Chapter – 10 Summary and Conclusion



SUMMARY

Lung cancer is the third most common cause of death after heart disease and pneumonia globally resulting in high mortality rates in cancer patients. Though conventional means of therapy such as radiation, surgery and chemotherapy are available, these prevalent therapeutic measures suffer from poor site specificity and selectivity. Failure of chemotherapeutic agents to differentiate among cancer and non cancer, healthy host cells predominantly restrains the success of conventional chemotherapy. Consequently, they unleash severe toxicities in patients undergoing cancer chemotherapy leading to poor quality of life in cancer patients. Since, last few decades, efforts have been concentrated in direction of using lung as a target organ for the treatment.

Designing colloidal vesicular drug delivery systems such as liposomes with sizes lesser than tumour size can be easily taken up and retained by tumours by virtue of Enhanced permeability and Retention (EPR) phenomenon and enhanced cellular uptake of cytotoxic drug via receptor mediated mechanisms. Targeting at the cellular level is highly desirable in tumour chemotherapy where therapeutic indices are insignificant with conventional therapy. Success seems to rely on some tumour cell associated receptors that are over expressed on the tumour cells and either downregulated or not expressed at all in normal, non cancerous healthy host cells. Ligand can be grafted onto the external surface of liposomal systems to render them site specific by directing ligand grafted liposomes to cancer cells over expressing such receptors. Efforts are on to investigate and develop the ligands that can be directed towards such over expressed receptors and thereby achieve a highly target specific drug delivery. Attachment of ligand on liposomal surface will coax the liposomal delivery system only to tumours and not the adjacent healthy, non cancerous tissues.

CD44 is generally found in low levels in epithelial, haemopoeitic and neuronal cells, however, their levels are very high in breast, colorectal and lung cancers. These cell surface receptors exhibit inherent high affinity towards hyaluronic acid.

Hyaluronic acid is a linear polysaccharide comprising of alternatively arranged D-glucoronic acid and N-acetyl glucosamine. Hyaluronic acid has got diverse physiological functions in body ranging from their effects on cell proliferation to triggering of inflammatory response.

Hyaluronic acid (HA) is a major component of extracellular fluid and CD44 has an important role in metabolism of dissolved HA. CD44 regulates lymphocyte adhesion to cells of high endothelial venules during lymphocytic migration as well as metastatic dissemination of solid tumours. CD44 role has been elucidated in proliferation of various carcinogenic and metastatic growths. HA grafted liposomes incorporated with bio active can excellently target the lung cancer.

Liposomes are used as carriers for drugs and antigens. Liposomes can prolong the duration of drug exposure, acting as a slow-release reservoir. This has been demonstrated in a number of studies. Liposomes can protect a drug against degradation (for example metabolic degradation). Conversely, liposomes can protect the patient against side effects of the encapsulated drug. Liposomes can be used to deliver biological agents either entrapped within the internal aqueous compartments, reconstituted in the lipid bilayer, or attached to the outer surface. Liposomes are artificial lipid vesicles composed of concentric lipid bilayers that alternate with aqueous compartments. They have permeability properties similar to those of biological membranes. The liposomal encapsulation has been shown to reduce the entry of the agent into the systemic circulation, compared with free drug and provide distribution throughout the airspace of the lung. One of the major advantages of liposomes over other carrier delivery systems of drugs is that they can be prepared from materials for which there is considerable data available regarding their fate *in vivo*.

One of the perceived benefits of liposomes as a drug carrier is based on their ability to alter favorably the pharmacokinetic profile of the encapsulated species and thus provide selective and prolonged pharmacological effects at these sites of administration. The resulting decrease in the frequency of drug dosing will significantly improve the quality of life for patients and at the same time reduce healthcare cost. The selective and controlled release of the drug is also expected to reduce or eliminate hypersensitivity and systemic toxicities. The challenging aspect still remains unanswered are the mode of delivery for liposomally encapsulated drug. Metered dose inhalers (MDI) are currently being reformulated as a result of the ban being implemented throughout the world by the United Nations on the use of chlorofluorocarbons (CFCs) to meet this challenge, one such alternative is the development of new and improved "Dry Powder Inhaler (DPI) system that will allow inhalation administration of all drugs presently delivered with MDIs. With constrain of propellant phase out and short-term stability of liposomal aqueous dispersion the most viable alternative would be to deliver the liposomally encapsulated drug in dry form.

Pharmaceutical aerosols, 1-5 µm once deposited may be removed by macrophage action before the dose is delivered, thereby reducing the bioavailability of the drug. It was hypothesized that ligand grafted liposomal ETP and DOC will show selective uptake in lung cancer cells along with controlling the release rate of the drug for longer duration at site of action and is expected to reduce systemic side effects and frequency of dosing. Hence, this investigation was focused on the pharmaceutical development of HA grafted liposomes of ETP and DOC followed by their evaluation in A549 cell lines for the former's effect on cell uptake, cytotoxicity and cell cycle. Optimized HA grafted drugs loaded liposomes were ultimately converted to DPIs followed by their evaluation, optimization and in vitro lung deposition.

Analytical Methods

The drug content in dosage form and in diffusion medium was analyzed by the reported analytical method with suitable modification whenever necessary to meet the requirement of this investigation.

ETP and DOC in solution, liposomes, in vitro drug release medium and liposomal DPIs were estimated by UV spectrophotometric method, Cell lysates, diffusion medium and other biological fluids by HPLC and BCA protein method.

Calibration curve was established for ETP and DOC which was observed to be linear in the concentration range of 5 to 120 μ g/ml for UV spectrophotometry and 50-800 ng/ml for HPLC. The drug concentration in diffusion media was estimated by withdrawing the aliquots of diffusion medium (PBS, pH=7.4 with 0.1% Polysorbate 80) and estimated by UV Spectrophotometric technique.

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The recovery of ETP from biological samples was evaluated at low, medium and high concentrations of 40, 200 and 800 ng/ml. The extraction efficiency was more than 95% in all the cases and calibration curves were linear in concentration range of 40-200 ng/ml.

Calibration curve was established for BCA protein assay and was found to be linear in concentration range of 10 to 1,000 μ g/ml with regression coefficient of 0.9851.

The surface density of HA grafted on liposomal surface can be determined by modified uronic acid carbazole reaction of Bitter and Muir based on DISCHE's carbazole reaction.

Preparation and Characterization of Liposomes

Multilamellar liposomes were prepared by TFH for both of the drugs. A full factorial design based mathematical approach was adopted for optimization of process variables. Percentage drug entrapment was chosen as a dependent variable and drug:lipid ratio, speed of rotation and solvent ratio were assigned independent variables. Prepared liposomes were sonicated to reduce their particle size suitable for inhalation delivery. The optimizations of various process variables like Vacuum (600 mm of Hg), hydration time (60 min), speed of rotation (100 rpm), no. of sonication cycles (3 Cycles of 80% amp, 0.5cycle), annealing time (60 min) and separation of unentrapped drug and formulation variables like composition of solvent system [chloroform: methanol (2:1), volume of hydration medium (4 ml) The optimum lipid composition for ETP was found to be 1:10 drug : lipid ratio (8:1:1 of HSPC: DPPE: Cholesterol) and 1: 15 (9:2:1:: HSPC: DPPE:Chol) Optimum speed of rotation was found to be 100 rpm for film drying and hydration. Liposomes of above composition showed good particle size, Polydispersity indices, zeta potential and percentage drug entrapment. Hence, the liposomes of above composition were considered to be optimized ones and subjected to further investigations.

Preparation and characterization of HA grafted drugs loaded liposomes

Hyaluronic acid (HA) was selected as a ligand to target CD44 receptors over expressed in lung cancers and hence, targeted drug loaded liposomes were prepared by grafting of HA to surface of Etoposide and Docetaxel loaded liposomes. HA was grafted to drug(s) loaded liposomes in different HA: drug loaded liposome weight ratios by carbodiimide coupling technique using EDC as a coupling agent in different amounts for different incubation periods. Carboxylic acid functional groups in HA were covalently bonded to free amine group of phosphatidylethanolamine of lipid component of liposomes resulting in formation of a stable amide bond. Success of HA grafting to liposomal surface was assessed and confirmed by correlating surface density of HA to zeta potential. HA grafting to drug loaded liposomal surface was optimized for different weight ratios of HA: liposomes and incubation time to determine the optimum surface density of HA to render drug(s) loaded liposomes site specific. 10:100 and 12: 100 HA:liposome weight ratios were found to be optimum for Etoposide loaded and Docetaxel loaded liposomes respectively. The amount of EDC required to achieve optimum surface HA density was found to be 10 µg in case of HAETPLIP and HADOCLIP. It was found that increase in HA amount beyond optimized HA:liposomes weight ratio resulted in significant rise in particle size of HAETPLIP and HADOCLIP as all HA binding sites on liposomal surface would be saturated with HA resulting in aggregation of excess HA.

FTIR analysis of HA non grafted and grafted drug loaded liposomes indicated characteristic amide peaks at 1690 cm⁻¹ in case of HAETPLIP and 1660 cm⁻¹ in case of HADOCLIP indicating formation of stable, primary amide bond indicating successful grafting of HA to surface of Etoposide and Docetaxel loaded liposomes. Optimized HA grafted liposomes of Etoposide and Docetaxel were subsequently converted to dry powder inhalers (DPIs) for local and site specific delivery to lung cancer.

The particle size of HAETPLIP and Etoposide loaded liposomes (non grafted ETPLIP) were found to be 217 ± 2.1 nm and 190 ± 3.7 nm respectively. A low polydispersity index of 0.187 and 0.109 was obtained for both the formulations indicating a narrow particle size distribution of the formed liposomes. The zeta potential of ETPLIP and HAETPLIP was observed to be $-10.7 \pm$ 0.57 mV and -20.2 \pm 0.37 mV respectively. The results are shown in Table 6.1. The zeta potential was observed to decrease on grafting of HA to liposomes owing to negative charge imparted to liposomal surface by HA.

Percentage drug entrapment was found to be 80.2 ± 3.4 % for ETPLIP and 73.1 ± 4.08 % for HAETPLIP. The reduced entrapment observed in HA grafted Etoposide liposomes (HAETPLIP) may be attributed to leaching out of some drug during HA attachment on liposomal surface.

The prepared liposomes were falling in nanometric size range with HADOCLIP liposomes of size range 235 ± 1.8 nm and Docetaxel loaded liposomes (non grafted DOCLIP) were found to be 195 ± 3.0 nm. A low polydispersity index of 0.197 and 0.210 was obtained for both the formulations indicating a narrow particle size distribution of the formed liposomes. The zeta potential of DOCLIP and HADOCLIP was observed to be -8.8 ± 1.2 mV and -16.2 ± 2.9 mV respectively. The zeta potential was observed to decrease on grafting of HA to liposomes owing to negative charge imparted to liposomal surface by HA.

Percentage entrapment of Docetaxel was found to be 70.1 ± 2.8 % for DOCLIP and 64.2 ± 3.04 % for HADOCLIP. The reduced entrapment in HA grafted Docetaxel liposomes (HADOCLIP) may be attributed to leaching out of some drug during HA attachment on liposomal surface. HA grafted and non grafted drug loaded liposomes were subjected to cell line studies in A549 cells where their effect on cell uptake, cell cytotoxicity, cell cycle and pharmacokinetic parameters was assessed. HA grafted liposomes of both the drugs were found to be superior in pharmacokinetic and biological performance as compared to non grafted liposomes. Hence, HA grafted drug loaded liposomes were further processed to Dry Powder Inhalers (DPIs) by lyophilization technique and were further assessed for solid state characterization and in vitro lung deposition.

Preparation and Characterization of Liposomal DPIs of HA grafted drugs loaded liposomes

The lyophilized powder yield was observed in between 65% and 72%. Lyophilized ETPLIP with lactose (LEDPIL), lyophilized ETPLIP with sucrose (LEDPIS) and lyophilized liposomes with mannitol (LEDPIM) were found to have VMD (Volume Mean Diameter) of 9.1 ± 1.76 , 11.43 ± 1.80 , and 5.9 ± 1.01 µm respectively, whereas LEPL was found to have VMD of 10.1 ± 2.1 µm, while lyophilized DOCLIP with lactose (LDDPIL), lyophilized DOCLIP with sucrose (LDDPIS) and lyophilized DOCLIP with mannitol (LDDPIM) were found to have VMD of 9.9 ± 0.8 µ, 10.5 ± 1.80 and 5.5 ± 1.2 µ respectively while that of lyophilized plain DOC with lactose (LDPL) was found to be 11.3 ± 2.5 µ indicating that minimum VMD was observed in case of Docetaxel DPIs prepared using mannitol which can ensure good penetration and retention in lungs as size of powder is around 5 µ in case of LDDPIM as compared to DPIs formulated using sucrose and lactose.

All the Dry Powder Inhaler formulations (DPIs) exhibited more than 95% drug retention on an average. Along with the afore discussed parameters, innovative means and techniques of developing aerodynamically light particles have been engineered and studied to facilitate drug optimum drug deposition in deeper parts of lungs. It has been observed that DPI formulations with tap density lesser than 0.4 g/cc and relatively large mean diameter falling in range of 5-30 μ m, however, possess MMAD (Mean Mass Aerodynamic diameter) of 1-5 μ m. The LEDPIM was found to have the lowest density (0.16±0.02 g/cc), good flowability (Angle of repose 25.0±1.16°, Carr's compressibility index 38.5±1.98 %), and low residual water content of 2.46±0.33. The LEDPIL was found to have density of 0.24±0.05 g/cc, angle of repose of 33.8±2.7°, Carr's compressibility index of 36.2± 2.3%, and residual water content of 5.1±0.85 and LEDPIS with tapped density of 0.38±0.14 g/cc, angle of repose of 35.1±1.9°, Carr's compressibility index of 30.9±3.1%, and residual water content of 5.44±0.86. Similarly, LDDPIM was found to have the lowest tap density of 0.20±0.04 g/cc, angle of repose of 26.1 ± 0.97 ° Carr's compressibility index of 39.1 ± 1.65 and residual water content of 2.46±0.84. LDPL was found to have tapped density of 0.81±0.2 g/cc, angle of repose of 45.6±3.1°, Carr's

compressibility index of 20.8 ± 2.2 and residual moisture content of 7.3 ± 0.98 . Liposomal DPIs developed in the investigation were reported to have low density, good flowability and low residual moisture content.

In Vitro Drug Release studies of Liposomal DPIs of ETP and DOC.

In vitro drug release study results indicated that DPIs of HA grafted liposomes of Etoposide and Docetaxel exhibited sustained release of Etoposide (97.3 \pm 1.3 % at the end of 48 h in case of LEDPIM as compared to > 93 % drug release in case of LEPL and Docetaxel (98.84 \pm 2.98 % at the end of 48 h in case of LDDPIM as compared to 97 % drug release within 3 h in case of LDPL respectively from developed liposomal DPI formulations gearing a testimonial to the fact that liposomal DPI based delivery systems can help to retain drug for longer time in tumours in contrast to rapid wash out and removal of conventional anti cancer drug preparations.

It was observed that the developed liposomal DPIs of ETP and DOC possessed volume mean diameter exceeding 5 μ m with a tap density lesser than 0.4 g/cm³ indicating the aerodynamic and light nature of DPI particles. Aerodynamic nature of these light and non massive particles renders the particles capable of escaping gravity induced and friction induced deposition in upper respiratory tract and can be targeted to deeper interiors of lungs and tumours prevailing therein.

L-Glycine was used as an anti adherent to reduce clumping of particle and thereby facilitates formation of aerodynamic, light particles with satisfactory flow properties and reduced probability of particle aggregation. The HA grafted drug(s) loaded liposomal DPIs developed in this investigation were found to exhibit tap density lesser than 0.4 g/cc, volume mean diameter exceeding 5μ and MMAD in range of 2-3 μ . This may be largely attributed to controlled particle size of liposomes and anti adherent nature of L-glycine. Non-reducing disaccharides are the most effective at protecting against drug leakage during freeze drying. Sugars that tend to crystallize more readily may not be as effective and so lactose despite of being disaccharides are not effective compared to sucrose. It was also observed that sucrose as a carrier led to highest particle size (>11 μ m) and even higher moisture content owing to its hygroscopic nature resulting in cohesiveness, poor flow and higher residual moisture content.

Stability studies

Optimized liposomal DPIs were further assessed for stability by determining drug leakage, particle size growth, *in-vitro* deposition characteristics, emission and the chemical stability of drugs at room condition ($28^{\circ}C\pm3^{\circ}C$, $60\%\pm5$ RH) and at refrigerated conditions ($2-8^{\circ}C$) up to twelve months.

Liposomal DPIs did not exhibit any significant changes in their assay values indicating the stability of drug encapsulated within the liposomal compartment. There was no significant increase in particle size of DPI formulations stored and the latter were found to be stable till 6 months however, particle size increase was observed during 12 months storage period which may be attributed to aggregation of particles. HA by virtue of its negative charge, imparts negative charge to liposomal surface and prevents particulate aggregation by electrostatic repulsion, however, at room temperature there was significant increase in particle size indicating the increased tendency of particles to aggregate at elevated temperatures and hence, it is preferable to store liposomal DPIs under refrigerating conditions.

Low residual moisture content is essential for maintaining free flow characteristics of DPIs. Increase in moisture content leads to formation of sticky and cohesive mass obstructing the penetration of formulation to deeper peripheries of lungs when used by the patient. The presence of certain sugars has been shown to enhance stability. The best evidence available suggests that there is a direct interaction between the sugar and the polar head group of the phospholipid, the result of which is a depression of the transition temperature of the lipid and its maintenance in a fluid state even in the absence of water. However, HA being hygroscopic in nature tends to absorb moisture. This was evident in case DPIs stored under room conditions (higher relative humidity) where increase in residual moisture content of DPIs was observed. The increased moisture uptake during storage period may be attributed to hygroscopic nature of HA.

Similarly, liposomal DPI formulations stored at room temperature conditions exhibited reduction in percentage drug retention. Decrease in PDR with corresponding increase in temperature may be attributed to fluidization of phospholipid membranes of liposomes. With increase in temperature or at temperatures closer to phase transition temperatures of lipid components, rigidity of lipid membranes reduces resulting in increased fluidity through which drug tends to leak out. In case of LDDPIM, there was remarkable decrease in percentage drug entrapment since Docetaxel tended to leak out from liposomal membranes to extra liposomal compartment and thus, exhibited lesser PDR as compared to that observed with LEDPIM.

No significant change in FPF % was observed in case of LEDPIM stored for 12 months at refrigerating condition; however, the one stored at room temperature exhibited significant reduction in FPF % which may be attributed to increased particle size due to aggregation and cohesion induced by moisture uptake, thus reducing the effective particle fraction falling in range of 1-5 μ m.

The DPI products did not exhibit any discolouration or change in organoleptic characters indicating fair stability of prepared DPIs, however, reduction in flow properties was observed which may be attributed to slight aggregation that occurred at higher temperature and increased cohesiveness due to moisture uptake under room temperature and humidity conditions.

On the whole, developed liposomal DPIs of Etoposide and Docetaxel were stable for 12 months when stored under refrigerating conditions on the whole and there was insignificant change in assay, PDR, residual moisture content, VMD, FPF and related parameters. Hence, one can arrive at a conclusion that in order to ensure maximum stability of liposomal DPIs, they ought to be stored under refrigerating conditions (2-8^oC).

Cell Line Studies

HA grafted, non grafted liposomes and plain solutions of ETP and DOC were assessed for their effect on cell cytotoxicity, cell uptake and cell cycle in A549 cell lines (epithelial lung carcinoma cell line derived from Caucasian adult male) Developed HA grafted and non grafted liposomal formulations of ETP and DOC were subjected to in vitro cell cytotoxicity studies by MTT assay, cell uptake studies by confocal laser scanning microscopy, cell cycle analysis by flow cytometry, assessment of intracellular pharmacokinetic parameters and establishment of mechanism for cell uptake in ex vivo experiments by incubating cells with liposomes for different time and temperature points.

In cell uptake studies by confocal laser scanning microscopy, a very little or no fluorescence in case of cells incubated with dye solution was observed indicating little or no internalization of that dye or simple drug solution. Fluorescence in cells was observed to increase with passage of time from 15 to 45 minutes indicating corresponding increase in cell uptake of non grafted and HA grafted drug loaded liposomes. Significantly higher fluorescence was associated with cells treated with liposomes and more with HA grafted liposomes than with non-grafted liposomes. Hence, one can conclude that increase in fluorescence intensity observed inside the cells was only due to higher cell uptake of drug in case of HA grafted ETP and DOC liposomes. This higher drug uptake in cells can be attributed to the fact that CD44 receptors-the target moiety is over expressed in A549 alveolar epithelial lung cancer cell line which resulted in higher cell uptake of drug and consequently, highly intense fluorescence was observed in case of cells incubated with HA grafted drug loaded liposomes.

A control experiment was performed by incubating cells with 6-coumarin solution released from liposomes in vitro for 2 hours. The intracellular fluorescence of the control was insignificant as compared to that in case of cells incubated with liposomes. Hence it can be inferred that the dye does not leak out from the liposomes during the course of experimental study.

The cytotoxicity of optimized non grafted and HA grafted liposomal formulations of ETP and DOC was assessed using A549 alveolar epithelial lung cancer cell line by MTT assay method. Reduced absorbance indicated either reduction or total aversion in conversion of MTT to formazan leading to inference that cells are dead.

Etoposide concentration in liposomes was adjusted to same as that of free drug. IC_{50} (Minimum Concentration of drug needed to inhibit 50% of viable cell population) was determined for free drug solution, non grafted liposomal formulations (ETPLIP) and HA grafted Etoposide liposomes (HAETPLIP)

Concentration of Etoposide inhibiting 50 % of experimental cell population of A549 cell lines (IC₅₀) was significantly lesser in case of HA grafted liposomes as compared to non grafted liposomes and etoposide solution. The IC₅₀ values for HAETPLIP, ETPLIP and ETPSOL were found to be 0.5 μ M, 1 μ M and 2 μ M respectively. Cytotoxicity of HA grafted Etoposide

liposomes was four times higher as compared to Etoposide drug solution (ETPSOL) in A549 cell lines. Blank liposomes (not loaded with Etoposide) did not exhibit any cytotoxicity against A549 cell line and were used as controls. Similarly, it was observed that IC_{50} in case of DOCSOL was 6 nM, while in case of DOCLIP it reduced to 4 nM and 2nM in case of HADOCLIP. There was nearly threefold increase in cytotoxicity of HA grafted Docetaxel liposomes as compared to Docetaxel drug solution (DOCSOL) in A549 cell lines.

Cellular drug levels for different formulations were estimated at different time intervals after treating A549 cells with plain drug, non grafted and HA grafted liposomes for 24 hours at 37^{9} C. The peak intracellular concentration of Etoposide was found to be 0.311 µg/mg and 0.397 µg/mg in case of ETPLIP and HAETPLIP respectively, which were significantly higher than those achieved with ETPSOL (0.288 µg/mg). The drug levels declined rapidly and Etoposide was detectable till day 4 (0.022 µg/mg) in the cells treated with Etoposide drug solution. For cells treated with ETPLIP and HAETPLIP, intracellular drug levels were detectable till day 6 and day 8 respectively. On the same track, A549 cells treated with DOCLIP and 0.393 µg/mg respectively) as compared to DOCSOL (peak intracellular concentration was 0.258 µg/mg). The cells treated with DOCSOL exhibited rapid decline in intracellular drug levels as compared to cells treated with DOCLIP and HADOCLIP where intracellular drug levels were detectable till day 6 and day 8 respectively.

Based on cellular drug concentration-time plot, pharmacokinetic parameters were calculated for conventional and developed Etoposide formulations. Liposomal Etoposide formulations exhibited higher Area Under the Curve (AUC) values than ETPSOL. Among liposomal formulations of Etoposide, HA grafted Etoposide liposomes exhibited higher AUC values as compared to non grafted Etoposide liposomes. AUCs were found to be 0.447, 0.893 and 1.46 for ETPSOL, ETPLIP and HAETPLIP respectively. Kel for ETPSOL was found to be 0.608, which is 1.77 times and 2.45 times higher than ETPLIP and HAETPLIP and HAETPLIP.

Higher AUC values and lower elimination rate constants observed in case of liposomal formulations (ETPLIP and HAETPLIP) as compared to Etoposide drug solution (ETPSOL) is a

testimonial to sustained release of liposomally entrapped Etoposide followed by its enhanced retention.

Similarly, higher AUC values were observed for liposomally entrapped Docetaxel as compared to Doetaxel plain drug solution (DOCSOL). Among liposomal formulations of Docetaxel, HADOCLIP exhibited higher AUC values as compared to non grafted Docetaxel liposomes. AUCs were found to be 0.465, 0.905 and 1.58 for DOCSOL, DOCLIP and HADOCLIP respectively. It was observed that drug elimination slowed down in case of liposomal formulations as compared to plain drug solution. Elimination rate constant for DOCSOL was found to be 0.338, which is 1.07 times and 1.41 times higher than DOCLIP and HADOCLIP respectively. Higher AUC values and lower elimination rate constants in case of liposomal formulations of ETP and DOC as compared to ETP/DOC solution respectively indicated that liposomal formulations of Docetaxel exhibited sustained drug release accompanied with enhanced drug retention.

The cell uptake studies and intracellular pharmacokinetic determinations of liposomal formulations clearly indicated that HA grafted liposomal ETP/DOC treated A549 cells not only showed enhanced cell uptake but also resulted in retention of higher intracellular concentrations of HA grafted ETP/DOC liposomes in A549 cells. Higher drug retention for prolonged time intervals in cells may be attributed to cytoadhesive nature of HA. HA is reported to bind specifically to CD44 receptors and N-acetyl D-glucosamine residues located at the surface of alveolar epithelium and hence, HA grafted liposomes (HADOCLIP) resulted in enhanced cell uptake followed by prolonged intracellular retention in A549 cells. Hence, apart from its well documented role as a targeting moiety for CD44 over expressing cell lines, cytoadhesive nature of HA also significantly retarded drug release leading to sustained drug release along with prolonged intracellular drug retention.

Effect of temperature and time of exposure on cellular uptake of vesicular systems was studied by conducting cell uptake experiments at different temperatures for different time periods. After 30 minutes of exposure, uptake of ETPLIP in cells incubated at 37°C was 1.62 times higher than when incubated 4°C and 3 fold higher when incubated for 120 minutes. Accumulation of Docetaxel loaded liposomes in cells incubated at 37^{0} C was 1.91 times higher than 4^{0} C when incubated for 30 min and 3.85 fold higher at 37^{0} C than 4^{0} C when incubated for 120 min. Thus, from above results it may be concluded that temperature and time of exposure are rate limiting steps for cellular uptake, since, incubation of cells at 37^{0} C for 120 minutes showed higher uptake of liposomes. Secondly, the temperature dependent cell uptake is an evidence to the fact that endocytic cell uptake mechanism was involved in cell uptake of Docetaxel liposomes (HA grafted and non grafted).

Cell cycle analysis was done to determine the phase of cell cycle arrest. Etoposide is a topoisomerase inhibitor and arrests the cell cycle mainly at G2/M phase leading to accumulation of cells in this phase.

Flow cytometric analysis of the effects of Etoposide on the cell cycle of the A549 cells showed that both Etoposide liposomes (ETPLIP) and HA grafted Etoposide liposomes (HAETPLIP) were able to accumulate in A549 cells and were able to arrest cells in G2/M phase. Higher proportion of cells was arrested in the G2/M arrest phase in case of treatment with HAETPLIP as compared to ETPLIP. The reason for low cell arrest in G2/M phase in case of ETPLIP may be attributed to the fact that non ligand grafted liposomes act as simple, non selective vesicular systems leading to slow cellular uptake of encapsulated drug. However, HA grafted Etoposide (HAETPLIP) showed enhanced rate and extent of cellular uptake which supports the hypothesis that grafting liposomes with HA rendered the liposomes more selective leading to increased Etoposide induced cell cycle arrest at G2/M phase in contrast to ETPLIP.

Docetaxel mainly inhibits the cell cycle during S Phase resulting in cell accumulation in S Phase. Flow cytometric analysis of the effects of Docetaxel on the cell cycle of the A549 cells showed that both Docetaxel liposomes (DOCLIP) and HA grafted Docetaxel liposomes (HADOCLIP) were able to accumulate in A549 cells and were able to arrest cells in S phase. The results revealed that with Docetaxel liposomes (DOCLIP), 18.67 % cells were in G0/ G1 phase, 11.6 % in S phase, 63.57 % in G2/M phase and 3.11% underwent apoptosis, whereas, in case of HA grafted Docetaxel liposomes (HADOCLIP), there are 16.50 % inG0/ G1, 40.17 % in S, 35.44 % in G2/M phase and 4.75% underwent apoptosis. The reason for low cell arrest in S phase in case

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of DOCLIP may be attributed to the fact that non ligand grafted liposomes act as simple, non selective vesicular systems and cell uptake of non grafted liposomally encapsulated drug would have been relatively slower. The results can be further justified by the fact that there was relatively higher cell arrest in G2/M which supports the fact that those cells that could escape the cytotoxic effect of Docetaxel due to relatively slower drug release from liposomes, could be successfully arrested at G2/M phase. The percentage apoptopic cell count was around 3.1%. However, HA grafted liposomes showed enhanced uptake of Docetaxel (40.17% cell arrest in S phase as compared to 11.6 % in case of Docetaxel liposomes- DOCLIP) which may be attributed to the hypothesis that grafting liposomes with HA rendered HADOCLIP more selective than DOCLIP and could easily target the A549 cells that over expressed CD44 receptors, leading to increased Docetaxel induced cell cycle arrest at S phase in contrast to DOCLIP. Cell percentage fraction undergoing apoptosis is also remarkably elevated to 4.75% as compared to 3.11% in case of DOCLIP corroborating the fact that HADOCLIP could show enhanced rate and extent of drug release and absorption in A549 cell line. Apart from increased cell fraction arrest in S phase, the apoptopic activity of Docetaxel in HA grafted liposomes (HADOCLIP) was also significantly higher as compared to plain, non grafted DOCLIP. Flow cytometric results for DOCLIP and HADOCLIP are consistent with MTT assay data which corroborates the hypothesis that HA renders the Docetaxel loaded liposomes more site specific leading to enhanced cell uptake and thereby cell cytotoxicity in A549 cells as compared to DOCSOL and DOCLIP. Blank liposomes had no effect on cell cycle distribution and the results were similar to those obtained with untreated control.

The results clearly demonstrated significantly higher cell uptake, achievement and sustenance of higher intracellular drug levels for prolonged time period, enhanced cell bioavailability and hence, greater cytotoxicity with Hyaluronic acid grafted liposomes as compared to non grafted liposomes and plain drug solutions.

CONCLUSION

Liposomal drug delivery to lung offers a number of advantages over conventional drug delivery systems such as localization of drug within the lungs, prolonged and controlled drug release, and enhanced cellular drug uptake. For achieving these objectives, liposomally entrapped drug must be obtained in a stable form that can be delivered conveniently and selectively to the targeted site in the lungs. Ligand grafting to liposomal surface enhances the site specificity of the liposomally entrapped cytotoxic drugs.

TFH method was used for the preparation of liposomes of ETP and DOC. Prepared liposomes were characterized for size, zeta potential and percentage drug entrapment followed by grafting these optimized liposomes with HA by carbodiimide coupling technique. Surface density of HA was optimized by correlating zeta potential of grafted liposomes with grafting efficiency and correlating HA incubation time with grafting efficiency. HA grafted and non grafted liposomes of ETP and DOC were subjected to cell line studies using A549 cell lines. The formulations were assessed for their effect on cell uptake, cytotoxicity, cell uptake, intracellular pharmacokinetic parameters etc. HA grafted liposomal formulations encapsulated with drugs exhibited manifold increase in cytotoxicity towards A549 cells, exhibited higher cell uptake, showed higher proportion of cell arrest (Higher percentage of cell arrest in G2/M phase with HAETPLIP and higher percentage of cell arrest in S phase with HADOCLIP). HA grafted liposomal formulations of ETP and DOC exhibited greater values for AUC, slower elimination constants and longer intracellular retention times indicating sustained intracellular drug delivery. HA grafted drug loaded liposomes were found to be superior during pharmacokinetic and other biological assessment and hence, were processed to DPIs by subjecting them to lyophilization using mannitol (as carrier and cryoprotectant) with 15% of glycine (as antiadherent). Highest PDR, lowest density, good MMAD (in the range of 4-5), lowest rehydrated liposomal size, angle of repose below 30°, tapped density below 0.40, lowest moisture content and highest values of % FPF, FPD, % Emission, ED, and % dispersibility were obtained with the batches prepared using mannitol as carrier. Hence, mannitol was found to be the best cryoprotectant and carrier amongst the three.

The stability studies were carried out as per ICH guidelines for countries falling under zone III (hot, dry) and zone IV (very hot, humid). It was apparent that the liposomal DPI formulations stored at higher temperatures showed increase in particle size mainly due to aggregation of liposomes mediated by cohesiveness of HA, increased fluidity of bilayered membranes leading to drug leakage and hence, decrease in percentage drug retention. However, liposomal DPI formulations were found to be stable at under refrigerated conditions and it should be prescribed to store them at $2-8^{\circ}$ C so as to ensure optimum stability and acceptable shelf life.

The studies supported the hypothesis that ligand grafted liposomal dry powder inhaler based pulmonary drug delivery for treatment of lung cancer exhibits enhanced site specificity, prolonged drug retention at targeted site and reduces the systemic exposure. Hence, ligand grafted liposomal Dry Powder Inhalers of cytotoxic drugs are expected to maximize the therapeutic index, reduce the systemic side effects, frequency of dosing and dose, and probably cost of therapy. However, the role of liposomal drug dry powder inhaler formulations in treatment of lung cancer can only be realized after detailed pharmacokinetic/pharmacodynamic and toxicological studies in at least two animal species followed by extensive clinical trials. The current investigation has provided an insight that delivery of ligand grafted liposomal dry powder inhaler of anti cancer agents can confine drug primarily to lung cancer cells only and hence expected to improve the therapeutic outcome, prognosis and overall quality of life in patients suffering from lung cancer.

CHAPTER 10 SUMMARY AND CONCLUSION

LIST OF PAPERS PUBLISHED/PRESENTED

1. HYALURONAN GRAFTED LIPOSOMAL ETOPOSIDE FOR TARGETING LUNG CANCER: CELL UPTAKE, CELL CYTOTOXICITY, CELL CYCLE ANALYSIS AND CELL PHARMACOKINETIC STUDIES.

Tapan R.Shah, Neelam Kedia-Mokashi, Nafisa Balasinor, Srabani Mukherjee, Himanshu Patel, Naazneen Surti and Ambikanandan Misra. (Communicated)

2. In vitro Cell Cytotoxicity and cell uptake studies for Hyaluronan grafted Docetaxel

Liposomes to target lung cancer.

Tapan R.Shah, Neelam Kedia-Mokashi, Nafisa Balasinor, Srabani Mukherjee, Himanshu Patel, Naazneen Surti and Ambikanandan Misra. (Communicated)

3. Targeted Delivery of Etoposide to Lung Cancer: Formulation, Characterization and

Stability Studies of Dry Powder Inhalers of Hyaluronan Grafted Liposomal Etoposide.

Tapan R.Shah, Himanshu Patel, Neelam Kedia Mokashi, Nafisa Balasinor, Srabani Mukherjee, Naazneen Surti and Ambikanandan Misra. (Communicated)

PRESENTATION:

1.Hyaluronic acid grafted Liposomal Dry Powder Inhalers of Etoposide in treatment of Lung Cancer at 2nd Winter School on "Nanotechnology in Advanced Drug Delivery", February, 2009, NIPER, Chandigarh, Punjab (India).