Chapter 1

INTRODUCTION

1.1 Introduction

Cancer is a major cause of death in India. Every year about 8,50,000 new cancer cases are diagnosed in India resulting in about 5,80,000 cancer related death every year. In females, Cervical (30%) and Breast cancers (19%) are the two main causes of cancer related illnesses and death. Despite the significant progress in the development of anticancer technology, there is still no common cure for patients with malignant diseases. In addition, the long-standing problem of chemotherapy is the lack of tumor-specific treatments. Traditional chemotherapy relies on the premise that rapidly proliferating cancer cells are more likely to be killed by a cytotoxic agent. In reality, however, cytotoxic agents have very little or no specificity as very less concentration of drug reaches tumor site, which leads to systemic toxicity.

The causes of breast cancer are still unknown, but there is a combination of risk factors including lifestyle factors, environmental factors, genetic factors, and hormonal factors that may be responsible for breast cancer. Although many risk factors are associated with breast cancer, it is not yet known exactly how these risk factors cause normal breast cells to become cancerous. The molecular biology of breast cancer is complex as multiple factors contribute in the development of breast cancer such as genetic mutations in BRCA1, BRCA2 and p53 and cross-talks between different signalling pathways. Cell-signalling pathways allow normal programs of proliferation, transcription, growth, migration, differentiation and death in the normal cell. But in the case of breast cancer cells, these normal programs are altered by altering cell-signalling pathways. Various signalling pathways that play an important role in development and progression of breast cancer are initiated by the interaction between growth factors and their receptors, such as human epidermal growth factor receptors (HER2 and VEGF) and their ligands, as well as insulin-like growth factor (IGF) and IGF-1R.

Specific molecular targets having critical roles in cancer proliferation are interfered by targeting drugs. Some important therapeutic targets in breast cancer are HER-2, vascular endothelial growth factor (VEGF), insulin-like growth factor binding proteins-3 (IGFBP-3), estrogen receptor (ER), gene silencing by siRNA and aptamer.

Estrogens and progesterone are important growth regulators in the development of breast cancer. In breast cancer, the majority of tumors found in postmenopausal women contain ER while tumors in younger women often lack this protein. Approximately 70% of all breast cancers retain the estrogen receptor α (ERα, encoded by ESR1) and the

progesterone receptor (PR) [Arpino 2005]. ER is a protein normally found in various reproduction-related tissues such as the breast and uterus. When estrogen receptor binds estrogen, it becomes activated and can interact with the genes of the cell, resulting in the activation of selected sets of responsive genes. This results in changes in the synthesis of specific RNA's and proteins involved in the regulation of cell proliferation, differentiation and physiologic function. Although normal breast tissue also makes ER, the amount of this protein produced in positive breast carcinomas is significantly higher. This may account for some of the differences seen in the abnormal growth of various tumors and tumor cell lines when compared to normal breast tissue development [Kocbek P 2007].

Clinical data indicates ER^+/PR^- breast cancer are more likely to have an aggressive phenotype to express HER-1 and over express HER-2. These tumors are more frequent in older patient, larger in size and have a higher S-phase fraction. Recurrence rate of tumor is higher in ER^+/PR^- than ER^+/PR^+ [Bonneterre J 2001]. There was little difference in the recurrence rate of PR^+ versus PR^- tumors in patients treated with anastrozole. The patients with ER^+/PR^- tumors respond nearly as well to anastrozole as those with ER^+/PR^+ tumors suggesting that the ER signalling pathway is functional in many ER^+/PR^- tumors and these tumors are still dependent on estrogen for growth despite having somewhat lower ER levels. ER^+/PR^- tumors were three times more likely than ER^+/PR^+ tumors to express HER-1 (25% versus 8%, respectively), and the levels of HER-1 in ER^+/PR^- tumors (40 fmol/mg protein) were nearly twice those in ER^+/PR^+ tumors (24 fmol/mg protein). ER^+/PR^- tumors were also statistically significantly more likely to over express HER-2 (21% for ER^+/PR^- versus 14% for ER^+/PR^+). Both HER-1 and HER-2 are markers of tumor aggressiveness, and therefore, are more likely to have an intermediate or high S-phase fraction than tumors negative for these two growth factors, regardless of PR status.

Endocrine therapies are effective in reducing recurrence, increasing overall survival, and reducing contralateral breast cancer up to 50%. However, about 50% of patients with ERα-positive breast cancer have intrinsic resistance to antiestrogen therapy and therefore do not benefit. In contrast to patients with intrinsically resistant tumors, there are patients who do initially respond to antiestrogen therapy; however, most of these patients develop acquired resistance during the treatment regimen. Therefore, the current goal in breast cancer research is to elucidate the mechanisms of both intrinsic

and acquired resistance to tamoxifen and the aromatase inhibitors in order to develop new therapeutic strategies to prevent and/or treat resistant breast cancer.

An alternate strategy to endocrine therapy, which specifically inhibits binding of E2 to the ER, is to inhibit the production of E2 by blocking the cytochrome p450 aromatase enzyme, the rate-limiting enzyme that converts androgens (i.e., testosterone and androstenedione) to estrogens (i.e., E2 and estrone) in the adrenal gland, surrounding stroma, and adipose tissue of the breast tumor. The main drugs of this type are aromatase inhibitors, which include Type I (steroidal) or Type II (nonsteroidal). Steroidal inhibitors are competitive-substrate mimics of androstenedione. These include formestane and exemestane, which are irreversible inhibitors that bind with high affinity to the binding site of aromatase and are converted to a covalently bound intermediate. Nonsteroidal inhibitors include the first-generation aromatase inhibitor aminoglutethimide and the second-generation compounds anastrozole and letrozole. All nonsteroidal aromatase inhibitors act by binding reversibly to the enzyme and competitively inhibiting binding of the substrate androstenedione. The benefits of using aromatase inhibitors over tamoxifen are believed to be the complete deprivation of E2 and thus better efficacy for ERα-positive breast cancer. Recent clinical data have clearly demonstrated that anastrozole is more effective than tamoxifen as first-line treatments in patients with metastatic breast cancer.

Two very small trials demonstrated a significantly increased clinical response rate in patients with ER-positive, HER2-positive cancers treated with preoperative aromatase inhibitors compared to preoperative tamoxifen. This led to a widely accepted hypothesis that aromatase inhibitors were a better choice than tamoxifen in patients with ER-positive, HER2-positive cancers. Treatment of breast cancer has included efforts to decrease estrogen levels, by ovariectomy pre-menopausally and by use of anti-estrogens and progestational agents both pre- and post-menopausally; and these interventions lead to decreased tumor mass or delayed progression of tumor growth in some women. Tamoxifen is a selective estrogen-receptor modulator [Leserman], which blocks estrogen from attaching to estrogen receptors on breast cancer cells and acts as an anti-estrogen. Tamoxifen increases the risks of uterine cancer, thromboembolism, and tamoxifen resistance. In postmenopausal women, ovaries do not produce estrogen. A small amount of estrogen is produced by the adrenal glands. Aromatase inhibitors block estrogen production [Sharaf 2006]. Anastrozole, a product of aromatase

inhibitors, has fewer side-effects than tamoxifen but it causes joint pain or bone fractures.

Currently, various conventional therapies like radiation therapy, chemotherapy, hormonal therapy, and immunotherapy are used for the treatment of breast cancer. Cancer cells that may not be seen during surgery can be killed by radiation to reduce the risk of local recurrence of cancer. Radiation therapy is a process in which cancer cells are exposed to high levels of radiation directly. Radiation therapy after surgery shrinks the tumor in combination with chemotherapy. But there are some side-effects of radiation therapy, such as decreased sensation in the breast tissue or under the arm, skin problems in the treated area (including soreness, itching, peeling, and/or redness) and at the end of treatment the skin may become moist and weepy. In chemotherapy cytotoxic drugs are administered to kill cancer cells. Chemotherapy may be recommended as adjuvant chemotherapy or neoadjuvant chemotherapy. Adjuvant chemotherapy is the systemic therapy given to patients after surgery to treat undetected breast cancer cells. Neoadjuvant chemotherapy is given before surgery to shrink large cancers so that they can easily be removed by lumpectomy. It is reported clinically that chemotherapy is most effective when given in combinations of more than one drug. The most common side-effects are hair loss, mouth sores, loss of appetite, nausea, vomiting, increased chance of infections (due to low white blood cell counts), easy bleeding (due to low blood platelet counts) and fatigue.

The purpose of hormonal therapy is either adding or blocking hormones. The female hormones estrogen and progesterone can promote the growth of some breast cancer cells. Therefore hormone therapy is required to block or lower the levels of estrogen and progesterone to prevent growth of cancer cells. Several types of hormonal drugs used for primary breast cancer include Tamoxifen, Toremifene, Arimidex, Zoladex, etc. These targeted therapies should allow action with high efficacy and less toxicity. Several nanotechnological approaches have been used to improve targeted delivery of a potent anticancer drug to breast cancer cells with minimum toxic effects on healthy tissues while maintaining efficacy. Nanotechnology is developing a new generation of more effective therapies by using nanocarriers that are capable of overcoming the biological, biophysical, and biomedical barriers in treatment of breast cancer. Nanocarriers show much promising breast cancer therapy by selectively reaching the desired specific sites due to their small size and surface modifying properties with multifunctionality. This

article highlights recent approaches that can be targeted and are clinically applicable for treatment of breast cancer.

An ideal drug-delivery system should possess two elements: the ability to target and to control the drug release. Targeting will ensure high efficiency of the drug and reduce the side effects, especially when dealing with drugs that are presumed to kill cancer cells but can also kill healthy cells when delivered to them. The reduction or prevention of side effects can also be achieved by controlled release. Nanoparticulate drug delivery systems provide a better penetration of the particles inside the body as their size allows delivery via intravenous injection or other routes. The nanoscale size of these particulate systems also minimizes the irritant reactions at the injection site. Nanoparticles (NPs) may be targeted to the growing vasculature serving the growing cancer or to the cancer cells themselves. Targeted delivery utilizes unique phenotypic features of diseased tissues and cells in order to concentrate the drug at the location where it is needed. Targeted delivery can be divided into passive and active targeting. Passive targeting tries to minimize nonspecific interactions between the drug carrier and nontarget sites in the body by detailing the physiochemical properties of the aberrant tissue such as size, morphology, hydrophilicity, and surface charge. When targeting tumor tissue, the enhanced permeability and retention effect (EPR) is an example of passive targeting approach; it allows passage of drug carriers ranging in size from 10 to 500 nm through the highly permeable blood vessels that supply growing tumors and leads to entrapment of large molecules as a result of deficient lymphatic drainage.

It is known that plasma retention time of the NPs is one of the primary driving forces for tumor accumulation by EPR [Modi et al. 2006]. In fact, one prerequisite for the EPR effect to manifest in mice is that the plasma concentration of the drug must remain high for more than 6 h [Iyer et al. 2006]. Because of their size, PEGylated NPs not only remain in circulation longer, giving them more time to accumulate in the tumor by EPR effect, but also take longer to leave the tumor and return to circulation. This extended tumor cell contact time can conceivably allow more of the ligand conjugated NP complex to bind to the tumor cells. Concomitantly, the polyethylene glycol (PEG) complex also takes longer to return to circulation [Bartlett et al. 2007; Kirpotin et al. 2006]. Support for this theory can be found in a publication by Khalid et al., who reported that tumor localization of a lipid NPs carrying docetaxel was not only

enhanced by inclusion of PEG but also increased with the PEG density on the particle over a range of 6 to 15 mol % [Khalid et al. 2006]. Similarly, Fang et al. reported that the peak tumor concentration, as well as the peak accumulation time, of NPs delivered ^{125}I labeled recombinant human tumor necrosis factor-a (rHuTNF-a) varied with the PEG molecular weight, surface density and the size of the NPs. In fact, it has been reported that the intra-cellular openings in vascular endothelium of tumor blood vessels can be up to 2 mm in diameter and that the vessel leakiness in tumor vasculature can be up to an order of magnitude higher than that of normal blood vessels [Fang 2006]. Active targeting utilizes biologically specific interactions including antigen–antibody and ligand–receptor binding and may seek drug uptake by receptor mediated endocytosis through association of the drug or drug carrier with such antigen or ligand.

Targeted therapy of cancer is based on the use of specific carriers to deliver cytotoxic agents, including chemotherapeutic drugs, radioisotopes, or toxins, to their preferred site of action (i.e., tumors). For targeted delivery of cytotoxic agents, it is important to select a carrier that can be delivered selectively to tumor cells. The most widely investigated and advanced polymers in regard to available toxicological and clinical data are the aliphatic polyesters based on lactic and glycolic acids, like poly(lactic acid) and poly (lactic co-glycolic acid) (PLGA). Other biodegradable polymers which are used in nanoparticulate drug delivery systems are poly (ε-caprolactone) (PCL), poly (alkylcyanoacrylate), etc. The drug loaded polymeric NPs can be attached to this carrier by a number of synthetic or biochemical means to form a tumor-selective conjugate. Administration of such conjugate should lead to the accumulation of drug loaded NPs preferentially in the tumor without significant distribution to normal tissues, followed by selective damage to the tumor cells. Several classes of specific carriers have been evaluated for the selective delivery of drugs to tumors. Such specific carriers include antibodies [Ghose et al. 1985], cytokines such as interleukin-2 (IL-2) and granulocyte-macrophage colony-stimulating factor (GM-CSF), growth factors such as epidermal growth factor (EGF) and vascular endothelial growth factor (VEGF), and hormones such as gonadotropin releasing hormone.

The use of antibodies as carriers of cytotoxic drugs is particularly attractive because of their unique specificity and high affinity for tumor antigens. The monoclonal antibodies (MAbs) are preferred over the conventional polyclonal antibodies (PAbs) due to defined specificity, homogeneity, and availability of MAbs in practically unlimited quantities.

These properties of MAbs render them as the most attractive carriers for the selective delivery of therapeutic agents to malignant tumors. To date, numerous MAbs have been produced against virtually every malignant tumor of human tissues. Many of these MAbs have been used as tumor specific carriers of cytotoxic agents and evaluated either in animal models and/or patients [Zhu Z. 2004].

Three major classes of MAbs have been developed as cancer therapeutics:

1. Antibodies that act as molecular antagonists that modulate the function of key regulatory molecules on tumor cells, such as blocking growth factor/receptor interaction and/or downregulating expression of oncogenic proteins (or receptors) on the cell surface;
2. Antibodies that recruit effector mechanisms of the immune system, such as the antibody-dependent cellular cytotoxicity (ADCC) and complement-mediated cytotoxicity (CMC), and
3. Antibodies used as targeting devices (immunoconjugates) to specifically deliver cytotoxic agents to tumor sites. Functional blockade is thought to be one of the main antitumor mechanisms for several antibodies, including those directed against EGF receptor (also called HER1) and HER2 (erbB2 / neu) on tumor-cell surface,[3] and receptors for VEGF on endothelial-cell surface. By interfering with important growth factor / receptor pathways, these antibodies can influence the growth and survival of tumor cells. In addition, antibodies that inhibit function of regulatory pathways may potentiate the cytotoxic effects of chemotherapeutic drugs and radiation.

Different conjugation techniques are used for targeting cancer cells using MAbs. Examples of such approaches are as follows;

Kocbek et al. applied two strategies for attaching MAbs to PLGA NPs: covalent and non-covalent [Kocbek P 2007]. In covalent attachment, a spacer or linker like EDC (1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide) can be used to conjugate the primary amine group of MAb with the free carboxylic end group of PLGA NPs, forming a connecting amide bond. Non-covalently, NPs were incubated with MAb to allow non-specific adsorption onto their surface. Lu et al. have developed a new surface tethering technology that can attach ligands to PLGA particles at very high density [Lu et al. 2011]. Using this technology doxorubicin was encapsulated in PLGA, and targeted thus making it more efficacious than untargeted doxorubicin particles or free drug in Non-

Hodgkins lymphoma in vitro studies. Kirpotin et al. have described evidence for a novel mechanism of monoclonal antibody (MAb)–directed NPs (immunoliposome) targeting to solid tumors in vivo [Kirpotin et al. 2006]. Long-circulating immunoliposomes targeted to HER2 (ErbB2, Neu) were prepared by the conjugation of anti-HER2 MAb fragments (Fab' or single chain Fv) to liposome-grafted polyethylene glycol chains. Sahoo et al prepared rapamycin loaded PLGA NPs that were surface conjugated with antibodies to epidermal growth factor receptor (EGFR), using EDC and N-hydroxysuccinimide (NHS) mediated cross linking agents [Acharya et al. 2009]. *In vitro* cytotoxicity of native rapamycin, rapamycin loaded NPs and EGFR antibody conjugated rapamycin loaded NPs were evaluated on malignant MCF7 breast cancer cell lines. IC50 doses as determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT) assay. Cell cycle arrest and cellular apoptosis induced by the formulations were confirmed by flow cytometry. Molecular basis of apoptosis was studied by western blotting. It was concluded that EGFR-Rapa-NPs provide an efficient and targeted delivery of anticancer drugs, presenting a promising active targeting carrier for tumor selective therapeutic treatment.

1.2 Aims to be achieved from the present study

The study aims;

i. at formulating a delivery system for Anastrozole and Exemestane, capable of delivering drugs to its site of action (breast) to improve disease condition.
ii. at developing an effective formulation to minimize the associated side effects with currently available drug treatment.
iii. to provide comparative evaluation of different nanoparticulate formulations, which will help to optimize formulation for breast tumor targeting.
iv. at developing a nanoparticulate formulation for anticancer drugs to provide better alternative to currently available tablet formulation, as these are not having site specificity.
v. to reduce dose of drugs and dosage frequency by sustaining the release of drug from nanoparticulate formulation.

1.3 Hypothesis

Targeted delivery of nanoparticulate formulation containing drug (anastrozole, or exemestane) surface engineered with antibody specifically to estrogen receptor positive breast tumor will facilitate high tumor uptake and retention. Targeting will improve therapy, reduce systemic side effects and prevent metastases.

1.4 Objectives

The prime objective of the study is to develop intravenously delivered nanoparticulate drug delivery system composed of synthetic polymers which are biocompatible and biodegradable (PLGA or PCL) conjugated with hetero-bifunctional PEG loaded with anticancer drugs (anastrozole or exemestane) for enhancing tumor uptake by ligand (antibody for estrogen receptor) specific breast tumor targeting with the following objectives;

Reducing systemic side effects,
Site specific drug delivery,
Sustained release of drug,
Reduced dose and dosing frequency,
Improved disease condition,
Patient compliance.

1.5 Plan of work

a. Literature survey, procurement of APIs and excipients.
b. Formulation and optimization: To prepare various nanoparticulate formulations using suitable formulation techniques and their optimization by applying statistical design of experiments for achieving optimum excipient concentration and process parameters to prepare best formulation.
c. Physicochemical characterization:
    i. Particle size and Zeta potential.
    ii. Assay/Entrapment efficiency.
    iii. Compatibility studies by Differential Scanning Calorimetry (DSC).
    iv. Transmission Electron Microscopy.
    v. In vitro release studies.

d. Conjugation of hetero-bifunctional PEG to polymers (PLGA or PCL) and confirmation of reaction steps using TLC, FTIR, NMR and GPC.
e. Phagocytic uptake studies on THP1 cells using flow cytometer.
f. Lyophilization.
g. Surface engineering of optimized formulation with monoclonal antibody.
h. Stability studies.
i. In vitro cytotoxic studies using MCF7 and MDAMB-231 cell lines.
j. Cellular uptake studies of native 6-Coumarin, 6-Coumarin loaded NPs and ER antibody conjugated 6-Coumarin NPs on MCF7 cell line using flow cytometer.
k. Cellular targeting of 6-Coumarin, 6-Coumarin loaded NPs and ER antibody conjugated 6-Coumarin NPs by fluorescence microscopy.
l. Cell cycle analysis and apoptosis studies on MCF7 cell line using flow cytometer.
m. In vivo biodistribution studies by radiolabelling in tumor bearing mice.

1.6 References

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