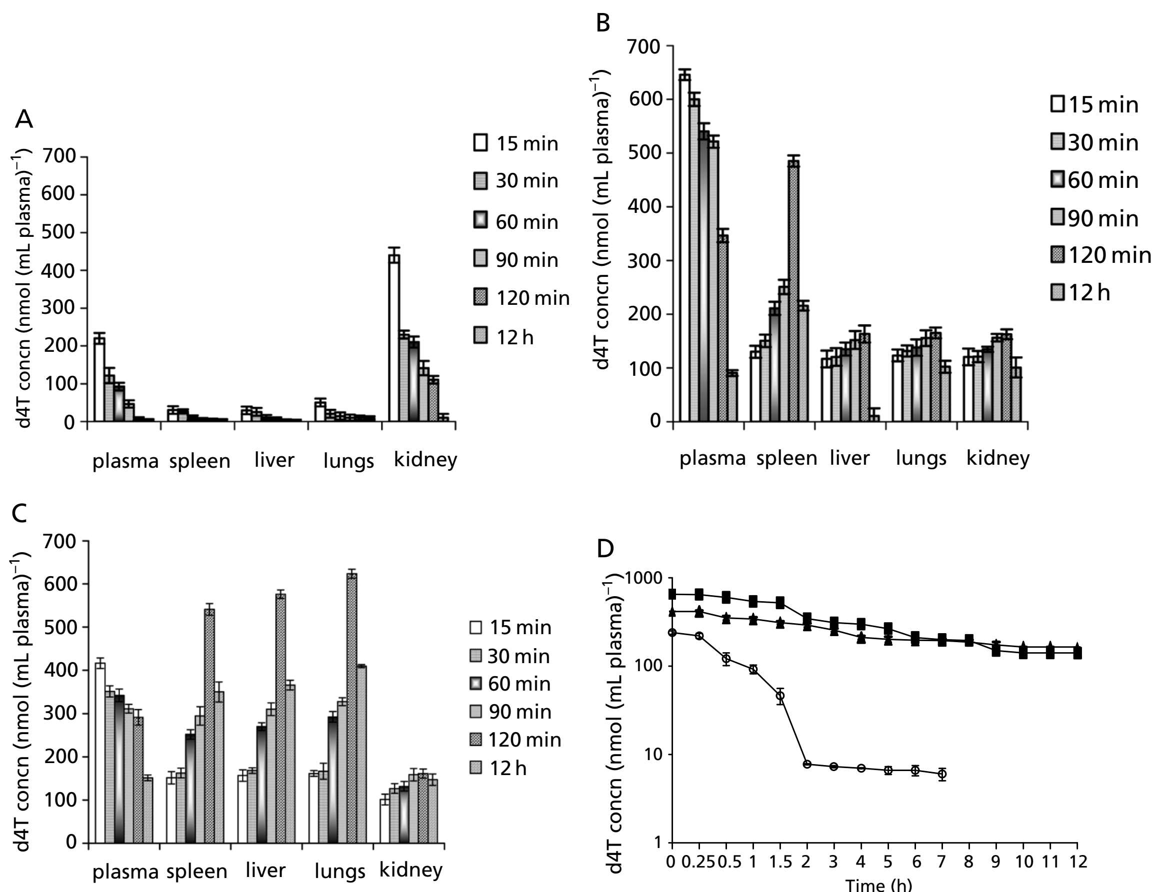

Figure 4 Distribution of free (A), uncoated liposome- (B) and mannosylated liposome-encapsulated d4T (C) in various tissues as a function of time after a single intravenous dose (0.1 mg kg^{-1}) to rats. D. Concentration–time curves of free d4T (●), uncoated liposome-encapsulated d4T (■) and mannosylated liposome-encapsulated d4T (▲) in plasma following the administration of a single intravenous dose (0.1 mg kg^{-1}). Values represent means ± s.d. obtained from three rats per time point.

the drug in liver, spleen and lungs up to 12 h (more than plasma concentration) compared with f d4T and L d4T. From the results it is very clear that coupling of mannose residues to the liposomal surface enhances their systemic clearance. The results indicate that delivery of anti-HIV drugs in liposomes may present certain advantages, particularly for potent compounds that do not have either high oral bioavailability or have a short elimination half-life. The results correlated well with the studies of Banerjee et al (1994) on mannosylated liposomes.

Pharmacokinetic study

The plasma level study of different formulations was carried out to determine the release profile in-vivo. The plasma concentration–time profiles for f d4T, L d4T and ML d4T formulations are shown in Figure 4D, which

Table 3 Pharmacokinetic parameters of free, uncoated liposomal and mannosylated liposomal d4T following the administration of a single intravenous dose ($0.1\,mg\,kg^{-1}$), in rats, determined using non-compartmental analysis

Pharmacokinetic parameter	f d4T	L d4T	ML d4T
t½ (h)	0.94 ± 0.002	2.41 ± 0.001	3.34 ± 0.005
AUC_{0-72} ($nmol\,h\,mL^{-1}$)	216.56 ± 3.11	3241.1 ± 6.21	2669.8 ± 150.34
K_{el} (h^{-1})	0.73 ± 0.001	0.29 ± 0.003	0.21 ± 0.004
Vd_{ss} ($L\,kg^{-1}$)	2.80 ± 0.02	0.48 ± 0.12	0.81 ± 0.31
Cl ($L\,h^{-1}\,kg^{-1}$)	2.05 ± 0.40	0.13 ± 0.031	0.17 ± 0.50
MRT (h)	1.36 ± 0.05	3.47 ± 0.21	4.82 ± 0.36

Values are expressed as mean ± s.d., n = 3. t½, half-life in plasma of the terminal elimination phase; AUC, area under the plasma concentration–time curve; K_{el}, elimination rate constant; Vd_{ss}, steady-state volume of distribution; Cl, systemic clearance; MRT, mean residence time.

demonstrates a rapid clearance of free drug from the plasma as compared with L d4T and ML d4T formulations. The concentration–time profile of L d4T and ML d4T depicted a similar sustained release pattern. With ML d4T, the plasma concentration of d4T was initially less, but enhanced significantly after 12 h ($P < 0.05$) as compared with L d4T, indicating the prolonged sustained release and the retention potential of ML d4T formulation. The pharmacokinetic parameters of f d4T, L d4T and ML d4T estimated from the concentration–time curves in plasma are shown in Table 3. Encapsulation of d4T in mannosylated liposomes profoundly modified its pharmacokinetics. The apparent clearance of the free drug was 15.8 and 12 times higher than that of d4T in uncoated liposomes and mannosylated liposomes, respectively. This resulted in a marked increase in the elimination half-life of encapsulated drug. The AUC of ML d4T was 12.3 times higher ($P < 0.05$) and 1.2 times lower ($P < 0.05$) than that of f d4T and L d4T formulations, respectively. For the ML d4T formulation, the MRT was found to be 3.5 and 1.4 times ($P < 0.05$) higher than that of f d4T and L d4T, respectively. Thus, the data projects that though ML d4T has greater systemic clearance than L d4T, it remains in the body for a longer period of time and is distributed to various organs of the body, as observed from the tissue distribution curve. The results indicate that the drug encapsulated in mannosylated liposomes is much more potent than normal liposome-encapsulated drug or free drug. The outcome is in good agreement with the previous studies of Medda et al (1993) for mannosylated liposomes.

Discussion

Macrophages are the major differentiating cells of the mononuclear phagocyte system. Macrophages colonize the liver (Kupffer cells), lungs (alveolar macrophages), spleen, lymph nodes, thymus, gut, marrow, brain, connective tissue and serous cavities (Vermon-Robert 1972). They play a critically prominent role in host defence against many infectious agents, including HIV. Mannose receptors on CD4 lymphocytes and macrophage surfaces have been exploited (Barrat et al 1986; Muller & Schuber 1989). The complement system of the body also helps in the opsonization of vesicular carrier system. One of the products of the complement cascade, C3b, strongly activates opsonization of vesicular carrier by macrophages. This phenomenon could be useful for uptake of liposomes by macrophages (one of the reservoirs for HIV). But for uptake by other cells such as hepatocytes (HIV reservoirs), which have mannose specific lectin receptors, mannosylated liposomes could be a useful approach (Vlahakis et al 2003). Liposomes are the most widely studied carrier in drug targeting to HIV-infected cells. Mannosylated liposomes can deliver the drug more efficiently to the MPS than the plain drug solution.

Mannose, being hydrophilic in nature, when adsorbed on to the liposomal surface is easily removed on dilution. Therefore it was necessary to chemically modify mannose by conjugating it to a hydrophobic group, which allows the mannose residue to interligitate with the liposomal membrane (Sato & Sunamoto 1992).

The minimum encapsulation efficiency of formulation L1 may be due to the formation of a leaky vesicle because the cholesterol content was relatively less in this formulation. In formulations L3 and L4 the cholesterol content was high beyond its optimum concentration ratio, resulting in closed packing with decreased fluidity. The larger vesicular size of L1 may be due to the formation of leaky and less rigid vesicles leading to the fusion of vesicles. Large deviation from the mean particle size was observed for L3 and L4, which may be due to the greater membrane area occupied by combination of acyl chains of lipids and cholesterol than that taken by the phosphocholine head group of lipids. Increased cholesterol gives more rigid liposomes, which are not easily broken down during the extrusion process.

The positive zeta-potential value is changed to a less positive value on addition of OPM, which indicates the masking of positive charge by stearylamine. On further addition of OPM (beyond a 10:5 lipid-to-ligand weight ratio) there is no change in zeta-potential. This is an indication of complete intrinsic charge quenching on OPM coating of liposomes. No significant change in zeta-potential after the 2nd hour can be attributed to the fact that the lipid bilayer gets saturated with OPM at the end of the 2nd hour.

Molecular recognition events in biological systems frequently make use of multivalent ligand–receptor interactions. Con A, a well-investigated lectin, is known to bind specifically with saccharides, such as mannose, fructose and glucose residues (Rademacher et al 1988; Polomino 1994). This lectin has multiple saccharide-binding sites located 65–70 apart (Habash et al 2000). Cross-linking the mannosylated vesicles can promote their agglutination. This may be the reason behind the increase in turbidity. Insignificant increase in turbidity beyond $500\,\mu g\,mL^{-1}$ may be due to saturation of the binding sites.

The release of liposome-encapsulated drug into tissues is based on the following mechanisms. The initial event in liposome–cell interaction is thought to be adsorption of intact vesicles to the cell surface followed by endocytosis (Pagano & Weinstein 1978). Other possible mechanisms include enzymatic degradation of the lipid bilayer and subsequent leakage of drug into the immediate vicinity of the cells, lipid exchange with the cells and non-facilitated diffusion of the drug across the lipid bilayer (Schroit et al 1986). Mannosylated liposomes could not deliver their content to plasma. After coming into contact with macrophages and other cells (HIV reservoirs), due to the presence of lectin they could release d4T in the vicinity of the target site. Macrophages also have lectin receptors on their surface. Because of macrophages' role in the pathogenesis of some diseases, targeting to macrophages could be potentially useful in the treatment of disorders such as AIDS (Mitra et al 1998).

The subtoxic concentration of free drug was high compared with the liposomal (uncoated and coated) formulations. This may be due to the sustained release potential of liposomal formulations. The delivery of d4T in mannosylated liposomes was more effective than the administration of free or uncoated liposome-encapsulated drug as shown by antiretroviral assay. This advantage of drug encapsulated in liposomes is most likely due to the receptor-mediated targeting of the mannose-coated system, which thereafter acts as a reservoir providing a high local concentration of drug in lymphocytes. The enhancement of the effect of d4T by encapsulation in mannosylated liposomes also indicates that the inhibitor does not merely leak out of the liposomes in the cell culture medium but is transported into lymphocytes via liposomes. The reduction in p24 production observed in our evaluation of the antiviral effect of d4T is consistent with observations made with the protease inhibitor L-689,502 (Pretzer et al 1997).

The haematological study was undertaken to assess the relative effects of the mannose-coated and uncoated liposomal systems, as compared with the plain drug, on various blood parameters. Lowering in haematological parameters was observed in the case of free or uncoated liposome-encapsulated d4T. This may be due to haemolytic toxicity and cytotoxicity of the positively charged liposomal surface (Duncan et al 1996). No toxicity was seen with mannosylated liposomal d4T. This is due to the coating of mannose, which bears a net negative charge that presents a neutral surface having a non-haemolytic nature (Jansen et al 1991).

The encapsulation in liposomes appears to slow down the renal elimination of d4T. Furthermore, L 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 the short elimination half-life of free d4T. With ML d4T, a significant ($P < 0.001$) amount of d4T was found in spleen, liver and lungs because mannose-specific receptors are present in abundance at these sites, which slowly localized the coated system in these organs. These localized mannosylated liposomes sustain the release of drug at these sites. gp120 glycoproteins of HIV bind to CD4 cell surface receptors located on CD4 T lymphocytes, monocytes and macrophages (Ho et al 1995; Perelson et al 1996). Lymphocytes that undergo constant recirculation between the blood, lymph, lymphoid organs and tissue spaces have cell-surface receptors. These receptors include mannose receptors on various tissue macrophages. The superiority of liposome encapsulation for drug delivery to macrophages may actually be enhanced in-vivo, due to the localization of liposomes in the reticuloendothelial system (the mononuclear phagocyte system) following intravenous injection (Poste et al 1982; Alving 1983; Popescu et al 1987; Senior 1987; Szoka 1991). Studies with liposome-encapsulated reverse-transcriptase inhibitors have shown that the drugs localize in the reticuloendothelial system (Desomeaux et al 1994; Makabi-Panzu et al 1994). The plasma half-life of liposome-encapsulated 2′,3′-dideoxyinosine (ddI) was shown to be 46 times higher than that of free drug (Harvie et al 1995). The systemic clearance of foscarnet encapsulated in conventional liposomes was found to be 77-fold lower than that of free drug (Dussere et al 1995).

Conclusions

Conclusively, d4T encapsulation in mannosylated liposomes improves cell uptake without increasing cytotoxicity. Mannosylated liposomal formulations deliver their content to MPS tissues, which are the reservoir of HIV. Encapsulation of d4T in mannosylated liposomes increases the elimination half-life and mean residence time of the drug. Low elimination and better distribution profile can be achieved by mannosylated liposomes. Encapsulation of anti-HIV drugs into mannosylated liposomes generates better pharmacological activity. The toxicity associated with antiviral drugs can be overcome by encapsulating them in mannosylated liposomes. The dose of the antiviral agent can be reduced due to the site-specific delivery from this carrier. Ligand-mediated biodisposition and cellular interaction of liposomes especially at the target sites would be a focal paradigm for upcoming research in the field of anti-HIV drug delivery. Mannosylated liposomes have paved the way for the biostable, site-specific and ligand-mediated delivery systems with desired therapeutics. Further studies using other antiviral drugs are in progress.

References

Alving, C. R. (1983) Delivery of liposome-encapsulated drugs to macrophages. *Pharmacol. Ther.* **22**: 407–422

Auger, M. J., Ross, J. A. (1992) The biology of macrophage. In: Lewis, C. E., Mcgee, J. O. D. (eds) *The natural immune system: the macrophage*. Oxford University Press, New York, pp 2–74

Bakker-Woudenberg, I. A. J. M. (1995) Liposomes in the treatment of parasitic, viral, fungal and bacterial infections. *J. Lipos. Res.* **5**: 169–191

Banerjee, G., Bhaduri, A. N., Basu, M. K. (1994) Mannose-coated liposomal hamycin in the treatment of experimental leishmaniasis in hamsters. *Biochem. Med. Metab. Biol.* **53**: 1–7

Barrat, G., Tenu, J. P., Yapo, A., Petit, J. F. (1986) Preparation and characterization of liposome containing mannosylated phospholipids capable of targeting to macrophages. *Biochim. Biophys. Acta* **862**: 153–164

Bedoya, L. M., Sanchez-Palomino, S., Abad, M. J., Bermejo, P., Alcami, J. J. (2001) Anti-HIV activity of medicinal plant extracts. *Ethnopharmacology* **77**: 113–116

Copland, M. J., Baird, M. A., Rades, T., McKenzie, J. L., Becker, B., Reck, F., Tyler, P. C., Davies, N. M. (2003) Liposomal delivery of antigen to human dendritic cells. *Vaccine* **21**: 883–890

Desomeaux, A., Harvie, P., Perron, S., Makabi-Panzu, B., Beauchamp, D., Tremblay, M., Poulin, L., Bergeron, M. G. (1994) Antiviral efficacy, intracellular uptake and pharmacokinetics of free and liposome encapsulated 2′,3′-dideoxyinosine. *AIDS* **8**: 1545–1553

Duncan, R., Dimitrijevic S., Eyagororou. E. G. (1996) The role of polymer conjugate in the diagnosis and treatment of cancer. *STP Pharm. Sci.* **6**: 237–263

Dussere, N., Lessard, C., Paquette, N., Perron, S., Poulin, L., Tremblay, M., Beauchamp, D., Desormeaux, A., Bergeron, M. G. (1995) Encapsulation of foscarnet in liposomes modifies intracellular accumulation, *in vitro* anti-hIV-1 activity, tissue distribution and pharmacokinetics. *AIDS* **9**: 833–841

Fidler, U., Brown, N. O., Hart, I. R. (1985) Species variability for toxicity of free and liposome-encapsulated muramyl peptides administered intravenously. *J. Biol. Resp. Modif.* **4**: 298–309

Fry, D. W., White, J. C., Goldman, I. D. (1978) Rapid separation of low molecular weight solutes from liposomes without dilution. *Anal. Biochem.* **90**: 809–815

Gregoriadis, G. (1988) Fate of injected liposomes: observation on entrapped solute tretinoin, vesicle clearance and tissue distribution in vivo. In: Gregoriadis, G. (ed.) *Liposome as drug carriers: recent trends and progress*. Wiley, Chichester, pp 3–18

Habash, J., Raftery, J., Nuttall, R., Price, H. J., Wilkinson, C., Kalb A. J., Helliwell, J. R. (2000) Direct determination of the positions of the deuterium atoms of the bound water in concanavalin a by neutron Laue crystallography. *Acta Crystallogr. D Biol. Crystallogr*. **D56**: 541–550

Hao, Y. L., Deng, Y. J., Chen, Y., Wang, K. Z., Hao, A. J., Zhang, Y. (2005) In-vitro cytotoxicity, in vivo biodistribution and antitumour effect of PEGylated liposomal topotecan. *J. Pharm. Pharmacol.* **57**: 1279–1287

Harvie, P., Desormeaux, A., Gagne, N., Tremblay, M., Poulin, L., Beauchamp, D., Bergeron, G. (1995) Lymphoid tissue targeting of liposome encapsulated 2′,3′-dideoxyinosine (ddI). *AIDS* **9**: 701–707

Herman, E., Rahman, A., Ferrans, V., Vick, J., Schein, P. (1983) Prevention of chronic doxorubicin cardiotoxicity in beagles by liposome entrapment. *Cancer Res.* **43**: 5427–5432

Ho, D. D., Neumann, A. U., Perelson, A. S., Chen, W., Leonard, J. M., Markowitz, M. (1995) Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection. *Nature* **373**: 123–126

Jansen, R. W., Molema, G., Ching, T. L., Oosting, R., Harms, G., Moolenaar, F., Hardonk, M. J., Meijer, D. K. (1991) Hepatic endocytosis of various types of mannose-terminated albumins. What is important, sugar recognition, net charge, or the combination of these features? *J. Biol. Chem.* **266**: 3343–3348

Kanter, P. M., Bullard, G. A., Pilkiewicz, F. G., Mayer, L. D., Cullis, P. R., Pavelic, J. P. (1993) Preclinical toxicology study of liposome encapsulated doxorubicin: comparison with doxorubicin and empty liposomes in mice and dogs. *In-Vivo* **7**: 85–95

Katragadda, A., Bridgman, R., Betageri, G. (2000) Effect of liposome composition and cholesterol on the cellular uptake of stavudine by human monocyte/macrophages. *Cell. Mol. Biol. Lett*. **5**: 483–493

Kawakami, S., Wong, J., Sato, A., Hattori, Y., Yamashita, F., Hashida, M. (2000) Biodistribution characteristics of mannosylated, fucosylated, and galactosylated liposomes in mice. *Biochim. Biophys. Acta* **1524**: 258–265

Konopka, K., Davis, B. R., Larsen, C. E., Alford, D. R., Debs, R. J., Duzgunes, N. (1990) Liposomes modulate human immunodeficiency virus infectivity. *J. Gen. Virol.* **71**: 2899–2907

Lasic, D. (1992) Liposomes. *Am. Scientist* **80**: 20–31

Lasic, D. D. (1993) *Liposomes: from physics to applications*. Elsevier, Amsterdam, pp 108–201

Levy, J. A. (1993) Pathogenesis of human immunodeficiency virus. *Microbiol. Rev*. **57**: 183–289

Lopez-Berestein, G., Kasi, L., Rosenblum, M. G., Haynie, T., Jahns, M., Glenn, H. (1984) Clinical pharmacology of ^{99m}Tc-labelled liposomes in patients with cancer. *Cancer Res.* **44**: 375–378

Lopez-Berestein, G., McQueen, T., Mehta, K. (1985) Protective effect of liposomal amphotericin B against C. albicans infection. *Cancer Drug Del.* **2**: 183–189

Lopez-Berestein, G., Bodey, G. P., Frankel, L. S., Mehat, K. (1987) Treatment of hepatosplenic candidiasis with liposomal amphotericin B. *J. Clin. Oncol.* **5**: 310–317

Makabi-Panzu, B., Lessard, C., Perron, S., Desomeaux, A., Tremblay, M., Poulin, L., Beauchamp, D., Bergeron, M. G. (1994) Comparison of cellular accumulation, tissue distribution and anti-HIV activity of free and liposomal 2′,3′-dideoxycytidine. *AIDS Res. Hum. Retroviruses* **10**: 1463–1470

Mayer, L., Tai, L., Bally, M. (1990) Characterization of liposomal systems containing doxorubicin. *Biochim. Biophys. Acta* **1025**: 143–148

Medda, S., Mukherjee, S., Das, N., Naskar, K., Mahato, S. B., Basu, M. K. (1993) Sugar-coated liposomes: a novel delivery system for increased drug efficacy and reduced drug toxicity. *Biotechnol. Appl. Biochem.* **17**: 37–47

Mehta, R. T., Hopfer, R. L., McQueen, T., Juliano, R. L., Lopez-Berestein, G. (1987) Toxicity and therapeutic effects in mice of liposome-encapsulated nystatin for systemic fungal infections. *Antimicrob. Agents Chemother.* **31**: 1901–1913

Meltzer, M. S., Skillman, D. R., Gendelman, H. E. (1990) Macrophage and the immunodeficiency virus. *Immunol. Today* **6**: 217–224

Mitra, S. K., Venkataranganna, M. V., Sundaram, R., Gopumadhavan, S. (1998) Protective effect of HD-03, a herbal formulation, against various hepatotoxic agents in rats. *J. Ethnopharmacol.* **63**: 181–186

Muller, C. D., Schuber, F. (1989) Neo-mannosylated liposomes: synthesis and interaction with mouse kupffer cells and resident peritoneal macrophages. *Biochim. Biophys. Acta Ser. Biomembr*. **986**: 97–105

Nassander, U. K., Storm, G., Peeters, P. A. M., Crommelin, D. J. A. (1990) Liposomes. In: Chasin, M., Langer, R. (eds) *Biodegradable polymers as drug delivery systems*. Marcel Dekker, New York, pp 261–338

New, R. R. C. (1990) Introduction and preparation of liposomes. In: New, R. R. C. (ed.) *Liposomes: a practical approach*. Oxford University Press, Oxford, pp 1–104

Pagano, R. E., Weinstein, J. N. (1978) Interactions of liposomes with mammalian cells. *Ann. Rev. Biophys. Bioeng.* **7**: 435–468

Perelson, A. S., Neumann, A. U., Markowitz, M., Leonard, J. M., Ho, D. D. (1996) HIV-1 dynamics *in vivo*: virion clearance rate, infected cell life-span, and viral generation time. *Science* **271**: 1582–1586

Polomino, E. (1994) Carbohydrate handles as natural resources in drug delivery. *Adv. Drug. Deliv. Rev.* **13**: 311–323

Popescu, M. C., Swensen, C. E., Ginsberg, R. S. (1987) Liposome mediated treatment of viral, bacterial and protozoal infections. In: Ostro, M. J. (ed.) *Liposomes: from biophysics to therapeutics*. Marcel Dekker, New York, pp 219–251

Poste, G., Bucana, C., Raz, A., Bugelski, P., Kirsh, R., Fidler, U. (1982) Analysis of the fate of systemically administered liposomes and implications for their use in drug delivery. *Cancer Res.* **42**: 1412–1422

Pretzer, E., Flasher, D., Duzgunes, N. (1997) Inhibition of human immunodeficiency virus type-1 replication in macrophages and h9 cells by free or liposomes encapsulated l-689,502, an inhibitor of the viral protease. *Antiviral Res.* **34**: 1–15

Rademacher, T. W., Parekh, R. B., Dwek, R. A. (1988) Glycobiology. *Annu. Rev. Biochem.* **57**: 785–838

Rahman, A., White, G., More, N., Schein, P. S. (1995) Pharmacological, toxicological and therapeutic evaluation of doxorubicin entrapped in cardiolipin liposomes. *Cancer Res.* **45**: 796–803

Roy, S., Wainberg, M. A. (1988) Role of mononuclear phagocyte system in the development of acquired immunodeficiency syndrome (AIDS). *J. Leukoc. Biol.* **43**: 91–97

Salvatori, D., Vincenzetti, S., Maury, G., Gosselin, G., Gaubert, G., Vita, A. (2001) Maedi-Visna virus, a model for in vitro testing of potential anti-hiv drugs. *Comp. Immunol. Microbiol. Infect. Dis.* **24**: 113–122

Sato, T., Sunamoto, J. (1992) Recent aspects in the use of liposomes in biotechnology and medicine. *Prog. Lipid Res.* **31**: 345–372

Schroit, A. J., Madsen, J., Nayar, R. (1986) Liposome-cell interactions: *in vitro* discrimination of uptake mechanism and *in vivo* targeting strategies to mononuclear phagocytes. *Chem. Phys. Lipids* **40**: 373–393

Senior, J. H. (1987) Fate and behavior of liposomes in vivo: a review of controlling factors. CRS *Crit. Rev. Ther. Drug Carrier Sys.* **3**: 123–193

Slidregt, L. A. J. M., Rensen, P. C. N., Rump, E. T., Santbrink, P. J. V., Bijsterbosch, M. K., Valentijn, A. R. P. M., Van der Marel, G. A., Van Boom, J. H., Van Berkel, T. J. C., Biessen, E. A. L. (1999) Design and synthesis of novel amphiphilic dendritic galactosides for selective targeting of liposomes to the hepatic asialoglycoprotein receptor. *J. Med. Chem.* **42**: 609–618

Stephen, C. P., Grace, K., Robert, E. W., Joseph, K., Judith, F., Richard T. T. D., Sangita, R., Henry, M., Michael, A. P. (1999) A multiple drug interaction study of stavudine with agents for opportunistic infections in human immunodeficiency virus-infected patients. *Antimicrob. Agents Chemother.* **43**: 647–650

Sunamoto, J., Gato, M., Lida, T., Hara, K., Saito, A., Tomonega, A. (1985) Polymer coated liposomes for drug delivery to target specific organs. In: Gregoriadis, G., Senior, J., Trout, A. (eds) *Receptor mediated targeting of drugs*. Plenum, New York, pp 359–371

Szoka, F. C. (1991) Liposomal drug delivery: current status and future prospects. In: Wilschut, J., Hoekstra, D. (eds) *Membrane fusion*. Marcel Dekker, New York, pp 845–890

Szoka, F. C., Papahadjopoulos, D. (1978) Procedure for preparation of liposomes with large aqueous space and high capture by reverse-phase evaporation. *Proc. Natl Acad. Sci. USA* **75**: 4194–4198

Szoka, F. C., Milhollan, D., Barza, M. (1987) Effect of lipid composition and liposome size on toxicity and in vitro fungicidal activity of liposome intercalated amphotericin B. *Antimicrob. Agents Chemother.* **31**: 421–429

Treat, J., Greenspan, A. R., Rahman, A. (1989) Liposome-encapsulated doxorubicin: preliminary results of phase I and phase II trials. In: Lopez-Berestein, G., Fidler, I. J. (eds) *Liposomes in the therapy of infectious diseases and cancer*. Alan R. Liss, New York, pp 353–365

Vermon-Robert, B. (1972) The macrophage. In: Harrison, R. J., Meminn, R. M. H. (eds) *Biological structure and function*. Vol. 2, Cambridge University Press, London

Vlahakis, S. R., Villasis-Keever, A., Gomez, T., Bren, G. D., Paya, C. V. (2003) Human immunodeficiency virus-induced apoptosis of human hepatocytes via CXCR4. *J. Infect. Dis.* **188**: 1455–1460

Wasan, K. M., Lopez-Berenstein, G. (1995) The past, present, and future uses of liposomes in treating infectious diseases. *Immunopharmacol. Immunotoxicol.* **17**: 1–15

Weatherman, R. V., Mortell, K. H., Chervenak, M., Kiessling, L. L., Toone, E. J. (1996) Specificity of *C*-glycoside complexation by mannose/glucose specific lectins. *Biochemistry* **35**: 3619–3624

Williams, C. L., Stancel, G. M. (1990) Antiviral agents. In: Gilman, A. G., Goodman, L. S., Rall, T. W., Murad, F. (eds) *The pharmacological basis of therapeutics*. 9th edn, Macmillan Publishing Co., New York, pp 1205