Chapter 10

SUMMARY AND

CONCLUSION

10.1 Summary

Cancer is a generic term for a large group of diseases that can affect any part of the body. Other terms used are malignant tumors and neoplasms. One defining feature of cancer is the rapid formation of abnormal cells that grow beyond their usual boundaries, and which can then invade adjoining parts of the body and spread to other organs. This process is referred to as metastasis. Metastasis is the major cause of death from cancer.

Cancer is the leading cause of death in economically developed countries and the second leading cause of death in developing countries. The burden of cancer is increasing in economically developing countries as a result of population ageing and growth as well as, numerous factors such as: i) Cancer is a leading cause of death worldwide, accounting for 14 million new cases and 8.8 million deaths in 2015. ii) Lung, stomach, liver, colon and breast cancer cause the most cancer deaths each year. iii) The most frequent types of cancer differ between men and women. iv) About 30% of cancer deaths are due to the some behavioural and dietary risks. v) Tobacco use is the most important risk factor for cancer causing 22% of global cancer deaths and 71% of global lung cancer deaths.

Lung cancer is a disease characterized by uncontrolled cell growth in tissues of the lung. If left untreated, this growth can spread beyond the lung in a process called metastasis into nearby tissue and, eventually, into other parts of the body. Most cancers that start in lung, known as primary lung cancers, are carcinomas that derive from epithelial cells. The main types of lung cancer are *small cell lung carcinoma* (SCLC), also called oat cell cancer, and *non-small cell lung carcinoma* (NSCLC).

The class of drugs used for treatment of lung cancer are alkylating agents eg. Ifosfamide, antimetabolites eg. Gemcitabine, antibiotic eg. Mitomycin, plant derived products eg. Vinblastine, miscellaneous agents eg. Cisplatin. Gemcitabine and Vinorelbine are first line agents used in the treatment of lung cancer and both show good cytotoxicity against lung tumour.

Gemcitabine, a nucleoside analog related to cytarabine, is a novel pyrimidine antimetabolite that shows significant cytotoxicity against lung tumours. Gemcitabine is marketed as “Gemzar” by Eli Lilly and Company. “Gemzar” is a white to off white solid

lyophilized powder and it is supplied in vials of either 200 mg or 1 gm administered by i.v. infusion over 30 minutes. It is formulated with mannitol and sodium acetate. Hydrochloric acid or Sodium hydroxide may have been added for pH adjustment.
Major limit for the use of Gemcitabine is represented by its rapid metabolic inactivation (deamination operated by deoxicytidine deaminase) responsible for its short half-life together with its low but still important systemic toxicity. The half-life and volume of distribution depends on age, gender and duration for infusion. The development of multidrug resistance in cells exposed to gemcitabine can limit its effectiveness. Gemcitabine HCl is effluxed by the Pgp (P glycol protein) and resistance is observed by MDR gene (Multi drug resistance gene). High Dose of Gemcitabine Hydrochloride 1000 to 1200 mg/m^2 is required to achieve therapeutically effective levels.

Another drug of interest is Vinorelbine. Vinorelbine (5'nor-anhydro-vinblastin) is a semisynthetic vinca alkaloid that is manufactured from alkaloids extracted from the rosy periwinkle, Catharanthus roseus. Vinorelbine is marketed by Abbott Healthcare under the name Navelbine. Navelbine is available in vial containing vinorelbine tartarate equivalent to 10 mg (1 mal vial) or 50 mg (5 ml vial) and formulated in water for injection administered by intravenous route over 6-10 minutes. Long half-life of Vinorelbine tartrate enables it to remain in systemic circulation for extended period of time. Vinorelbine tartrate has high degree of plasma protein binding of 80-90%.
Both Gemcitabine hydrochloride (Vd: i.v. 50 L/m2) and Vinorelbine tartarate (Vd: i.v. 25.4-40.1 L/kg) due to their non-specific distribution throughout the body, are associated with serious side effects like anaemia, thrombocytopenia, cardiac arrhythmia, alopecia etc. Development of a targeted drug delivery systems containing these drugs reduces its distribution to the other organs and tissues resulting into decrease in the side effects.

Hence the aim of the present investigation was to target the drug encapsulated carrier i.e. spherulites passively to the lungs by intravenous route to achieve maximum therapeutic effect by reducing the dose and thereby lowering the chances of side effects associated with the therapy of Non-small cell lung cancer.

Analytical methods for the estimation of drugs

UV-Visible Spectrophotometric methods were developed for both Gemcitabine Hydrochloride and Vinorelbine Tartrate separately. Gemcitabine Hydrochloride obeyed Beers law in the range of 5-30 µg/mL with R^2 value of 0.9999 in both distilled water and Phosphate-buffered saline (PBS) pH 7.4. Analytical Method was validated and found to be accurate and precise. LOD and LOQ values for Gemcitabine Hydrochloride were 0.67 µg/mL and 2.24 µg/mL, respectively, in distilled water, whereas in PBS pH 7.4, LOD and LOQ values were found to be 0.81 µg/mL and 2.70 µg/mL, respectively.

LCMS-MS analytical method for estimation of Gemcitabine Hydrochloride in rat plasma was also developed. Analytical Method was found to be linear in the range of 20-2000 ng/mL with R^2 value of 0.999. The retention time obtained was 2.89 minutes. Method validation revealed that it was accurate. Moreover, LCMS-MS analytical method was developed for the estimation of drug from homogenate of various highly perfused organs of rat like heart, lungs, liver, kidney and spleen. Analytical methods were found to obey Beers law with R^2 value near to 1.

Vinorelbine Tartrate obeyed Beer's law in the range of 5-30 µg/mL with R^2 value of 0.9951 and 0.9936 in distilled water and Phosphate-buffered saline (PBS) pH 7.4, respectively. Analytical Method was validated and found to be accurate and precise. LOD and LOQ values for Vinorelbine Tartrate were 0.22 µg/mL and 0.74 µg/mL, respectively, in distilled water, whereas in PBS pH 7.4, LOD and LOQ values were found to be 0.22 µg/mL and 0.75 µg/mL, respectively.

LCMS-MS analytical method for estimation of Vinorelbine Tartrate from rat plasma was also developed. Analytical Method was found to be linear in the range of 5-500 ng/mL with R^2 value of 0.999. The retention time obtained was 2.72 minutes. Method validation revealed that it was accurate. Moreover, LCMS-MS analytical method was developed for the estimation of drug from homogenate of various highly perfused organs of rat like heart, lungs, liver, kidney and spleen. Analytical methods were found to obey Beer's law with R^2 value near to 1.

Preformulation and Preliminary Optimization

Gemcitabine Hydrochloride and Vinorelbine Tartrate were identified and authenticated in preformulation study. FTIR and DSC analysis showed that both drugs were pure and authentic. Drug-excipient compatibility study revealed that there was no interaction. Preliminary trails were carried out to formulate spherulites. Various formulation variables were optimized. Screening of lipids was performed and upon assessing the results, Soyabean Phosphatidylcholine 95 % was chosen as lipid of choice. SPC concentration range was optimized and 48-62% was found to be useful in formulating the spherulites. Lipid: cholesterol ratio was also optimized and it was found to be 1:1 M ratio. For PEGylation, DSPE PEG 2000 was optimized and 2mol % was found to be optimum. Various process variables were also optimized. Effect of hydration time on lipid film was assessed. Lipid film with no hydration time as well as long hydration time yielded spherulites of large size with less entrapment, hence, it needed to be optimized. Type of homogenization assembly to be used for grinding of lamellar phase was chosen based on the size of spherulites and entrapment efficiency obtained. Homogenization time and speed were optimized and results showed that 60 minutes of homogenization time with 65 min^{-1} speed were optimum. Extrusion method was used to downsize the spherulites. Extrusion cycle was optimized and 5 cycles were found to be optimum.

Formulation and Optimization of Spherulites

GCH loaded spherulites were prepared and optimized by applying 3^3 Full Factorial design. Design Expert® (Version 7.0.0, State-Ease Inc., Minneapolis, USA) was used to apply the 3^3 Full Factorial design. Spherulites were composed of SPC, Chol and Potassium oleate. Independent variables were chosen viz. Phospholipid concentration (%w/w), Hydration time (hrs) and Probe-Cylinder distance (mm). While, spherulites size and %EE were response variables. Optimized values of independent variables were found to be Phospholipid concentration (%w/w) 42 %w/w, Hydration time (hrs) 19.66 hrs and Probe-Cylinder distance (mm) 0.57 mm. Optimized batch of GCH loaded spherulites exhibited size of 204.9±1.2 and 76.28±1.1 % entrapment efficiency with overall desirability of 0.91. Optimized batch of GCH loaded spherulites was PEGylated in order to improve the circulation time of the administered formulation in vivo. For that purpose DSPE-PEG 2000 (2 mole % of lipid phase) was used. DSPE-PEG 2000 was incorporated

in the lipid phase of the formulation and remaining procedure was carried out in the same manner

VLB loaded spherulites were prepared and optimized by applying Box-Behnken design. Design Expert® (Version 7.0.0, State-Ease Inc., Minneapolis, USA) was used to apply the Box-Behnken design. Spherulites were composed of SPC, Chol and Potassium oleate. Independent variables were chosen viz. Phospholipid concentration (%w/w), Hydration time (hrs) and Probe-Cylinder distance (mm). While, spherulites size and %EE were response variables. Optimized values of independent variables were found to be Phospholipid concentration (%w/w) 42 %w/w, Hydration time (hrs) 12 hrs and Probe-Cylinder distance (mm) 0.53 mm. Optimized batch of VLB loaded spherulites exhibited size of 122.4±1.6nm and 95.65±0.86 % entrapment efficiency with overall desirability of 0.99. Optimized batch of VLB loaded spherulites was PEGylated in order to improve the circulation time of the administered formulation *in vivo*. For that purpose DSPE-PEG 2000 (2 mole % of lipid phase) was used. DSPE-PEG 2000 was incorporated in the lipid phase of the formulation and remaining procedure was carried out in the same manner

Characterization of formulations

All four optimized formulations i.e. Gemcitabine Hydrochloride (GCH) Loaded Non-PEGylated Spherulites, GCH Loaded PEGylated Spherulites, Vinorelbine Tartrate (VLB) Loaded Non-PEGylated Spherulites and VLB Loaded PEGylated Spherulites were characterized for size (nm), zeta (ζ) potential, % Entrapment efficiency (%EE), % drug loading (% w/w), morphological analysis by Scanning electron microscope (SEM) and Transmission electron microscope (TEM), *in vitro* drug release, Osmolality (mOsm/Kg) and electrolyte-induced flocculation.

GCH Loaded Non-PEGylated Spherulites and GCH Loaded PEGylated Spherulites exhibited particle size of 204.9±1.2 nm and 209.2±1.4 nm, respectively. Zeta potential of Non-PEGylated spherulites was found to be -26.5±1.3 mV and -33.3±1.6 mV for PEGylated spherulites. Non-PEGylated and PEGylated spherulites exhibited %EE of 76.28±1.1 % and 77.42±1.5 %, respectively. Whereas, % drug loading in Non-PEGylated spherulites was 9.38±0.94 %w/w and 9.07±0.89 %w/w for PEGylated spherulites. SEM and TEM analysis revealed that the spherulites were discrete, uniformly spherical in

shape with no sign of aggregation. In vitro drug release showed that entire Standard GCH solution diffused through the dialysis bag within 2 hours. Whereas, GCH loaded Non-PEGylated spherulites (93.09±1.21 % drug release) and GCH loaded PEGylated spherulites (89.49±1.65 % drug release) displayed sustained release upto 48 hours. The kinetics of drug release was evaluated by fitting the release data in various mathematical model. First order model fitted the best with highest (near 1) R^2 value. Osmolality of GCH loaded Non-PEGylated and PEGylated spherulites was measured and it was found to be 282±1.2 mOsm/Kg and 291±1.4 mOsm/Kg, respectively. Electrolyte-induced flocculation study showed that steric barrier around the spherulites inhibited the flocculation induced by addition of an electrolyte. However, increase in electrolyte concentration above certain level disrupted the steric barrier and caused flocculation or aggregation of spherulites.

VLB Loaded Non-PEGylated Spherulites and VLB Loaded PEGylated Spherulites exhibited particle size of 122.4±1.6 nm and 131.6±1.9 nm, respectively. Zeta potential of Non-PEGylated spherulites was found to be -26.9±2.4 mV and -37.8±2.1 mV for PEGylated spherulites. Non-PEGylated and PEGylated spherulites exhibited %EE of 95.65±0.86 % and 94.20±0.74 %, respectively. Whereas, % drug loading in Non-PEGylated spherulites was 11.76±0.59 %w/w and 11.04±0.76 %w/w for PEGylated spherulites. SEM and TEM analysis revealed that the spherulites were discrete, uniformly spherical in shape with no sign of aggregation. In vitro drug release showed that entire Standard VLB solution diffused through the dialysis bag within 24 hours. Whereas, VLB loaded Non-PEGylated spherulites (90.84±1.45 % drug release) and VLB loaded PEGylated spherulites (86.85±1.20 % drug release) displayed sustained release upto 48 hours. The kinetics of drug release was evaluated by fitting the release data in various mathematical model. First order model fitted the best with highest (near 1) R^2 value. Osmolality of VLB loaded Non-PEGylated and PEGylated spherulites was measured and it was found to be 284±1.4 mOsm/Kg and 292±1.6 mOsm/Kg, respectively. Electrolyte-induced flocculation study showed that steric barrier around the spherulites inhibited the flocculation induced by addition of an electrolyte. However, increase in electrolyte concentration above certain level disrupted the steric barrier and caused flocculation or aggregation of spherulites.

***In vitro* cytotoxicity study**

In vitro cytotoxicity studies (MTT Assay) were performed on MCF-7 cell line at different concentrations (0.001, 0.01, 0.1, 1, 10 µg/ml) of drugs and drug loaded liposomal formulations (std. GCH, GCH Spherulites, std. VLB and VLB Spherulites) at three different incubation times (8, 16 and 24 hours). Results showed that GCH spherulites showed (87.43 % cell inhibition after 24 hours for 10 µg/mL concentration) more cytotoxicity than standard GCH (78.42 % cell inhibition after 24 hours for 10 µg/mL concentration). Further it was confirmed by IC50 values that GCH spherulites (0.04 µg/mL after 24 hours) exhibited more cytotoxicity than standard GCH (0.2 µg/mL after 24 hours). Results clearly indicated that the spherulites formulation loaded with GCH have lower IC50 values than that of the standard drug. Lower dose ($1/5^{th}$) was required for spherulites formulation for inhibition of 50% total cell population as compared to standard drug. Apoptosis study and cell cycle analysis was also performed by FACS analysis. Results showed that cells treated with GCH spherulites displayed high (93.48 %) intensity of cell counts in apoptotic region than standard GCH (73.71 %). These results indicated that high cellular uptake of GCH spherulites was responsible for augmented apoptosis in cells than standard GCH.

Results showed that VLB spherulites showed (83.27 % cell inhibition after 24 hours for 10 µg/mL concentration) more cytotoxicity than standard VLB (78.94 % cell inhibition after 24 hours for 10 µg/mL concentration). Further it was confirmed by IC50 values that VLB spherulites (0.04 µg/mL after 24 hours) exhibited more cytotoxicity than standard VLB (0.3 µg/mL after 24 hours). Results clearly indicated that the spherulites formulation loaded with VLB have lower IC50 values than that of the standard drug. Lower dose ($1/5^{th}$) was required for spherulites formulation for inhibition of 50% total cell population as compared to standard drug. Apoptosis study and cell cycle analysis was also performed by FACS analysis. Results showed that cells treated with VLB spherulites displayed high (37.13 %) intensity of cell counts in apoptotic region than standard VLB (22.21 %). These results indicated that high cellular uptake of VLB spherulites was responsible for augmented apoptosis in cells than standard VLB.

***In vivo* studies**

In vivo studies like hemocompatibility study, acute toxicity study, pharmacokinetic study, biodistribution study by LCMS-MS method and biodistribution study by gamma scintigraphy were performed.

Results of hemocompatibility study showed that GCH loaded spherulites were safe for in vivo use as no alteration in the morphology of RBCs was observed.

Results of acute toxicity study (study was performed in Female Swiss Albino Mice) revealed that GCH loaded spherulites were well tolerated at higher dose than standard GCH solution. 500 mg/kg was found to be the LD_{50} of Standard GCH solution. Whereas, LD_{50} of GCH loaded spherulites was found to be > 500 mg/kg.

Pharmacokinetic data revealed the difference between the in vivo fate of standard GCH solution, GCH loaded non-PEGylated and GCH loaded PEGylated formulation. Study was performed in Female Sprague-Dawley (SD) rats. PEGylated formulation displayed significantly ($p<0.001$) higher AUC followed by non-PEGylated formulation and standard drug solution. Moreover, PEGylated formulation showed increased half-life and mean residence time than non-PEGylated formulation followed by standard drug solution. Also, Clearance rate of PEGylated formulation was much reduced when compared with non-PEGylated formulation followed by standard drug solution.

Results of Biodistribution (study was performed in Female Sprague-Dawley (SD) rats) by LCMS-MS method showed that Standard GCH solution showed non-specific biodistribution. Standard GCH was found in Heart, Lungs, Liver, Spleen and Kidneys. Initially the drug concentration in all the highly perfused organs except Heart was high, however, in the later time points it declined due to rapid metabolism of drug. GCH loaded non-PEGylated Spherulites displayed that the formulation was reaching to the organ of interest i.e. Lungs, however, it started to decline after 2 hours. Initial accumulation of formulation in Lungs can be seen which shows that Spherulites could reach the target organ. Another observation was that formulation showed accumulation in Liver, Spleen and Kidneys. This could be because of identification of Non-PEGylated spherulites by RES. PEGylated formulation reached and retained at the target organ i.e. Lungs till 12 hours. Liver, Spleen and Kidney showed minimal drug disposition. This

could be due to the presence of PEG chains over the vesicles surface enabling them to remain undetected from RES.

Biodistribution (study was performed in Female Sprague-Dawley (SD) rats) was also studied by gamma scintigraphy. ^{99m}Tc-GCH complex was found to be stable when incubated in presence of saline and serum. Results of Transchelation study by DTPA challenge showed that the radiotracer-drug complex was highly stable as evidenced by low degree of transchelation. Gamma scintigraphy revealed that the initial distribution, within 1 hour of administration, of PEGylated Spherulites to lungs was significantly higher (18.73%) than that of Non PEGylated (12.17%) and plain drug solution (14.62%). Even after 4 hours of administration, lung retention of PEGylated Spherulites (9.11%) exceeded that of non-PEGylated (4.31%) and plain drug solution (1.20%).

Results of hemocompatibility study showed that VLB loaded spherulites were safe for in vivo use as no alteration in the morphology of RBCs was observed.

Results of acute toxicity study (study was performed in Female Swiss Albino Mice) revealed that VLB loaded spherulites were well tolerated at higher dose than standard VLB solution. 42 mg/kg was found to be the LD_{50} of Standard VLB solution. Whereas, LD_{50} of VLB loaded spherulites was found to be 75 mg/kg.

Pharmacokinetic data revealed the difference between the in vivo fate of standard VLB solution, VLB loaded non-PEGylated and VLB loaded PEGylated formulation. Study was performed in Female Sprague-Dawley (SD) rats. PEGylated formulation displayed significantly ($p<0.001$) higher AUC followed by non-PEGylated formulation and standard drug solution. Moreover, PEGylated formulation showed increased half-life and mean residence time than non-PEGylated formulation followed by standard drug solution. Also, Clearance rate of PEGylated formulation was much reduced when compared with non-PEGylated formulation followed by standard drug solution.

Results of Biodistribution (study was performed in Female Sprague-Dawley (SD) rats) by LCMS-MS method showed that Standard VLB solution showed non-specific biodistribution. VLB was found in Heart, Lungs, Liver, Spleen and Kidneys. VLB concentration in all the isolated highly perfused organs was found to be persistent from 0.25 hour to 12 hours. The reason for this is that VLB has long half-life as a results of which it gets accumulated in highly perfused organs. VLB loaded non-PEGylated

Spherulites showed increased lung accumulation till 1 hour, however, it started to decline at later time points. This result indicates that the formulation is able to reach target organ i.e. Lungs at initial time points. Moreover, the results showed that the non-PEGylated formulation was found to be accumulated in Liver, Spleen i.e. RES system and Kidneys and it was found to be increasing with time. Non-PEGylation of Spherulites could be responsible for the uptake by RES system and consequently elimination from systemic circulation. VLB loaded PEGylated Spherulites reached and retained in the Lungs i.e. target organ at higher concentration than other organs till 12 hours. Moreover, the PEGylated Spherulites showed minimal disposition in Liver, Spleen and Kidneys. The attributable reason for this could be the PEGylation of vesicles which enabled the formulation to remain in the circulation for longer period of time by bypassing the RES system.

Biodistribution (study was performed in Female Sprague-Dawley (SD) rats) was also studied by gamma scintigraphy. ^{99m}Tc-VLB complex was found to be stable when incubated in presence of saline and serum. Results of Transchelation study by DTPA challenge showed that the radiotracer-drug complex was highly stable as evidenced by low degree of transchelation. Gamma scintigraphy revealed that the initial distribution, within 1 hour of administration, of PEGylated Spherulites to lungs was significantly higher (18.48%) than that of Non PEGylated (10.90%) and plain drug solution (14.23%). Even after 4 hours of administration, lung retention of PEGylated Spherulites (14.73%) exceeded that of non-PEGylated (3.83%) and plain drug solution (7.36%).

Stability study

Short-term accelerated stability study for three months was performed according to the ICH Q1A (R2) guidelines for all four spherulites formulations. Formulations were characterized for all critical parameters viz. visual appearance, % Entrapment efficiency (%EE), % drug loading (%w/w), particle size with polydispersity index (PDI), zeta potential and cumulative drug release at 0, 1, 2 and 3 months to study the stability. Results showed that there was no significant difference between the measured parameters at initial time and after three months indicating the stability of developed formulations.

10.2 Conclusion

GCH loaded spherulites and VLB loaded spherulites were successfully prepared and optimized using 3^3 Full Factorial design and Box-Behnken design, respectively. PEGylation of both formulation was also performed. All four Spherulites formulations had desired size range with high drug entrapment efficiency and loading. SEM and TEM analysis confirmed that the spherulites were spherical and discrete. Drug release from all the formulations was sustained up to 48 hours. Electrolyte-induced flocculation study revealed that the spherulites were stable. *In vitro* cell line study and apoptosis study showed that the formulations were inhibiting cell growth at much lower dose than standard drug solution. In vivo acute toxicity study showed that drug loaded spherulites were well tolerated at high dose than standard drug solution. In vivo pharmacokinetic study showed that PEGylated formulation of both the drugs remained in systemic circulation for extended period of time than non-PEGylated formulation. Biodistribution study by LCMS-MS method revealed that the formulation was able to reach target organ i.e. lungs. Moreover, same has been confirmed by biodistribution by gamma scintigraphy. PEGylated spherulites of both the drugs could efficiently reach the lungs by bypassing the RES. Hence, Spherulites proved to be a promising drug delivery system for drug targeting to the lungs for the treatment of Non-small cell lung cancer. Moreover, encapsulation of cytotoxic drug in a carrier system could result into lesser side effects compared to the plain drug solution which has non-specific biodistribution throughout the body. Extended research involving preclinical and clinical trials may further prove the potential of the formulation in drug targeting to the lungs.