CHAPTER 13

SUMMARY AND CONCLUSION

13.1 INTRODUCTION

Recently, with the advance of biotechnology, an increasing number of proteins and peptides have been developed as therapeutic drugs. Biotechnology has enabled several new categories of molecules to be used as therapeutic agents. For example, peptide based drugs and DNA based therapeutic agents (gene therapy, DNA vaccines and antisense oligonucleotides) are now being routinely investigated. Due to the low bioavailability after oral delivery, these drugs are usually administered by the parenteral route. However, parenteral administration has some limitations. First, the short biological half-life of peptide drugs and DNA result in an inconveniently high dosing frequency. Second, the high blood concentrations of some peptide drugs after intravenous administration or after chronic dosing could cause significant toxicity. To prolong the biological half life of the drug, to prevent from enzymatic degradation and to reduce the injection frequency and toxicity of intravenously administered drugs, it would be necessary to develop safe and sustained injectable delivery systems containing peptide drugs and DNA.

Particulate systems, such as liposomes, micro or nanoparticles, have attracted a great deal of attention for peptide drugs and DNA as safe and sustainable drug delivery systems. Liposomes have considerable potential as drug carriers for peptide drugs and DNA to prevent them from enzymatic degradation. Liposomes are biodegradable and essentially non toxic, can encapsulate hydrophilic, hydrophobic and amphipathic drugs. Delivery of agents to the reticuloendothelial system (RES) is easily achieved since most conventional liposomes are trapped by RES. As one approach to avoid the phagocytosis of liposomes by RES, coating the liposomes with chains of polyethylene glycol (PEG) has been used to prolong the circulation time of the liposomes, based on a sterical hindrance or an increase of liposomal surface hydrophilicity. For the purpose of delivery of agents to target organs other than RES, long circulating liposomes have been developed by modifying the liposomal surface and its surface charge for targeting liposomes to specific tissues or cells.

The aim of the present study was to develop a sustained and a safe injectable drug delivery system for peptide drugs (cyclosporine and

leuprolide acetate) and DNA by exploring the potential of charged and sterically stabilized liposomes.

13.2 CYCLOSPORINE

Cyclosporine (also known as Cyclosporin A), a poorly water soluble cyclic peptide comprising 11 amino acids, has been utilized clinically as a potent immunosuppressive agent, such as the prevention of rejection following transplantation of kidney, liver, bone marrow and pancreas. The use of CsA has been limited due to a broad toxicity profile, including both acute and chronic nephrotoxicity. Liposomes are microscopic lipid spheres that accumulate preferentially at sites of infection and inflammation and also at the tumor sites after intravenous administration.

13.3 LEUPROLIDE ACETATE

Leuprolide acetate a highly water soluble nonapeptide and a potent GnRH analogue utilized clinically for the treatment of prostate carcinoma in men, ovarian and breast carcinoma in women. Leuprolide acetate has been used extensively in clinical oncology because these agents exert anti tumor effects against a variety of tumor cells both *in vitro* and *in vivo*. The direct action of GnRH agonists via the GnRH-receptors, which is expressed in malignant tumors that differs from the pathway mediated via the pituitary, promotes the antiproliferative effect of these agents on the tumors. However, the direct antiproliferative activity of GnRH analogue requires the continuous presence of this molecule at the site of tumor, therefore a delivery system that prolongs the biological half-life of the drug, prolongs the exposure of target cells (such as malignant cells) to GnRH analogue into malignant cells would be advantageous, such goals can be achieved by liposomal delivery system.

13.4 DNA

Calf thymus DNA was encapsulated in the liposomes as a model DNA. Upon systemic administration, naked DNA was rapidly cleared from the circulation. Within the short period, DNA will be available as a pharmaceutical for gene delivery, vaccination and other applications in molecular medicine. The generally poor efficiency of delivery remains one of the limitations to the development of gene therapy and vaccination. Much

attention is therefore being paid to the design of new formulations of DNA using liposomes, an attractive alternate for the controlled delivery of therapeutic agents (including DNA). Cationic liposomes have been widely used for gene delivery systems and various cationic liposomes are commercially available. A major limitation in development and *in vivo* applications of the cationic liposomes is the rapid uptake of particulate liposomes, following intravenous administration, by the RES. Therefore a delivery system that prolongs the circulation in blood and diminished uptake by the liver and spleen cells would be advantageous, such goals can be achieved by sterically stabilized liposomal delivery system.

13.5 PREPARATION OF LIPOSOMES CONTAINING CYCLOSPORINE A, LEUPROLIDE ACETATE AND DNA

13.5.1 CYCLOSPORINE (CsA)

Multilamellar vesicles (MLVs) were prepared by dissolving different molar ratios of lipids, DL-α-tocopherol (0.5 ml of 0.1 % w/v solution in chloroform) and CsA in Chloroform: Methanol solvent mixture (1:1 ratio). Positive (CPL), negative (CNL) and neutral liposomes (CL) were prepared by using Hydrogenated Soya Phosphatidyl Choline (HSPC), Cholesterol (Ch), Stearylamine (SA) (positive charge), Distearoyl Phosphatidyl Glycerol (DSPG) (negative charge), in the molar ratio of 1: 0.17: 0.3 by thin film hydration method. The process and the formulation parameters were optimized by 3^3 factorial design to achieve maximum entrapment efficiency. The lipid: drug ratio used in all the formulations was 1: 0.04 (by molar ratio). The organic solvent was evaporated at a temperature of 60°C on a rotary flash evaporator to yield a thin, dry lipid film. The lipid film was then hydrated with phosphate buffer saline (PBS) of pH 7.8 at 60°C for 1h to form a stable liposomal CsA formulation. The liposomal suspension so formed was then transferred to a suitable glass container and sonicated for 15 min using a probe sonicator (model – RR-120, Ralsonics, Mumbai) at 60°C. The sonicated dispersion was then allowed to stand undisturbed for about 2 h at room temperature for complete hydration. The multilamellar vesicles formed were sequentially extruded two times through 1μm Nucleopore polycarbonate track-etch membrane filters (Whatman Inc. New Jersey, USA)

at 60°C. The process was repeated through two stacked 0.4μm and 0.2μm membranes. The unentrapped drug was removed by centrifugation at 15,000 rpm for 30 min, the supernatant was removed and the pellet was resuspended in PBS. The liposomal suspension was freeze dried along with sucrose or lactose as cryoprotectant (2.75 times by weight of total lipids) overnight at -20°C, lyophilized for 24 h and stored in vials at 2 - 8°C. The lyophilized powder was rehydrated with PBS before use.

The unentrapped drug was removed from the liposomal suspension by centrifugation (Remi, C-24, India) at 15,000 rpm for 30 min. The supernatant was diluted appropriately using methanol and tetra hydro furan (1:1) and absorbance was measured at 238nm on Shimadzu 1601 UV- Visible spectrophotometer with respect to similarly treated empty liposome as blank. Entrapment efficiency (EE) was calculated from the difference between the initial amount of CsA added and that present in the supernatant and was expressed as % of total amount of CsA added. EE of the prepared liposome formulations was also confirmed by dissolving the liposomes using methanol and measuring the absorbance at 238nm on Shimadzu 1601 UV- Visible Spectrophotometer.

13.5.2 LEUPROLIDE ACETATE

Conventional (LL) and sterically stabilized (methoxypolyethylene glycol of mol wt. 5000 and methoxy polyethylene glycol of mol wt. 2,000 activated with cyanuric chloride coupled with phosphatidyl ethanolamine [mPEG5000-CC-PE, mPEG2000-CC-PE]) leuprolide acetate liposomes [SLL5000, SLL2000] were prepared by the reverse phase evaporation technique. The lipid: drug ratio used in all the formulations was 1: 0.2 (by molar ratio). The sterically stabilized agents investigated were mPEG5000-CC-PE, mPEG2000-CC-PE. In the synthesis of mPEG-CC-PE, first the methoxypolyethylene glycols (mPEG-5000 & mPEG-2000) were activated using cyanuric chloride (2, 4, 6-trichloro-s-triazine) in presence of potassium carbonate as per the previously reported method, which was modified suitably. The second step involved the conjugation of the activated mPEG with phosphotidyl ethanolamine in presence of triethylamine based on the earlier reported procedure. The course of the reaction was monitored by thin layer chromatography. In both the steps, the products were

characterized by infrared spectroscopy, nuclear magnetic resonance spectroscopy and by ultra violet spectroscopy.

The amount of the agents required for steric stabilization was optimized using the electrolyte induced flocculation test. Here, a fixed quantity of the liposomal suspension was diluted to a predetermined volume using different molar concentrations of an electrolyte, sodium sulphate. The absorbance of the resulting suspensions was measured spectrophotometrically. The concentration of the steric stabilizing agent, which did not allow any changes in the absorbance when different molar concentrations of the electrolyte were added to the liposomes was selected as the optimum concentration and used for further studies. 6 mol % of the total lipids of both mPEG5000-CC-PE and mPEG2000-CC-PE were found to be adequate for steric stabilization of the liposomes.

The unentrapped drug was removed from the liposomal suspension by ficoll- gradient centrifugation (New, 1990). 0.5ml of liposomal suspension was mixed with 1ml of 30% ficoll solution (in saline). It was transferred to an ultracentrifuge tube; 3ml of 10% ficoll solution was layered gently on top of the above mixture. The upper ficoll layer was covered with a layer of buffered saline, centrifuged (Remi, C-24, India) at 15,000 rpm for 30 min. The unentrapped drug present in the lowest ficoll layer was diluted appropriately using 0.1N sodium hydroxide and the absorbance was measured at 282nm on Shimadzu 1601 UV- Visible Spectrophotometer. The liposomes were collected at the interface between the saline and 10% ficoll layers. The drug present in the liposomes was estimated by Modified Bligh-Dyer extraction method. 0.5ml of liposome suspension, 2ml of chloroform, 1ml of methanol and 1ml of saturated sodium chloride solution were taken in a centrifuge tube, the mixture was spun at 1000 rpm for about 10 minutes. The aqueous layer was removed and the drug content was estimated by UV- Visible Spectrophotometer at 282nm. Entrapment efficiency was calculated from the difference between the initial amount of leuprolide acetate added and that presents in the unentrapped form and was expressed as % of total amount of leuprolide acetate added.

The proportion of the system components i.e. HSPC, Chol, charge and drug as well as the process parameters such as the volume of hydration medium,

hydration time, vacuum applied, speed of the rotary flash evaporator, sonication time were optimized based on the percent drug entrapment by 3^3 factorial design. It was observed that a molar ratio of HSPC: Chol: DSPG (1: 0.3: 0.2) and HSPC: Chol: DSPG: mPEG 5000 or mPEG 2000 (1:1:0.15:0.15) gave liposomal suspensions with better entrapment efficiency.

13.5.3 DNA

Conventional (DL-HSPC: Chol: DSPG) and sterically stabilized (methoxy polyethylene glycol of mol wt. 5,000 and 2,000 (SDL5000-HSPC: Chol: DSPG: mPEG5000, SDL2000 -HSPC: Chol: DSPG (anionic lipid): mPEG2000, CSDL2000-HSPC: Chol: DOTAP (cationic lipid) [Dioleyl trimethyl amino propane]: mPEG2000) liposomes were prepared by the Freeze dried empty liposomal method [FDEL]. The multilamellar vesicles formed were sequentially extruded two times through 1μm Nucleopore polycarbonate track-etch membrane filters (Whatman Inc. New Jersey, USA) at 60°C. The process was repeated through two stacked 0.4μm and 0.2μm membranes. The liposomal suspension was freeze dried overnight at -20°C, lyophilized for 24 h and stored in vials at 2 - 8°C. To the lyophilized powder, aqueous solution of DNA (0.77%w/w of total lipids) was added and rehydrated by gently shaking the suspension at 60°C for 15 minutes. 5mol% of the total lipids of both mPEG5000-CC-PE and mPEG2000-CC-PE were found to be adequate for steric stabilization of the liposomes.

The unentrapped drug was removed from the liposomal suspension by ficoll- gradient centrifugation described as before. The unentrapped drug was estimated spectrophotometrically by measuring the absorbance at 260nm. The DNA present in the liposomes was estimated by Modified Bligh-Dyer extraction method as described earlier. It was observed that a molar ratio of HSPC: Chol: DSPG (1:0.9:0.6), HSPC: Chol: DSPG: mPEG 5000 (1:0.9:0.6:0.13) or mPEG 2000 (1:0.9:0.6:0.13) and HSPC: Chol: DOTAP: mPEG2000 (1:0.3:0.9:0.13) gave liposomal suspensions with better entrapment efficiency.

In all the above cases, the PC and Cholesterol content were also analyzed and the ratio of PC: Chol in the liposomes was almost the same as taken initially for the drying down process. Phosphatidyl choline was estimated in

the liposomes using Stewart assay in conjunction with a the organic layer obtained by Bligh-Dyer two phase extraction method Phosphatidyl choline was estimated in the liposomes using Stewart and Cholesterol was estimated using the Zlatkis, Zak and Boyle method involving color development with ferric chloride and concentrated sulphuric acid.

13.6 CHARACTERIZATION OF THE LIPOSOMAL FORMULATIONS

The particle size distribution of the prepared liposomes was estimated by the laser diffraction Malvern particle size analyzer that follows Mie's theory of light scattering. Morphology and lamellarity of the liposomes prior to extrusion through polycarbonate filters were ascertained through photomicrographs of the suspensions taken using an Olympus microscope. After lyophilization, the lyophilized liposome powder was coated with gold and then kept in the sampling unit as a thin film and then the photographs were taken at various magnifications using Jeol Scanning Electron Microscope.

All the liposomal preparations were found to have a mean particle size diameter between 150-250 nm. Photomicrographs of the formulated liposomal suspensions before extrusion indicated that the liposomes prepared were spherical in shape. It was observed that the majority of the liposomes formed in each case were multilamellar. However small unilamellar vesicles (SUVs) were also seen and the presence of these vesicles is due to the sonication process used for size reduction during preparation. The scanning electron microscopy photographs of the lyophilized liposomal formulations indicated that the liposomes were spherical in nature and the particle size were in the range of 200 nm after extrusion through 0.2 μm nuclepore polycarbonate filters.

The sterically stabilized liposomes of leuprolide acetate and DNA were characterized for entrapped volume, steric stabilization and the amount of PEG incorporated in the liposomal bilayers.

Entrapped volume of the liposomes was determined by measuring the water content of a tight pellet of the prepared liposomes. Pelletization was achieved by centrifugation at an appropriate g'value.

Steric stabilization of the liposomes was studied by subjecting them to the electrolyte induced flocculation test. The electrolyte induced flocculation test was carried out by using different concentrations of sodium sulphate solutions. The liposomal formulations were mixed with the electrolyte solutions and the resulting suspensions were measured at 400 nm to check the extent of steric barrier present around the liposomes.

The amount of incorporated polyethylene glycol derivatized lipid present in the liposomes was determined by the earlier reported method using sodium nitrate picrate solution.

All the liposomal preparations, both conventional and sterically stabilized liposomes were found to have a mean particle size diameter between 150-250 nm. However, as compared to the conventional liposomes, sterically stabilized liposomes were found to exhibit a smaller mean particle size and a narrower distribution. These observations could be correlated with the surfactant properties associated with these steric stabilizing agents.

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13.7 DRUG RELEASE KINETIC STUDY

Drug release kinetic studies were performed by suspending the liposomes in suitable medium at controlled conditions of temperature (37°C) and agitation. At a predetermined time point aliquot of suspension was removed, replaced with the same amount of medium, centrifuged and the drug

present in the supernatant was determined. The data obtained was analysed using the equation of Eyring.

$f = f_l (1-e^{-k_l t}) + f_f (1-e^{-k_f t})$

where

f = cumulative amount of drug released at time t normalized to the total amount in the preparation at time = 0.

f_l = fraction of drug entrapped in liposomes at time = 0.

f_f = fraction of drug free in the preparation at time = 0.

k_l = rate constant for drug release by diffusion, from liposomes.

k_f = rate constant for free drug release by diffusion.

t = time

$f_f + f_l = 1$.

The raw data was plotted on semi log paper and the curve was subjected to curve stripping to get initial estimates of the unknown variables f_l, f_f, k_l and k_f. These initial estimates were then taken for non linear regression analysis to obtain the final regressed values.

All the above liposomes showed biphasic release pattern –a relatively more rapid initial phase extended over a few hours, followed by a relatively slower phase. A faster release profile was obtained for leuprolide acetate and DNA containing liposomes. In case of CsA containing liposomes, the release was slower compared to other two drugs, leuprolide acetate and DNA. The second phase of release extended over to a time even up to several days. Between the conventional and the sterically stabilized liposomes containing leuprolide acetate and DNA, conventional liposomes exhibited a slower rate of release as against that of the sterically stabilized liposomes. Being water-soluble drugs, leuprolide acetate and DNA localizes in the aqueous compartments of the liposomes. Introduction of steric stabilizing agents tends to increase the bilayer hydrophilicity making it more permeable to leuprolide acetate and DNA accounting for the faster release of the drug from these types of liposomes.

13.8 STABILITY STUDY OF THE PREPARED LIPOSOMES

Pharmaceutically acceptable liposome formulation should retain encapsulated drug for long periods, so that it can be stored in a form ready

for injection. Lyophilization has a great potential as a method to solve long-term stability problems of liposomes. The use of cryoprotectants during lyophilisation of liposomes was evaluated and compared. The liposomal suspensions were freeze dried along with the sucrose, lactose or trehalose (gm of cryoprotectant/gm of lipid) overnight at -20°C, lyophilized for 24 h. The lyophilized liposomal products were also tested for its stability at two different temperatures viz. refrigerator (2-8°C) and room temperature (30 ± 2°C). At predetermined intervals of time, samples were removed; rehydrated using suitable medium and studied for parameters such as change in particle size distribution, steric stabilization and percent drug retention.

13.8.1 CYCLOSPORINE LIPOSOMES

A mass ratio of sucrose and lactose (2.75 times of lipid) was found to be the most effective. Sucrose gave more than 90% of drug retention. Lactose is not particularly effective like sucrose, which retains only 80% of drug at 1:2.75 mass ratio. The freeze-dried powder was stable for a period of 6 months and the size of the liposomes was maintained. The process of lyophilisation-rehydration did not significantly alter the entrapment levels of CsA indicating that 100 % of the entrapped drug remains associated with liposomal membranes.

13.8.2 LEUPROLIDE ACETATE LIPOSOMES

It was found that sucrose and trehalose proved to be better cryoprotectants providing maximum retention of the drug in the liposomes. The liposomal suspensions were relatively more stable at 2-8°C in comparison to liposomal suspension kept at RT. The lyophilized liposomal products were tested for its stability at two different temperatures viz. refrigerator (2-8°C) and room temperature (30°±2°C / 60±5% R.H) stored for a period of 6 months. At predetermined time points, samples were removed; rehydrated using PBS and evaluated for the particle size distribution, steric stabilization and percent drug retained. The particle size of the rehydrated lyophilised liposomes was not altered much after storing at different temperatures over a period of 6 months and showed no significant reduction in the percentage drug retained after rehydration of the lyophilised liposomes and proved to be highly stable during storage. The steric stabilizing property of the

liposomes was also preserved in the lyophilised form was proved from the results obtained from the electrolyte induced flocculation tests.

13.8.3 DNA LIPOSOMES

The lyophilized liposomal products were also tested for its stability at two different temperatures viz. refrigerator (2-8°C) and room temperature (30°±2°C / 60±5% R.H) stored for a period of 6 months. At predetermined time points, samples were removed; rehydrated using DNA aqueous solution and evaluated for the particle size distribution, steric stabilization and percent entrapment efficiency. The particle size of the rehydrated lyophilised liposomes was not altered much after storing at different temperatures over a period of 6 months and showed no significant reduction in the percentage drug retained after rehydration of the lyophilised liposomes and proved to be highly stable during storage. The steric stabilizing property of the liposomes was also preserved in the lyophilised form was proved from the results obtained from the electrolyte induced flocculation tests.

13.9 *IN VITRO* EVALUATION OF CSA LIPOSOMAL SUSPENSIONS ON MOUSE SPLENOCYTES

Splenocytes ($1X10^6$ cells/ml) were either incubated in PD-35 petri plates containing 2ml of RPMI-1640 medium supplemented with 10% fetal calf serum, 4mm L-glutamine, 50 units/ml penicillin and 50µg/ml streptomycin at 37°C in a 5% CO_2 and 95% air-humidified incubator for several periods (6, 12 and 24h) with different concentration of CsA or cultured for 24h with different doses of liposomes encapsulated CsA. After 24hr incubation cells were washed twice, resuspended in PBS. Untreated splenocytes incubated for the same periods served as controls. Analysis of the nuclear DNA content as well as CD4 and CD8 status were carried out by flow cytometry according to a method reported previously. Experiments were carried out with either freshly isolated cells or cells cultured for 24h. Flow cytometric measurements were made using a FACS Calibur flow cytometer (Becton-Dickinson, USA) having a 488nm laser excitation. Data from 10,000 cells were was acquired using the "Cell Quest" software and analyzed using the Mod-fit program supplied by the manufacturer. For the analysis of CD4 and

CD8, approximately 0.5-1 x 10^6 cells were incubated with 200µl rabbit anti-mouse CD4 or CD8 antibody (Pharmingen, USA) for 30 minutes at 4°C and washed twice with PBS. The pellet was dislodged with PBS and incubated with the 200µl of FITC-labeled anti-rabbit IgG. The green fluorescence was measured on a FACS- Calibur flow cytometer (Becton-Dickinson, USA) using the Cell Quest software as described earlier. Analysis of CD4 and CD8 positive cells was carried out with appropriate off-line gating.

Untreated splenocytes cultured for the same periods also displayed apoptotic characteristics, suggesting that the untreated splenocytes underwent spontaneous apoptosis. However, the fraction of apoptotic nuclei was significantly lower than that of the treated splenocytes. The induction of apoptosis by CsA was found to be time and CsA concentration dependent. Since changes in the concentration of drug could alter the kinetics of cell death induced by drug, we studied the kinetics of apoptosis at different drug concentration. The DNA analysis showed that the percentage of apoptotic nuclei (hypo diploid) in the CsA treated splenocytes increased with the incubation time (6, 12 and 24h) and CsA dose. CPL induced a marginally higher level of apoptosis at 1.0µM as compared to free CsA, whereas CNL and CL exhibited similar as free CsA at both these concentrations, suggesting the efficiency of liposome mediated drug delivery containing CsA.

Incubation of splenocytes obtained from the four weeks old mice with 1.0µM of free CsA and its liposomal preparation induced a decrease in the levels of both CD4 and CD8 subsets. The fraction of CD4+ cells decreased from 42% to 25%, while the CD8+ cells dropped from 36% to 27%. However, the ratio of CD4 to CD8 cells did not significantly change under these conditions. Significant differences could not be observed between the effects of liposomal preparation and the free CsA on CD4 as well as CD8 cells.

13.10 *IN VITRO* CYTOTOXICITY STUDIES OF LEUPROLIDE ACETATE LIPOSOMES

The *in vitro* cytotoxicity of leuprolide acetate and its liposomal formulations were evaluated by using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay. MTT cell proliferation assay offers a quantitative, convenient method for evaluating a cell population's response

to external factors, whether it is an increase in cell growth, no effect, or a decrease in growth due to necrosis or apoptosis. The yellow tetrazolium salt (MTT) is reduced in metabolically active cells to form insoluble purple formazan crystals, which are solubilized by the addition of a detergent. The color can then be quantified by spectrophotometric means. For each cell type a linear relationship between cell number and absorbance is established, enabling accurate, straightforward quantification of changes in proliferation.

BMG-1 human cerebral glioma cancer cell lines were used for the study. Free drug and liposomes containing the drug were incubated at various concentrations with a specific number of cells for a period of 48 hrs at 37°C in a humidified incubator (5 % CO_2 and 95 % air). The result of the MTT assay showed that there was a concentration dependant cytotoxic effect with the leuprolide acetate (0.001μM, 0.01μM, 0.1μM, 1μM, 10μM and 100μM) used. The cytotoxicity was evaluated at three time points (0, 24 and 48h). The cytotoxicity effect of leuprolide acetate was found (78 to 95% of cell viability) after 24h, but appreciable amount of cytotoxicity was found after 48h of incubation. After 48h of incubation, there was a reduction of almost 50% of cell viability at a concentration of 100μM of leuprolide acetate. Hence, evaluation of the cytotoxic potential of the liposome encapsulated leuprolide acetate was carried out after 48h. The liposomes containing leuprolide acetate also showed similar cytotoxicity effect as that of the free drug after a period of 48 hrs.

The cytotoxicity produced by the leuprolide acetate and by its liposomes was almost similar and no significant difference was found between them. The reason for the similar cytotoxicity effect may be due to the drug release from the liposomes when incubated at 37°C.

13.11 *IN VIVO* BIODISTRIBUTION STUDIES BY RADIOLABELING TECHNIQUE

The *in vivo* behaviour of the liposomes containing CsA, leuprolide acetate and DNA was studied by carrying out biodistribution studies in balb/c mice and blood kinetic studies in New Zealand rabbits by radiolabeling technique. Liposomes were labeled with high efficiency by the direct labeling

technique using reduced ^{99m}Tc with a slight modification of the earlier method. The pertechnetate used for the study was first reduced to its lower valency state using stannous chloride dihydrate and then pH was adjusted to neutral before mixing with the drug/liposome suspension. The radiolabeling was optimized by taking three factors into account. i.e. pH of the complex, stannous chloride dihydrate concentration and incubation time. The optimum conditions required for maximum labeling efficiency of all the liposomal formulations were established.

Stability of the labeled complex with time was studied in saline and in serum (rabbit) at 37°C. Even after a period of 24 h incubation the presence of > 90 % labeled compound and only 1-10% decrease of the labeled product signifies not only the high stability of the radio labeled product but also its suitability for in vivo use. High binding affinity of the ^{99m}Tc-labeled drug and its liposomes was ascertained by incubating the tagged compound with DTPA at different molar ratios. DTPA at a molar concentration of 25 mmol decreased labeling by only 1-3 %. However, when DTPA was increased to 100mmol, the labeling % was reduced by about less than 10%. The observation could be appreciated due to higher strength and binding affinity of ^{99m}Tc with all three drugs and their liposomal formulations.

Blood kinetics data of liposomal formulations were studied in healthy New Zealand rabbits at different time intervals after intravenous administration of the labeled complex through the ear vein. Biodistribution studies were carried out for the free drugs (CsA, leuprolide acetate and DNA) and their liposomes in 2-3 months old balb/c mice (weighing about 25-30 gm). Biodistribution studies of leuprolide acetate and its liposomes were carried out in 2-3 months old ehrlich ascites tumour bearing balb/c mice (weighing about 25-30 gm). The mice were sacrificed at different time intervals and blood was obtained by cardiac puncture. The blood was weighed and the radioactivity present in the whole blood was calculated by keeping 7.3 % of the body weight as total blood weight. Subsequently, tissues (heart, lung, liver, spleen, kidney, stomach, intestine and bone or tumor) were dissected, washed with normal saline, made free from adhering tissues, weighed and their radioactivity was measured in a shielded well gamma scintillation counter.

Scintigraphic studies were carried out in healthy New Zealand rabbits at different time intervals after intravenous administration of the labeled complex through the ear vein. The rabbits were fixed on a board and imaging was performed using a Single Photon Emission Computerized Tomography (SPECT) gamma camera.

Scintigraphic studies were carried out in EAT bearing mice after administration of fixed amount of ^{99m}Tc labeled leuprolide acetate/liposome complexes through the tail vein. The mice were fixed on a board and imaging was performed using a Single Photon Emission Computerized Tomography (SPECT) gamma camera.

The results of ^{99m}Tc-cyclosporine and ^{99m}Tc-cyclosporine liposomes proved that the free drug was eliminated from the blood very quickly whereas the conventional liposomes showed increased circulation time compared to free drug. The blood clearance studies of ^{99m}Tc-cyclosporine and ^{99m}Tc-cyclosporine liposomes conducted in rabbits showed that the half-life of the drug, when entrapped in liposomes was greater than the drug in its free state. Incorporation of positive charge in the lipid bilayer shows a very significant increase in the blood residence time compared to the free drug ($P<0.05$). CPL and CL shows a very significant increase in the blood residence time when compared to CNL ($P<0.05$). The gamma scintigraphic study of charged liposomes showed very clearly the increased accumulation in liver, spleen and bone marrow. No significant activity was observed in kidney, thyroid and stomach. Results of the *in vivo* biodistribution studies indicated that these liposomes can be used for targeting of the drug to liver, spleen and bone marrow and gamma imaging also confirms that an appreciable amount of these labeled complexes goes to bone marrow with higher targeting seen for the positive liposomes compared to the neutral and negative liposomes, implying the possibility of using these formulations for liver and bone marrow transplantation. The residence time of positive liposomes in blood at 24 h was twofold greater than negative and neutral liposomes, which are in agreement with the previous reports. Through out the period of experimentation, it was found that the distribution of charged cyclosporine liposomes to liver, spleen, kidney, bone and blood varied extremely significantly ($P < 0.001$) when compared to the free drug. Analysis

of variance was carried out to compare the three liposomal formulations (CL, CNL and CPL) at 4 h post injection at a significance level of $\alpha = 0.05$ and it was found that the difference between the three liposomal formulations was extremely significant in blood (F=518.9), liver (F=44.44), spleen (F=361.88), kidney (F=76.27) and bone (F=4283.34).

The results of ^{99m}Tc-leuprolide acetate and ^{99m}Tc-leuprolide acetate liposomes proved that the blood clearance studies of free drug and liposome entrapped drug conducted in rabbits shows that the half-life of the drug, when entrapped in liposomes was greater than the drug in its free state. Incorporation of 6 mol% of mPEG in the lipid bilayer markedly increase the blood residence time, as evidenced by the threefold and fourfold higher level at 24 h post injection of SLL5000 and SLL2000 in comparison to LL. The sterically stabilized liposomes prepared using polyethylene glycol of mol wt.2000 showed enhanced circulation compared to the liposomes prepared using polyethylene glycol of mol wt 5000, which showed that the polyethylene glycol of low mol wt provides equally, or little higher resistance to opsonization and also provides prolonged blood circulation. Results of the *in vivo* biodistribution studies indicated that the polymers used produced steric stabilization of the liposomes. Free drugs were found to be cleared from blood quite rapidly. Conventional liposome also cleared from the blood stream quickly when compared to the sterically stabilized liposomes. The sterically stabilized liposomes showed enhanced blood circulation and also increase in accumulation in tumour. There was around 65 times increase in tumour accumulation of drug from LL compared to leuprolide acetate and around 200-225 times increase in tumour drug concentration from SLL5000/SLL2000 compared to leuprolide acetate. When compared to sterically stabilized liposomes, conventional liposomes showed extremely significant increase in accumulation in liver and spleen. It could be accounted for as the combined activity of the circulating blood passing through the organs as well as that due to particle uptake by cells of reticuloendothelial system (RES). The presence of very low amount of radioactivity in stomach after 24 h proved the in vivo stability of the radiolabeled complexes. The conventional liposomes showed increased liver and spleen accumulation with less tumour accumulation in comparison

with the sterically stabilized liposomes because of increase in opsonization. The increase in circulation time may be due to the smaller particle size (<200 nm) of the liposomes or due to the surface modification made by polyethylene glycol. The gamma scintigraphic study of conventional and sterically stabilized liposomes showed very clearly the accumulation of labeled complexes in various organs including liver, spleen and tumour present in the mouse.

The liver/tumor uptake ratio of free drug, conventional and sterically stabilized liposomes (SLL5000 and SLL2000) was found to be 20, 7.99, 1.63 and 1.23 respectively, which showed the increased accumulation of sterically stabilized liposomes in tumor compared to the free drug and conventional liposomes at 24 h post injection. Liver uptake of sterically stabilized liposomes was still 7 fold less than the conventional liposomes. We were able to obtain very low liver to tumor ratio in the range of 1 to 1.6 for PEG coated liposomes in comparison to 7.9 for conventional liposomes.

The results of ^{99m}Tc-DNA and ^{99m}Tc-DNA liposomes proved that the blood clearance studies of free drug and liposome entrapped drug conducted in rabbits shows that the half-life of the drug, when entrapped in liposomes was greater than the drug in its free state. Incorporation of 5 mol% of mPEG in the lipid bilayer markedly increased the blood residence time SDL5000, SDL2000 and CSDL2000 in comparison to DL. The enhanced blood circulation of the liposomal formulations containing DNA may be attributed to the increase in bilayer rigidity by incorporation of high melting lipids and incorporation of PEG with lipids.

The biodistribution of ^{99m}Tc-DNA after 15 min, 30 min, 1 h and 4 h of intravenous injection in Balb/c mice was studied. Blood was obtained by cardiac puncture, weighed and the radioactivity present in the whole blood was calculated by keeping 7.3 % of the body weight as total blood weight. The percent-injected dose/ gm and dose/whole organ in various organs of mice at different times after i.v administration of ^{99m}Tc-DL, ^{99m}Tc-SDL5000, ^{99m}Tc-SDL2000 and ^{99m}Tc-CSDL2000 after 1h, 4 h, 24 h and 48 h of intravenous injection in Balb/c mice were studied. Various organs / tissues like lung,. liver, spleen, kidney, stomach, intestine and heart were removed and analyzed for labeling content. In case of ^{99m}Tc-DNA, the radioactivity

present in whole organ, 4 h of post injection were found to be as follows: blood (0.98 %), liver (2.66 %), spleen (0.20%) and kidney (0.59%). DNA was found to be cleared from blood quite rapidly. Conventional liposome also cleared from the blood stream quickly when compared to the sterically stabilized liposomes.

In case of conventional liposomes (DL), liver and spleen accumulated a major portion of the administered radioactivity. This type of distribution is normally found with the non sterically stabilized liposomes. Thus conventional liposomes of DNA behaved as expected *in vivo*. There was an inverse relationship between liposome clearance by the reticuloendothelial system (RES) and a prolonged circulation time of liposomes. The biodistribution data reveals that initial rapid uptake by liver, 23.4 %, 10.8%, 9.48 and 9.49% at 1h and 16.6%, 9.26%, 8.09% and 8.9% at 4 h post injection for DL, SDL5000, SDL2000 and CSDL2000 respectively. The spleen accumulates significant radioactivity at 1 h and 4 h post injection for DL, SDL5000, SDL2000 and CSDL2000 respectively. The distribution of the sterically stabilized liposomes to liver and spleen was considerably reduced as compared to the conventional liposomes. This indicates that steric stabilization of liposomes containing DNA successfully alters the distribution pattern as compared to the conventional liposomes. Hardly any activity appeared to clear via intestine. The constancy of radioactivity in stomach also pointed to the fact that there is no in vivo leaching of radioactivity as free Technetium. The radioactivity in whole blood was found to be 2.89 %, 10.71 %, 16.56% and 18.41% at 24 h and 1.39%, 4.42%, 5.89% and 7.74% at 48 h of post injection for DL, SDL5000, SDL2000 and CSDL2000 respectively, which proves the enhanced circulation of sterically stabilized liposomes in comparison to the conventional liposomes. Through out the period of experimentation, it was found that the distribution of DNA liposomes to liver, spleen, and blood varied extremely significantly ($P < 0.001$) when compared to the free DNA. Analysis of variance was carried out to compare the four liposomal formulations (DL, SDL5000, SDL2000 and CSDL2000) at 4h and 24 h post injection at a significance level of $\alpha = 0.05$ and it was found that the difference between the four liposomal formulations was extremely significant in blood (F=7703.7 and 40364), liver

(F=7016.6 and 1012.8) and spleen (F=1156.0 and 199.24). The residence time of sterically stabilized liposomes in blood at 24 h was five to nine folds greater than conventional liposomes. The lipid composition, the addition of sterically stabilizing agents (mPEG5000-CC-PE and mPEG2000-CC-PE), and the particle size of the liposomes, which was maintained around 200nm, played a major role in prolonging the blood circulation in case of sterically stabilized liposomes. It has been hypothesized that the decreased uptake of sterically stabilized liposomes by MPS was possibly due to the presence of steric barrier, which decreases the adsorption of plasma proteins (opsonins) on the surface of the liposomes. The liposomes prepared using polyethylene glycol derivative of low molecular weight (mPEG2000-CC-PE) showed higher blood concentration compared to liposomes prepared using polyethylene glycol derivatives of higher molecular weight (mPEG5000-CC-PE), which showed that the polyethylene glycol of low mol wt provides higher resistance to opsonization and also provides prolonged blood circulation. It was quite evident that the ratio of RES: Blood for sterically stabilized liposomes were significantly low compared to free DNA and DL. Hence these findings indicate when compared with free DNA, PEG coated liposomes, particularly showed a significant increase in circulation half-life in addition to low uptake by RES. Thus the avoidance of RES and prolonged circulation time of sterically stabilized liposomes encapsulating DNA is confirmed by the biodistribution and kinetic studies.

The above observations of long circulation have been corroborated by analyzing the gamma images of rabbit after 4 h post injection with radio labeled complexes. Images are shown for rabbit at 1h and 4h post injection of free DNA and its liposomes (DL, SDL5000, SDL2000 and CSDL2000) respectively in figures 11.18 –11.22. There is an increasing accumulation in liver, spleen and a clear observation of the entire blood pool. We confirm our studies that in general, the presence of a PEG along with the charged lipids induces definite changes in the organ distribution of liposomes and prolonged circulation.

13.12 NEPHROTOXICITY STUDIES OF THE CYCLOSPORINE LIPOSOMAL SUSPENSIONS

Nephrotoxicity studies were conducted in rats to find out the comparison between the toxicity profile of CsA and the prepared liposomal suspensions. The serum parameters like urea, uric acid and creatinine were estimated and histopathology of kidney was studied. Administration of cyclosporine resulted in a significant ($p<0.001$) elevation in serum creatinine, urea, uric acid and BUN levels, the markers of renal injury, as compared to control group. The administration of liposomal formulations (CPL, CL, CNL) showed significant decrease in these levels. The nephrotoxicity of cyclosporine, characterized by the elevation of serum creatinine, urea, uric acid and BUN, was also reversed to a significant extent by the liposomal formulations containing cyclosporine (CPL, CL, and CNL).

13.13 CONCLUSIONS

Liposomes have considerable potential as drug carriers for peptide drugs and DNA to prolong the biological half-life and to reduce the injection frequency and toxicity of intravenously administered peptide drugs. A systematic attempt was made to make a safe and sustainable delivery by incorporating the drugs and DNA in liposomes by modifying the liposomal surface and its surface charge for prolonged circulation in blood and targeting to specific tissues or cells. The use of liposomes as drug carriers requires the liposomal preparations with various clearance rates and biodistribution patterns to better fit the specifics of each particular application. Liposome charge and liposome coating with different polymers, such as PEG, are among the parameters known to strongly affect biological properties of liposomes. It was repeatedly demonstrated that the incorporation of charged phospholipids into liposomes accelerates their clearance, while grafting liposomes with PEG and similar polymers makes liposomes long circulating. Thus the incorporation of Phosphatidyl serine (PS) or dicetyl phosphate (DCP) into PC/Chol liposomes dramatically enhances enhances liposome uptake by the perfused mouse liver. The fact that the negative charge strongly increases the clearance of liposomes. Negatively charged PS was found to abolish the longevity of liposomes

prepared of a lipid composition resembling that of erythrocyte membrane. The major mechanism behind the charge facilitated liposome clearance is an interaction of charged phospholipids head groups with certain opsonizing proteins. Liposomes of different charge and composition exhibit different binding centers for plasma proteins. Liposome grafted PEG prevents liposome clearance by neutralizing the surface charge of liposomes and shielding various opsonins. Hence our aim was to compare the liposome biodistribution with negative or positive surface charge additionally coated with a PEG moiety with different molecular weight, in order to investigate the relative role of the liposome charge and the length of the liposome attached PEG chains on the liposome circulation time and liver accumulation.

The use of CsA has been limited due to its nephrotoxicity. Other side effects associated with the intravenous delivery of CsA are due to the intravenous carrier vehicle, Cremophor-EL. Cremophor-EL is a polyoxyethylated castor oil that is one of the best ionic surfactants used to dissolve the lipophilic drugs. It is also believed that part of the nephrotoxicity associated with CsA treatment may be enhanced by Cremophor-EL deposition and crystal formation within the kidney tubules. Efforts have been made to eliminate the toxicity of CsA by incorporating the drug into liposomes.

Leuprolide acetate a potent GnRH analogue utilized clinically for the treatment of prostate ovarian and breast cancers. A liposomal delivery system that prolongs the biological half-life of the drug, prolongs the exposure of GnRH analogue into target cells (such as malignant cells) would be advantageous for the delivery of leuprolide acetate. DNA was rapidly cleared from the circulation due to its short biological half-life. It was hypothesized that the addition of mPEG-PE helps to prolong the circulation of the liposome in the blood by shielding the positive charge and by providing a more hydrophilic surface. This combination of sustained and targeted delivery would help to reduce side effects of these drugs and would also lead to a significantly lower dose being required for achieving therapeutic efficacy. The sterically stabilized liposomes were prepared using agents like methoxypolyethylene glycol (mol wt.5000) activated with cyanuric chloride coupled with phosphatidyl ethanolamine (mPEG5000-CC-

PE) and methoxypolyethylene glycol (mol wt.2000) activated with cyanuric chloride coupled with phosphatidyl ethanolamine conjugate (mPEG2000-CC-PE). The concentration of these agents, which provide steric stabilization, was optimized using the electrolyte induced flocculation test. 5-6 mol % of the total lipids of both mPEG5000-CC-PE and mPEG2000-CC-PE were found to be adequate for steric stabilization of the liposomes.

Drug release kinetics from the liposomes was studied under controlled conditions of temperature and stirring, and the data analysis was done using the equation of Eyring. It was observed that drug release from the liposomes was of biphasic nature with an initial, rapid phase followed by a subsequent slow release phase. Location of the drug within the liposomes and the solubility characteristics of the drugs were shown to play important role in the rate of drug release. Stability studies of the formulated liposomes were found to be quite stable for long time at both refrigeration and room temperatures. The induction of apoptosis on mouse splenocytes confirms that the CsA encapsulated in liposomes showed similar apoptotic effects *in vitro* on the mouse splenocytes when compared to that of the free drug. The MTT assay corroborates that the leuprolide acetate encapsulated in liposomes showed similar cytotoxic effects on the *in vitro* BMG-1 cell lines when compared to that of the free drug.

Radiolabeling of the three drugs and their liposomes were carried out successfully with high labeling efficiency. The radiolabeled complexes were found to be highly stable in both *in vitro* and *in vivo* conditions. In general, the biodistribution studies revealed that the presence of surface charge induces definite changes in the organ distribution of liposomes. Hence the results indicate the utility of this novel cyclosporine intercalated charged liposomal systems for liver and bone marrow targeting and thereby reducing the accumulation of drug in kidney and the possibility of nephrotoxicity generally seen in free cyclosporine. The biodistribution studies of conventional and sterically stabilized liposomes containing leuprolide acetate and DNA revealed that sterically stabilized liposomes led to increased residence times in blood with significant reduction in the distribution of the drugs to the organs of the mononuclear phagocyte system such as the liver and the spleen. The sterically stabilized liposomes

also offered increased drug accumulation in tumor compared to conventional liposomes containing drug and the free drug. The tumor accumulation of the sterically stabilized liposomes was also proved by γ imaging studies.

Thus the study presents some new findings, which may be exploited in improving the therapeutic efficacy of cyclosporine and leuprolide acetate using liposomes as delivery systems. However these improvements are still not enough to obtain a liposomal peptide and DNA delivery system, which remains circulating for an extended period of time. Extensive clinical trials have to be performed to establish the efficacy and safety of the formulated liposomes in clinical practice. In future experiments, this new liposome containing peptides and DNA need to be investigated further to improve the drug retention, for the attachment of targeting ligands to allow specific drug delivery to target cells.