CHAPTER 8
SUMMARY AND CONCLUSION

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8.1 Summary

8.1.1 Introduction

In recent years, significant improvement in the field of colloidal carriers has been achieved and liposomes have emerged as versatile tools for delivering active compounds, such as antitumor drugs. Current efforts to selectively deliver chemotherapeutic agents to improve their sites of action have focused on exploiting the natural endocytosis pathway by targeting an over-expressed receptor or antigen on the surface of target cell. Receptor-mediated targeting of long circulating carriers has been accomplished by attachment of ligands to the distal end of the polymer chains. Interaction between the targeting ligand and the cell surface receptors allows the carrier to be bound to the cell surface for a time sufficient to facilitate internalization of the vehicle and the drug that it contains. A number of ligand-presenting delivery systems targeting differentially expressed receptors on tumor cells are used to improve the drug uptake.

Liposomes have been investigated as potential drug delivery vehicles because of their large cargo capacity and their compatibility. Liposomal drugs currently used in clinical oncology were successfully stabilized to allow for durable drug encapsulation, liposomal carriers have a protective effect on incorporated drugs by preventing their enzymatic degradation. However, liposomes can also suffer from short blood circulation times and low tissue specificity. We have attempted to solve both these problems by using stable lipids and linking these lipids to folic acid via a polyethylene glycol (PEG) bridge. The resulting liposomes are both stable in serum and tumor-specific. Thus folate-PEG-liposomes display no tendency to fuse with or otherwise enter FBP-negative cells. However, the same liposomes are avidly internalized by receptor-bearing tumor cells. In contrast, liposomes similarly derivatized with PEG alone (no folate) showed no tendency to enter the same cancer cells. The accumulation of the liposomal drugs was shown to further improve their specific targeting to the tumor, i.e. by attaching tumor-specific molecules folate to the liposome surface.

Most of ligands delivered into cells by endocytic pathways are internalized for the purpose of destruction. That is, hormones that have already transduced their signals, as well as foreign particles that could be harmful to a cell if allowed to remain, are endocytosed and rapidly trafficked to lysosomes where they are digested. In contrast, folic acid is internalized not for destruction, but rather for consumption. Not surprisingly, folate conjugates have been observed to remain stable and functional for hours following uptake by cancer cells. Thus, proteins and nucleic acids remain undigested, enzymes retain their activities and liposomes resist disruption (unless engineered to disintegrate at endosomal pH) following uptake by FBP. The folate receptor (FR) is a family of GPI-anchored proteins that bind folic acid with high affinity (K_d>10^{-10}M) and shuttle the molecule inside cells via endocytic mechanism. The FR is expressed at low level in a limited number of epithelial cells but is markedly increased in carcinomas. The folate receptor as a tumor marker and simultaneously suggested that the folate-mediated delivery technology that we had been studying in cultured cells might be useful to target agents to cancer cells *in vivo*. Those cancers that were found to most prominently overexpress the α-isoform of the folate receptor included carcinoma's of the ovary, kidney, uterus, testis, brain, colon, and adeno carcinomas of the lung.

High tumor selectivity has been achieved *in vitro* and *in vivo* with folate-targeted antineoplastic drugs, compared to the more commonly exploited tumor-specific monoclonal antibodies; the vitamin folic acid has many advantages as a tumor targeting agent. These include its low cost, high chemical and biological stability, compatibility with organic solvents, non-immunogenicity, stronger affinity for its receptor and small size for fast tumor extravasation and thorough intercellular infiltration. Folate-mediated targeting systems should consequently have significant clinical potential, since overexpression of folate receptors has been found in a large fraction of human cancers but rarely in normal tissues, allowing high tumor specificity in the diagnosis and treatment of cancer.

In the recent years, Gemcitabine (trade name Gemzar) has emerged as a very potent anti-tumour drug and it is currently used, alone or in combination, in the treatment of

patients with different malignancies, including ovarian, pancreatic, colon, non-small cell lung and other cancers. Gemcitabine is a novel deoxycytidine analogue, a pyrimidine antimetabolite related to cytarabine. Gemcitabine exhibits cell phase specificity, primarily killing cells undergoing DNA synthesis (S-phase) and also blocking the progression of cells through the G1/S-phase boundary. Gemcitabine is prodrug converted intracellularly via deoxycytidine kinase to difluoro deoxycytidine monophosphate, which is further converted to two active metabolites, dFdCDP and dFdCTP, di– and triphosphate, respectively. Firstly, dFdCDP inhibits the catalysing enzyme ribonucleotide reductase resulting in a reduced amount of deoxynucleotide, deoxycytidine triphosphate (dCTP), available for DNA synthesis. Secondly, dFdCTP competes with dCTP for incorporation into DNA. Incorporating dFdCTP results in chain termination after the further addition of one more nucleotide and thus to apoptosis. Thus dFdC affects the synthesis phase of cell metabolism in two different ways and exhibits a self potentiating effect.

Unfortunately, gemcitabine possesses a rapid body clearance that limits its efficacy, a drawback due to kidney excretion and metabolism by the plasmatic enzyme cytidine-deaminase, which yields the inactive metabolite 2′,2′-difluorodeoxyuridine (dFdU). The problem with GEM is its short plasma $t_{½}$ and its quick metabolism into dFdU followed by elimination from the body. Thus, a frequent administration schedule at high drug doses is required and this leads to significant side effects.

A high amount of dFdC in the aqueous compartment of liposomes with a negligible amount of dFdC outside the liposomes could increase the potency and decrease the toxicity associated with GEM treatment thereby improves the tumor targeting.

8.1.2 Analytical Method

The UV spectroscopic method described in the USP was used for the GEM estimation. GEM liposomal formulations were estimated at 268nm. The method was validated for linearity, accuracy and precision. The validation parameters were found to meet, "readily pass criteria" specified in the USP.

8.1.3 Formulation and Characterization

Liposomes were prepared by thin film hydration method. GEM was remote-loaded into the liposomes by a transmembrane pH gradient method. The lipid compositions of the conventional (CL), Stealth (SL) and the Folate targeted (FT) liposomes were HSPC/CHOL at molar ratio of 8:2, HSPC/CHOL/mPEG-DSPE at molar ratio of 7.5:2:0.5 and HSPC/CHOL/F-PEG-DEPE at molar ratio of 7.75:2:0.25, respectively. Briefly, the lipids (45 mg total) were dissolved in 3 mL $CHCl_3$: methanol and dried into a thin film by rotary evaporation and then further dried under vacuum. The lipid film was hydrated with 2mL of 200mM ammonium sulfate ($(NH_4)_2SO_4$) for 60 min at 57±3°C with vortex mixing. The liposomal suspension was then probesonicated (2×2min×0.6cycle×80amplitude) using a serotorius probsonicator to produce small unilamellar vesicles (SUVs). The $(NH_4)_2SO_4$ outside of the liposomes was removed by centrifugation method by using HEPES buffer at 25000RPM, 4°C at 30 min, 3 cycles. The mean diameter of the liposomes was determined by dynamic light scattering using malvern mastersizer. GEMHCl (6 mg/mL) was dissolved deionized H_2O and added to the liposomes at a GEM-to-lipid ratio of 0.25 M, followed by a 30 min incubation at 57±3°C. Residual free GEM in the liposomal preparation was removed by size exclusion chromatography on a Sephodex G25 column. GEM concentration in the liposomes was determined by measuring absorption at 268 nm on a Shimadzu UV–spectrophotometer following liposome lysis in methanol.

After optimizing all the parameter like rotation speed, vacuum, hydration time, sonication cycles, drug: lipid, lipid: cholesterol ratio and selection of lipids in the formulation CL, SL and FT liposomes, the EE was found to be 74.83 ± 2.65, 73.36± 2.34, 75.16± 2.92; zeta potential was 4.71 ± 0.97, -7.33 ± 2.13, 3.61 ± 1.31, size was 103.60± 4.58, 120.30±6.46, 98.43±4.18 and PDI was 0.074 ± 0.010, 0.097 ± 0.017, 0.085 ± 0.013 for the CL, SL and FT liposomes respectively. The formulations were characterized by TEM for surface morphology, FITR and DSC for drug to lipid interaction studies. By *in vitro* diffusion study at pH 7.4 buffer shows a reduction in release from the FT and SL in comparison to conventional liposomes.

8.1.4 Stability Studies

Liposomal formulations were stable when stored at 2-8°C as aqueous dispersion by size and EE, whereas aqueous dispersion stored at 30±5°C showed increase in particle size and more leakage occurred during storage.

8.1.5. *In Vitro* Cell Line Studies

8.1.5.1 Cytotoxicity: Cytotoxicity of FT, SL and CL liposomes and GEM was determined on both HeLa and A549 cells using an MTT assay. The results shows that enhancement in cytotoxicity exhibited by FT liposomal GEM had approximately two times lower IC_{50} value compared to that of GEM. The enhancement in cytotoxicity exhibited by FT liposomal suspension over CL and SL. The results nonetheless demonstrated a FR-dependence of the cytotoxicity.

8.1.5.2 Cell Cycle Analysis: Cell cycle analysis based on measurements of DNA content generates a clear pattern of distribution: G0/G1 phase (one set of paired chromosomes per cell), S phase (DNA synthesis with variable amount of DNA), and G2/M phase (two sets of paired chromosomes per cell, prior to cell division), DNA content can be measured using FACs analyzer. Gemcitabine were able to affect the cell cycle of cervical (HeLa) and NSCL (A549) cancer cells. In HeLa cells liposomal suspension caused an increase in the population of cells in G1 and S phase by this it shows apoptosis in later G1 and S phase compare to pure drug it shows only G1 phase arrest, among this FT liposomes will be more effective. In case of A549 cell line the liposomal suspension shows greater arrest in the latter G1 and S phase compare to pure drug. By comparing both the cell lines the FT liposomal suspension shows an improved targeting efficiency, specific targeting to HeLa cervical cell line by arresting at latter G1 and S phase at 4hrs compare to PD, CL and SL this may be because of folate receptor which is a tumor marker that is consistently over expressed in HeLa then in A549 cell line.

8.1.5.3 Cellular Uptake: cellular uptake facilitates penetration of various molecular cargo through the cell and is measured by FACs and live fluorescent imaging technique. In HeLa cells liposomal suspension shows small increase in uptake within 15 min but after 15 min it shows an "exponential" increase in fluorescence intensity at

30 min, indicating rapid entry of liposomal suspension in the cell compare to free drug. In A549 cells also it shows exponential increase in uptake by liposomal suspension at 30 min, However, For GEM -6coumarin conjugates increase in intensity was gradual over a period of 30 min, with a gradual shift in peak along the fluorescence intensity axis in both the HeLa and A549 cell lines. From flow cytometry it was observed that the cell uptake peaked at 30 min for liposomal conjugates. There was statistical difference ($p > 0.05$) in the cell entry dynamics of liposomal and free drug conjugates and there was no significant difference between FT and SL. FT liposomes showed much greater cellular uptake and liposome internalization compared to SL and CL liposomes. These results indicated that these liposomes have efficient interactions with the cellular folate receptor.

8.1.5.4 Western Blotting: Caspase-3 is a frequently activated cell death protease enzyme important for cell death, Gemcitabine liposomal solution (FT) increases caspase-3 activity in A 549 cell has been shown by western blotting analysis as compared with plain drug.

8.1.6 Radiolabeling and Biodistribution Studies

Labeling of the liposomes with radioisotopes is carried out by entrapping the suitable gamma emitting radioisotope such as ^{99m}Tc by direct conjugation method. The stability studies of ^{99m}Tc- CL/SL/FT/PD were carried out *in vitro* using normal saline and mice serum by ascending ITLC. The bonding strength of ^{99m}Tc- CL/SL/FT/PD was assessed by DTPA (Diethylene triamine penta acetic acid) challenging test. Biodistribution of ^{99m}Tc- CL/SL/FT/PD following i.v. administration in swiss mice and tumor induced swiss mice were performed and the radioactivity was estimated at predetermined time intervals up to 24hours. The pharmacokinetic parameters AUC, terminal elimination rate constant, MRT and half life were calculated using standard pharmacokinetic principles. The PD shows more accumulation in RES and liposomal suspension shows an increased blood circulation half life confirmed by both biodistribution and gamma imaging study. The results revealed that the liposomal suspensions show more tumor uptake, long span in the blood and increase in AUC. Among this liposomal suspension, the FT liposomes show an improved tumor uptake,

tumor to muscle uptake ratio (nearly 5 fold higher) compared to CL indicating an improved targeting efficiency to tumor.

8.2 Conclusion

A novel folate receptor targeted GEM liposomal formulation of FT liposomes was prepared by thin film hydration method. GEM was loaded into the liposomes by a transmembrane pH gradient method. Encapsulation of the drug into liposomal formulation was thought to prevent problems associated with free drug and by targeting to improve tumor specificity. A high amount of dFdC in the aqueous compartment of liposomes with a negligible amount of dFdC outside the liposomes could increase the potency and decrease the toxicity associated with GEM. The prepared liposomal suspension is spherical in nature as confirmed by TEM studies, with the diameter of around 100nm, PDI of around 0.08 and EE was around 75%. There was no chemical interaction between the drug and lipids used are confirmed by FTIR and DSC studies. The *in vitro* study shows sustain release of FT liposomal suspension at pH 7.4 buffer. Liposomal suspension was stable when stored at 2-8°C as aqueous dispersion for 3 months. The *in vitro* cytotoxixity shows an enhanced cytotoxicity of FT liposomal suspension on both the cell lines. In cell cycle studies FT liposomes shows an enhanced apoptosis in latter G1 and S phase on both the cell line, FT liposomal suspension shows an improved specific targeting to HeLa cervical cell line by arresting at latter G1 and S phase at 4hrs, this may be because of folate receptor is a tumor marker that is consistently over expressed HeLa then A549. FT liposomes shows improved cellular uptake and liposomal internalization compared to SL and CL liposomes. These results indicated that FT liposomes have efficient interactions with the cellular folate receptor. FT liposomes shows an increases caspase-3 activity in A 549 cell line, Caspase-3 is a frequently activated death protease important for cell death. From the pharmacokinetic, gamma imaging and radiolabeling biodistribution studies, FT liposomes shows an increase circulation half life, AUC, tumor uptake by this it has been concluded the FT liposomes shows an improved in tumor specific delivery.

There would a considerable improvement over existing therapies because of putative advantage in dose, dosing schedule, patient compliance and reducing toxicity.

8.3 Future Direction

- ✓ Pharmacodynamamic studies would be conducted in mice model to investigate the *in vivo* behavior of liposomal formulation.
- ✓ Alternative surface modulation to improve the targeting efficiency by transferrin-conjugated or by conjugating Fab' fragments of a recombinant humanized anti-HER2 MAb to the distal termini of poly(ethylene glycol) chains on the surface of unilamellar liposomes.