



Chapter 1
Introduction



1.1 Introduction

Despite of intense research and recent advances in drug delivery, the effective and non-toxic delivery of chemotherapeutic agents for the treatment of cancer remains a challenge for pharmaceutical industry. Cancer is one of the most disastrous diseases for mankind from centuries even after exhaustive research in this arena. American cancer society (ACS) estimated that in 2014 about 1,665,540 new cancer cases are estimated to be diagnosed and about 585,720 are expected to die of cancer, almost 1,600 people per day in United States (US) (1). There are more than 100 types of cancer, including cancer of breast, lung, skin, blood, colon and prostate.

Breast cancer is most frequently diagnosed cancer and now the leading cause of cancer deaths among women (2). It is a malignant tumor that starts in the cells of the breast and grows into nearby tissues or spread to distant areas of the body. All women are susceptible to breast cancer, but men can get it too. ACS estimated that in 2014 about 235,030 (232,670 women and 2,360 men) new cases of invasive breast cancer are expected to be diagnosed and about 40,430 (40,000 women, 430 men) are expected to die of cancer in US (1). Lifetime risk of a woman developing invasive breast cancer is 12.6 % and one out of eight females in US developing breast cancer (3). Looking at 2011 Indian statistics, more than 40,000 breast cancer deaths were recorded. It is estimated that around 100,000 to 125,000 new breast cancer cases would develop in India every year (4) and number of breast cancer cases in India would be doubled by 2025 (5).

Cancer can be treated by surgery, radiation therapy, hormone therapy, chemotherapy, biologicals, and targeted therapies. Chemotherapy involves the use of anti-cancer drugs to destroy cancer cells. However the cancer chemotherapy is generally accompanied by lack of specificity and is often associated with numerous severe toxicities including bone marrow depression, aplastic anaemia, lymphocytopenia and inhibition of lymphocyte function resulting in suppression of host immunity. If an anticancer drug is delivered at the right site of action in the right concentration at the right time, cancer can be treated without any lateral effects (6). Breast cancer can be treated by surgery, chemotherapy, radiation therapy, hormone therapy, targeted, biological and bone directed therapies. These therapies are classified based on how they work and when they are used such as local versus systemic therapy and adjuvant versus neoadjuvant therapy. Various anticancer drugs like docetaxel, paclitaxel, doxorubicin, vinorelbine, epirubicin and tamoxifen are commonly used in treatment of breast cancer.

In this project, docetaxel and vinorelbine have been selected for development of protein nanoparticulate system. Currently marketed formulations of these drugs are limited by high toxicity, rapid elimination from the systemic circulation, accumulation in non-targeted organs and tissues, enzymatic and hydrolytic degradation, and/or inefficient cell entry. Additionally polysorbate 80 used in commercial formulation of docetaxel (Taxotere®) is reported to exhibit serious adverse effects including hypersensitivity reactions and peripheral neuropathy in human (7) whereas the commercial formulation of vinorelbine tartarate (Navelbine®) are known to cause injection-site reactions including transient local pain, swelling, erythema and the local reactions including phlebitis (8).

Albumin (Protein) nanoparticles encapsulating above cancer therapeutics is an appropriate option to overcome the limitations of currently available chemotherapy. Because protein nanoparticulate system offers various advantages including protection of therapeutic agents from degradation in the biological environment and enhancement of cellular uptake. By developing protein nanoparticulate system problems associated with free drug and solvents/surfactants used in delivery of chemotherapeutic agents can be prevented. Binding/encapsulation of anticancer drug to protein nanoparticles may diminish their toxicity, increase bioavailability, optimize their body distribution and may overcome multidrug resistance. Further surface modification of protein nanoparticulate system using receptor specific ligand help targeting cytotoxic drug to tumor and reduce the toxicity to the surrounding tissues. This would be of considerable improvement over existing therapies because of putative advantage in dose, dosing schedule, patient compliance and reducing toxicity (9).

Albumin based nanoparticles offer various possibilities for surface modification due to the presence of functional groups (i.e. carboxylic and amino groups) on the surface of the nanoparticles (10) which help tumor specific targeting. This inturn leads to better pharmacokinetics and pharmacodynamics profiles providing controlled and sustained release of drugs. Also improved site specific delivery, increased internalization, intracellular delivery and most importantly lower systemic toxicity. The tumor targeting consists of “passive targeting” and “active targeting”; however, the active targeting process cannot be separated from the passive targeting because active targeting occurs only after passive accumulation in tumors. Mechanism of passive and active targeting is shown in **Figure 1.1**.

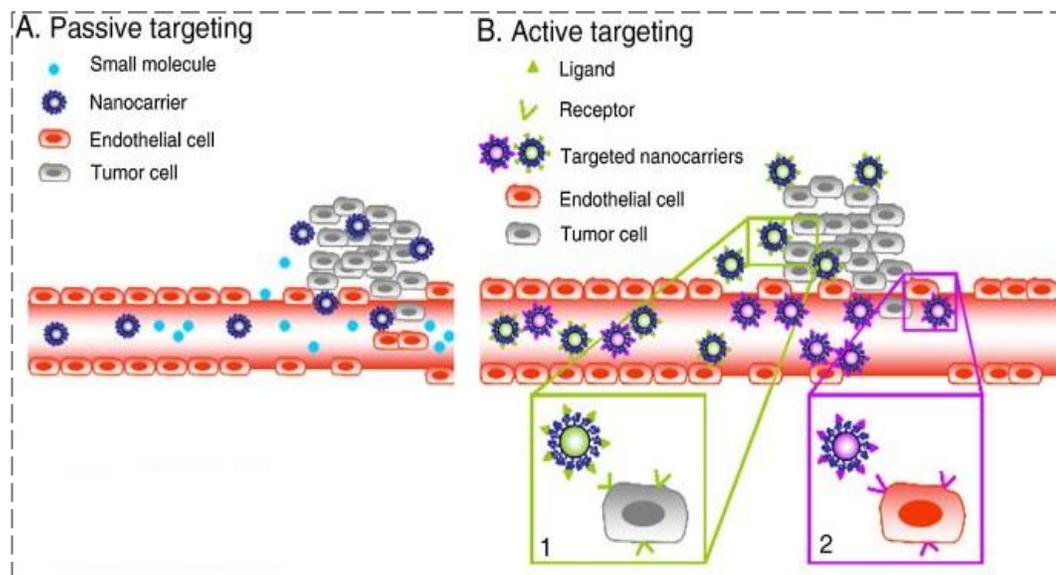


Figure 1. 1 Mechanism of passive and active targeting

Active targeting involves exploiting the presence of overexpressed receptors on cell surface of tissues being targeted. The epidermal growth factor receptor (EGFR) is a cell-surface receptor belonging to ErbB family of tyrosine kinase and it plays a key role in the regulation of cell proliferation, survival and differentiation. EGFR is aberrantly activated by several mechanisms including receptor overexpression, mutation, ligand-dependent receptor dimerization and ligand-independent activation which lead to development of variety of tumors (11). Tumors expressing EGFR in breast cancer is 14 to 91% (12). Therefore, specific EGFR inhibition is one of the key targets for cancer therapy. The receptor has several natural ligands including epidermal growth factor (EGF) and transforming growth factor- α (TGF- α). Binding of ligand to EGFR promotes receptor autophosphorylation that stimulates cell proliferation. EGFR comprises of an extracellular ligand-binding domain, a hydrophobic membrane-spanning region, and an intracellular tyrosine kinase domain. Monoclonal antibody-mediated blockade of the extracellular ligand-binding domain and small-molecule inhibition of the intracellular tyrosine kinase domain are the most clinically advanced EGFR inhibition strategies. Blocking EGFR by means of a monoclonal antibody directed against the receptor inhibits tumor growth *in vivo*. Monoclonal antibody that binds to the EGFR with high affinity blocks the ligand binding and induces receptor internalization and degradation, resulting in downregulation of surface EGFR expression. By combination of EGFR targeting agents and cytotoxics synergy can be attributed to a great extent on cell division, apoptosis and angiogenesis (13).

Several anti-EGFR monoclonal antibodies have been used for cancer treatment in patients but they have a comparatively large size [Immunoglobulin G (IgG) antibody has an average size of $14.5 \times 8.5 \times 4 \text{ nm}^3$ and a molecular weight of 160 kDa (14, 15)] limiting the number of ligands that can be linked to the surface of a nanoparticle and obstructs the intratumoral distribution owing to interstitial tumor pressure. Generation of single-chain fragment variable (scFv) consisting of antibody heavy and light-chain variable domains coupled with a flexible peptide linker for EGFR targeting is a virtuous option, as the single chain anti-EGFR antibody (scFvEGFR) provides a much smaller ligand for targeting. The resulting antibody fragment (scFvEGFR) having molecular weight 25 to 28 kDa which is smaller than 20% of an intact antibody but retains a high binding affinity and specificity (16, 17). This is the pioneering work carried out by conjugating single chain anti-EGFR antibody with protein nanoparticles for breast cancer targeting and can be classified as SMART drug delivery.

Docetaxel (DTX)

DTX belonging to the taxane class of anticancer agent is the most active chemotherapeutic agent for treatment of breast cancer (18, 19). The cytotoxic activity of DTX is exerted by promoting and stabilizing the cellular microtubule assembly thus preventing the physiological microtubule depolymerization essential during cell division (20). 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 (21, 22). DTX (trademarked as Taxotere[®]) was approved by the FDA in April 1994 for the treatment of advanced ovarian cancer and in December 1996 for the treatment with locally advanced or metastatic breast cancer. The clinical applications of DTX are limited by its poor aqueous solubility ($7 \mu\text{g/mL}$) (23, 24) and high toxicity. Clinically used Taxotere[®] and Duopafei[®] formulations of DTX contain high concentration of non-ionic surfactant tween-80 and having various reported adverse reactions including hypersensitivity, fluid retention, neurotoxicity, musculoskeletal toxicity and neutropenia etc (25). In order to eliminate the tween-80-based vehicle and to increase the drug solubility, various alternative novel dosage forms have been developed, such as liposomes, polymeric nanoparticles, solid lipid nanoparticles, micelles and nanostructured lipid carriers. Among these forms, nanoparticles have favorable characteristics to enhance therapeutic efficacy and reduce toxicity.

Vinorelbine Tartrate (VBT)

Vincristine, vinblastine and vinorelbine are the three kinds of vinca alkaloids and are one of the most widely used classes of antineoplastic agents (26). Vinca alkaloids act by binding to tubulin and preventing its assembly into microtubules, ultimately leading to inhibition of cell growth at metaphase and induction of apoptosis (27). Vinorelbine (5 nor-anhydro-vinblastine) is a clinically used in the treatment of various cancers, including metastatic breast cancer (28) and non-small cell lung cancer (29). In vinca alkaloids, vinorelbine is better tolerated because of reduced neurotoxicity (30) due to its reduced affinity for axonal microtubules (31).

An injectable formulation of VBT (Navelbine[®] IV) developed by Pierre Fabre Medicament France, is widely marketed for the treatment of cancer in many countries (32, 33). However, short-duration and non-cumulative granulocytopenia are the major dose-limiting toxicities, and other side effects, such as nausea and vomiting, constipation, peripheral neuropathy and mild reversible alopecia (34). Moreover, Navelbine[®] IV is not an optimal drug delivery system because of its vesicant action. It is very well known that this action tends to cause venous irritation and phlebitis when Navelbine[®] IV is directly administered intravenously as an aqueous solution. Venous irritation is reported as injection site reactions, local reactions, and superficial phlebitis, whereby the symptoms include erythema, pain at the injection site, vein discoloration and tenderness along the vein (34). Thus, it is necessary to find a new strategy to reduce the venous irritation produced by aqueous injections of VBT. The albumin nanoparticles have favorable characteristics to reduce the severe venous irritation, enhance the anti-tumor activity and improve patient tolerance.

1.2 Objectives

The prime objective of this study is to develop protein conjugated nanoparticulate formulation for safe and effective management of breast cancer.

- To develop biocompatible and biodegradable protein nanoparticulate drug delivery system encapsulated/entrapped with cytotoxic drug to improve the safety and efficacy of drugs.
- To increase drug concentration in tumor through active targeting of nanoparticles by conjugation of single chain anti-EGFR antibody to drug loaded protein nanoparticles.
- To characterize and evaluate the designed drug delivery system for their physicochemical parameters, stability and *in-vitro* drug release characteristics.

- To study the cell uptake, cell viability, cell cycle analysis and apoptosis of developed nanoparticulate formulation in breast cancer cell lines.

1.3 Hypothesis of Treatment

- Developed stable albumin nanoparticulate formulation can pass through the leaky capillary junctions in the tumor bed more easily than through the normal vessels in healthy tissue and taken selectively by the tumor tissues and cells because various proliferating tumors are known to accumulate albumin as source of energy (nitrogen source for de nova protein synthesis).
- The single-chain anti-EGFR antibody (scFv EGFR) conjugated albumin nanoparticles bind to and are internalized by EGFR-expressing tumor cells.
- Accumulated Immunonanoparticles (INPs) in solid tumor will undergo endocytosis, lysosomal damage, and release of loaded drug in the cytoplasm may cause cytotoxicity.
- The accumulated INPs function as a sustained release system, resulting in direct cell kill, including cytotoxicity against cells that are at the tumor periphery.

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

1.4 Plan of Work

1. Literature review, procurement of APIs and excipients.
2. Development of analytical method for the estimation of drugs.
3. Preliminary trials for screening of process and formulation variables.
4. Formulation of nanoparticles by high pressure homogenization and desolvation method.
5. Optimization of nanoparticles by application of statistical designs.
6. Characterization (Particle size, Zeta potential, TEM, XRD and DSC) and *in vitro* drug release studies of formulation in comparison with marketed formulations.
7. Cell line studies: Quantitative uptake studies by FACS, Qualitative uptake studies by confocal laser microscopy, Cytotoxicity studies by MTT assay and Apoptosis studies by FACS in breast cancer cell lines.
8. Stability study of optimized nanoparticulate formulation.
9. *In vivo* pharmacokinetic and toxicity studies of drug loaded albumin nanoparticles.

1.5 References

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