

# Chapter - 1

## *Introduction*



### 1.1. Introduction

Cancer being one of the disastrous diseases to mankind from centuries even after a lot of research work carried out in this field. Solid tumors have historically provided many challenges to systemic therapy. Theoretical barriers to drug penetration within solid tumors interstitium results from high interstitial pressures and large interstitial space compared with normal tissue, particularly in necrotic core. Vascular permeability in tumors is heterogeneous with respect to tumor type, location of the vessel within the tumor, and the tumor microenvironment (Jain, 1989; Yuan et al., 1994) will help engineer liposomes for more effective delivery of their contents to the tumor.

Antineoplastic agents used in the treatment of lung cancer, solid tumor, testicular cancer, breast cancer, several types of leukemia, lymphoma and etc. have often associated with number of severe toxicities such as bone marrow depression results in granulocytopenia, agranulocytosis, thrombocytopenia, and aplastic anaemia, lymphocytopenia and inhibition of lymphocyte function results in suppression of host immunity (Tripathi, 2004).

Cancer chemotherapy is generally accompanied by lack of specificity and systemic side effects. If an anticancer drug could deliver only the right site in the right concentration at the right time, cancer could be cured without side effects. For such delivering system, liposomal formulation is thought to be useful since liposomes are essentially non-toxic and biodegradable, their size, components, and modifications with various molecules are easily controlled, and they could deliver the large amount of either hydrophilic or hydrophobic agents (Oku, 1999).

The concept of site specific drug delivery for treatment of localized disease in the body to improve therapeutic index of the drug is considered as perennial challenge to the formulator in modern formulation design. Constant efforts have been pursued in designing such an ideal drug delivery system which can effectively overcome dose related toxicity and adverse side effects and thus improve patient compliance (Chien, 1992). One such area which has attracted ever growing attention of pharmaceutical scientist and has shown tremendous potential and promise is colloidal drug carrier system (Crommelin and Schreier, 1994).

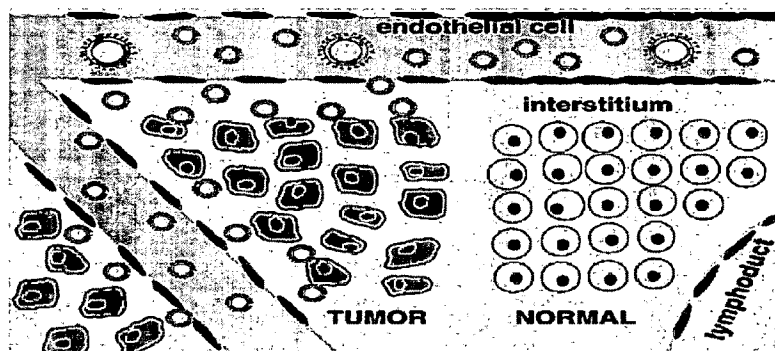
The idea of drug carrier with targeted specificity has fascinated scientists for number of years and in the last decade successful efforts have been made to achieve this goal (Justin et al., 2003). The ultimate form of targeted drug delivery system should be realization of Paul Ehrlich's "magic bullet concept" (Vyas and Khar, 1997) which documents the delivery of drug exclusively to a preselected targeted cell type.

Amongst all targeted drug delivery systems, liposomes have gained popularity because of their biological inert nature, freedom from antigenic, pyrogenic or allergic reaction and their enhanced stability (Jain and Jain, 1997). Liposomes are micro-particulate or colloidal carriers which form spontaneously when certain lipids are hydrated in aqueous media (Sharma and Sharma, 1997). Liposomes are composed of relatively biocompatible and biodegradable material and they consist of aqueous volume trapped

by one or more bilayers of natural or synthetic lipids. Highly lipophilic drugs, with partition coefficient greater than 5 are entrapped almost completely in the lipid bilayer of liposomes.

The delivery of liposomes at the appropriate site, however, is still not achieved. For this purpose, both active targeting and passive targeting are considered. Conventional liposomes, however, tend to be trapped by the reticuloendothelial system (RES) such as liver and spleen before encountering the target. On the contrary, passive targeting, especially targeting to tumor tissues, could be achieved by reducing the RES trapping, since the vasculature in the tumor tissues is leaky enough to extravasate liposomes and circulating liposomes may accumulate passively in tumor tissues (Figure 1.1) (Oku, 1999). The development of liposomes containing lipid derivatives of PEG or saturated phospholipids such as DSPC with cholesterol has made targeted liposomal therapy more feasible by reducing the uptake by the RES system and there by prolonging the circulation time (Lundberg et al., 2000).

Particularly, PEG is useful because of its ease of preparation, relatively low cost, controllability of molecular weight and linkability to lipids or protein including the antibody by a variety of methods. The presence of PEG reduces binding of serum protein, i.e. opsonins marking the liposome for clearance by macrophages. It has been proposed that antibodies should be attached to the distal end of PEG chains which are already bound to the liposome membrane (Maruyama et al., 1999). In the proposed research, we have selected PEG derivative, DSPE-PEG<sub>2000</sub>, in order to prepare long circulating (RES avoiding) liposomes.

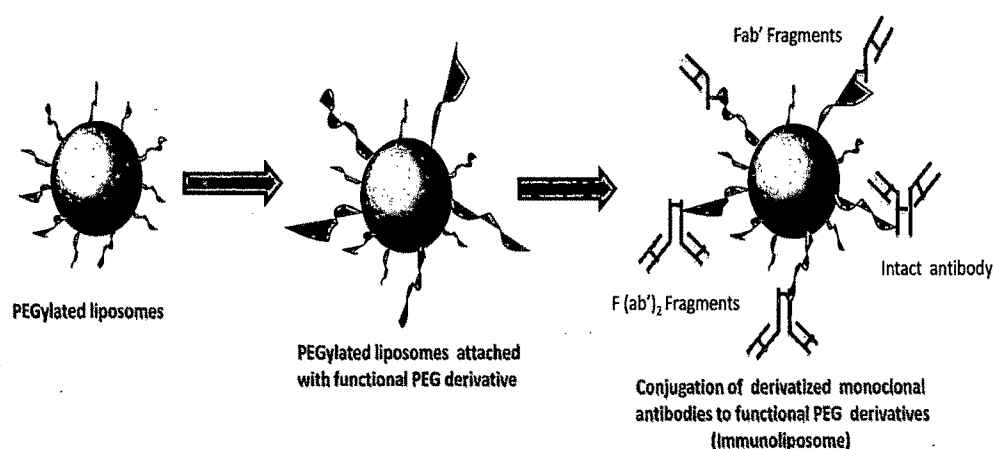


**Figure 1.1.** Passive accumulation of liposomes at tumor through leaky tumor endothelium

Active targeting of liposomes to tumor cells is generally attempted by conjugating ligands to the liposomal surface which allow a specific interaction with the tumor cells. Several types of ligands have been used for this purpose, including antibodies or antibody fragments, vitamins, glycoproteins, peptides (RGD-sequences), and oligonucleotide aptamers. Among the different approaches of active targeting, immunoliposome using antibody or antibody fragment as a targeting ligand and a lipid

vesicle as a carrier for both hydrophilic and hydrophobic drugs, is a fascinating prospect in cancer therapy (Figure 1.2). The process of targeted drug delivery with immunoliposomes can be roughly divided into two phases: the transport phase, in which the immunoliposomes travel from the site of administration (often i.v. administration) to the target cells, and the effector phase that includes the specific binding of immunoliposomes to the target cells and the subsequent delivery of entrapped drugs (Mastrobattista et al., 1999).

Immunoliposomes for the treatment of tumor should satisfy a number of requirements aimed at maximum targeting effect of immunoliposome administered systemically in the bloodstream. Antigen binding site of the liposome-conjugated antibody must be accessible for unperturbed interaction with antigen on the surface of target cells. The blood clearance of immunoliposomes must be minimized in comparison with rate of extravasation in the tumor. Immunoliposome must allow efficient loading and retention of a selected anticancer drug. And finally, the drug and antibody incorporation must be stable enough to permit liposomal entry into the tumor tissue without the loss of either of these agents (Maruyama et al., 1999).



**Figure 1.2.** Preparation of PEGylated liposomes and conjugation of intact or derivatized monoclonal antibodies (PEGylated Immunoliposomes) through various functionalized PEG derivatives (Manjappa et al., 2011)

**DOCETAXEL:** The cytotoxic activity of docetaxel is exerted by promoting and stabilising microtubule assembly, while preventing physiological microtubule depolymerisation/disassembly in the absence of GTP. This leads to a significant decrease in free tubulin, needed for microtubule formation and results in inhibition of mitotic cell division between metaphase and anaphase, preventing further cancer cell progeny (Tripathi, 2004).

Docetaxel (trademarked as Taxotere® by Rhone-Poulenc Rorer now it is Sanofi Aventis) was approved by the FDA for the treatment of advanced ovarian cancer in April 1994

and in December 1999 for the treatment of patients with locally advanced or metastatic non-small cell lung cancer. Taxotere is approximately twice as potent as taxol in inhibiting cold and calcium-induced depolymerization of microtubules (Gueritte et al. 1993). The clinical application of docetaxel (DTX) is limited by the poor aqueous solubility (7 $\mu$ g/mL) (Du et al., 2007; Lili et al., 2011), low bioavailability and high toxicity. Presently used Taxotere® and Duopafei® in clinical contain high concentration of non-ionic surfactant tween-80. The adverse reactions due to either the drug itself or the solvent system have been reported in patients (e.g., hypersensitivity, fluid retention, neurotoxicity, musculoskeletal toxicity and neutropenia) (Chu et al., 2000). In order to eliminate the tween-80-based vehicle and increase the drug solubility, alternative dosage forms have been developed, such as microparticulate lipoidal vesicles (liposomes) (Naik et al., 2010; Zhai et al., 2010), cyclodextrins (Grosse et al., 1998), polymeric nanoparticles (Hwang et al., 2008), micelles (Li et al., 2008), solid lipid nanoparticles (SLN) (Xu et al., 2009) and nanostructured lipid carriers (NLC) (Li et al., 2009). Among these forms, liposomes, NLC and SLN, belong to lipid-based nanocarriers, have favourable characteristics such as: (a) improved drug dispersibility; (b) enhanced drug solubilization; (c) enhanced drug transmembrane transport capability and (d) increased therapeutic efficacy and reduced toxicity.

Therefore, to overcome conventional chemotherapy and Taxotere associated problems, in the present study, we have prepared DTX loaded PEGylated liposomes composed of mixture of phospholipids (HSPC, DPPC and DPPG), as the combination of more than one lipid can increase the hydrophobic drug loading (Chen et al., 2006; Kan et al., 2011).

Angiogenesis is defined as the formation of new blood vessels from existing ones. For solid tumors (1-2mm<sup>3</sup>), oxygen and nutrients can reach the center of the tumor by simple diffusion. Because of their non-functional or non-existent vasculature, non-angiogenic tumors are highly dependent on their microenvironment for oxygen and the supply of nutrients. When tumors reach 2mm<sup>3</sup>, a state of cellular hypoxia begins, initiating angiogenesis (Fabienne et al., 2010). Neuropilins (NRP-1 and NRP-2) are membranous receptors capable of binding two disparate ligands, class 3 semaphorins (SEMA 3A) and vascular endothelial growth factors (VEGF-A165), and regulating two diverse systems, neuronal guidance and angiogenesis. NRP-1 is expressed by a wide variety of human tumour cell lines and diverse human neoplasms, and are implicated in mediating effects of VEGF and Semaphorins on the proliferation, survival and migration of cancer cells. NRP1 is expressed in patient specimens from lung, breast, prostate, pancreatic and colon carcinomas. NRP1 has also been found in several other tumors including melanoma, astrocytoma and neuroblastoma. These findings taken together with the expression of NRPs in diverse neoplasms, suggests a possible role for this molecule in tumour invasion and metastasis in addition to its involvement in tumour vascularisation (Bielenberg et al., 2006).

In the present research project, we have formulated long circulating immunoliposomes, by attaching PEG derivatives (DSPE-mPEG<sub>2000</sub>) and then by attaching anti-neuropilin-1

antibody to the distal end of functionalized PEG chain (DSPE-PEG<sub>2000</sub>-Maleimide) through thioether linkage, for site specific delivery of docetaxel. Developed long circulating immunoliposomes are expected to have the following advantages over conventional chemotherapy and conventional liposomes.

- Reduction in toxicity of encapsulated agent
- Increased efficacy and therapeutic index
- Decreased RES uptake and prolonged circulation time
- Selective active targeting to tumor tissues
- Reduced Taxotere vehicle related toxicities

### **Hypothesis of Treatment**

- The neuropilin-1(NRP-1) antibody conjugated liposomes bind to NRP-1 receptor on endothelial cells of tumor blood vessels and undergo endocytosis. The lysosomal lysis of immunoliposomes thereby releasing of loaded docetaxel causes cytotoxicity. This vascular damage will enhances the leaky nature of tumor blood vessels and leads to excessive accumulation of immunoliposomes in solid tumor.
- By destroying tumor endothelial cells (blood vessels), they indirectly kill the tumor cells that these vessel support by supplying nutrients and oxygen.
- The accumulated immunoliposomes function as a sustained release system, resulting in direct cell kill, including cytotoxicity against cells that are at the tumor periphery and are independent of the tumor vasculature.
- The most of the cancer cells express neuropilin-1 receptor. Therefore, the accumulated immunoliposomes in solid tumor undergo endocytosis, lysosomal damage, and release of loaded drug in the cytoplasm cause cytotoxicity.

This combined strategy has the potential to overcome some major limitations of conventional chemotherapy.

## 1.2. Objectives of the work

The prime objectives of the present research are as follows.

1. Formulation optimization of docetaxel loaded conventional liposomes using saturated phospholipids by thin film hydration method
2. Formulation of docetaxel loaded long circulating liposomes using DSPE-mPEG<sub>2000</sub> by pre-insertion technique
3. Optimization of DSPE-mPEG<sub>2000</sub> concentration on liposomal surface for enhanced *in vitro* steric stability
4. Development of immunoliposomes by conjugating Fab' fragments of anti-neuropilin-1 antibody, as the recognition moiety, over the PEGylated liposomes using functionalized PEG derivative (DSPE-mPEG<sub>2000</sub>-Maleimide) *via* thioether linkage
5. Characterization of developed PEGylated liposomes and anti-neuropilin-1 immunoliposomes for mean particle size, zeta potential, drug content, and *in vitro* drug release
6. Determination of neuropilin-1 receptor expression on human adenocarcinoma cell line (A549) and mouse melanoma cell line (B16F10)
7. *In vitro* cell culture studies on human adenocarcinoma cell line (A549) and mouse melanoma cell line (B16F10) to evaluate PEGylated liposomes, and anti-neuropilin-1 immunoliposomes for cytotoxicity, intracellular uptake, interaction with matrix metalloproteinases (MMP 9 and MMP 2), anti-metastatic activity, and effect on apoptosis and cell cycle
8. *In vivo* evaluation of docetaxel loaded PEGylated liposomes, and anti-neuropilin -1 immunoliposomes for tumor regression and anti-angiogenesis performance in tumor (B16F10 melanoma) bearing mice.

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