Chapter 2

LITERATURE REVIEW

2.1 Cancer

Cancer begins in cells, the body's basic unit of life. The body is made up of different types of cells. These cells grow and divide in a controlled way to produce more cells which are needed to keep the body in healthy conditions. When cells become old or when they are damaged, they die and are replaced with new cells. However, sometimes this orderly process goes wrong. When the genetic material (DNA) of a cell is damaged or changed, producing mutations that affect normal cell growth and division. When mutation occurs, cells do not die when they should and the new cells form when the body does not need them (Figure 2.1). These extra cells forms a mass of tissue called a tumor. Not all tumors are cancerous; tumors can be benign or malignant [Anand 2008].

- Benign tumors aren't cancerous. They can often be removed, and, in most cases, they do not come back. Cells in benign tumors do not spread to other parts of the body.
- Malignant tumors are cancerous. Cells in these tumors can invade nearby tissues and spread to other parts of the body. The spread of cancer from one part of the body to another is called metastasis.

Some cancers do not form tumors. For example, leukemia is a cancer of the bone marrow and blood.

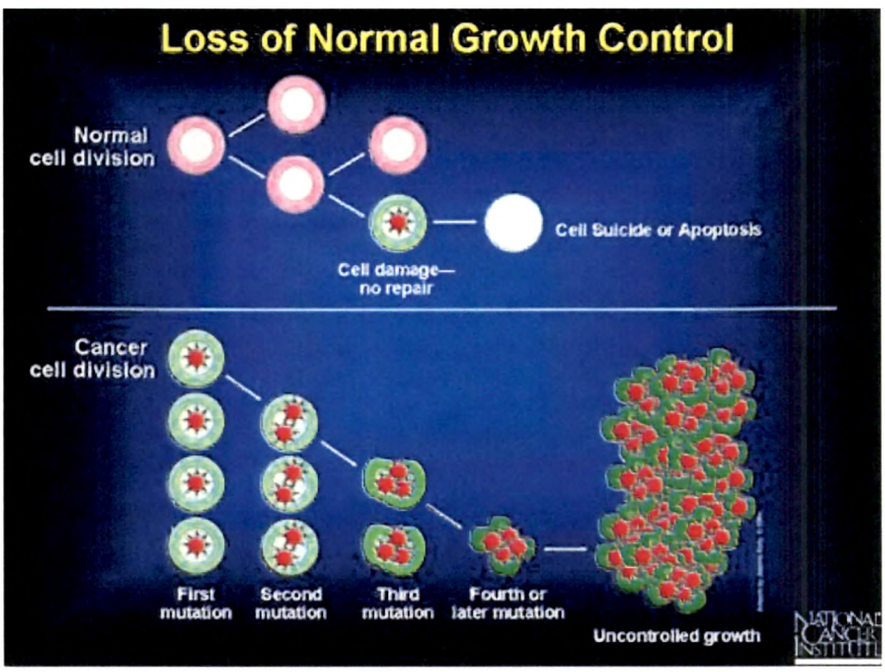


Figure 2.1 Growth of cells

Cancer is a disease in which abnormal cells divide without control and are able to invade other tissues. Cancer cells can spread to other parts of the body through the blood and lymph systems. Cancer is not just one disease but many diseases. There are more than 100 different types of cancer. Most cancers are named for the organ or type of cell in which they start - for example, cancer that begins in the breast is called breast cancer; cancer that begins in melanocytes of the skin is called melanoma [Jemal 2011]. Cancer types can be grouped into broader categories. The main categories of cancer include:

- Carcinoma - cancer that begins in the skin or in tissues that line or cover internal organs. There are a number of subtypes of carcinoma, including adenocarcinoma, basal cell carcinoma, squamous cell carcinoma, and transitional cell carcinoma.
- Sarcoma - cancer that begins in bone, cartilage, fat, muscle, blood vessels, or other connective or supportive tissue.
- Leukemia - cancer that starts in blood-forming tissue such as the bone marrow and causes large numbers of abnormal blood cells to be produced and enter the blood.
- Lymphoma and myeloma - cancers that begin in the cells of the immune system.
- Central nervous system cancers - cancers that begin in the tissues of the brain and spinal cord.

Cancer cells can also spread to other parts of the body. For example, cancer cells in the lung can travel to the bones and grow there. When cancer cells spread, it is called metastasis. Cancers can sometimes recur (or come back) after treatment, mostly at the site where they started but sometimes at a distant site like lungs, liver, brain, or bone. Some cancers tend to grow and spread very quickly. Others grow more slowly. Cancers also respond to treatment in different ways. Some types are best treated with surgery. Others do better with drugs called chemotherapy. Often 2 or more treatments are used to get the best results. Most cancers form a lump that doctors call a tumor or a growth. Not all tumors (lumps) are cancer. Doctors have to take out a piece of the lump and look at it to find out if it is cancer. Lumps that are not cancer are called benign. Lumps that are cancer are called malignant. There are also a few kinds of cancer, like leukemia (cancer of the blood), that do not form tumors. They grow in the blood or other cells of the body. The most common treatments for cancer are surgery, chemotherapy, and

radiation. Surgery is used to remove the cancer when it is confined to the organ where it started. The surgeon might also take part or the entire organ it affects. For breast cancer, part (or all) of the breast might be removed. For prostate cancer, the prostate gland might be removed. But surgery is not used for all types of cancer.

Chemotherapy is the use of drugs to kill cancer cells or to slow their growth. Some chemotherapy is given by IV (put into a vein using a needle), some as a shot, and others are swallowed as a pill or liquid. Because chemotherapeutic drugs travel to nearly all parts of the body, they are useful for cancer that has spread. Radiation treatment is also used to kill or slow the growth of cancer cells. It can be used alone or with surgery or chemo. Radiation treatment is like getting x-rays. Or sometimes it can be given by placing "seeds" that give off radiation inside the tumor.

2.1.1 Cancer statistics

Cancer is one of the leading causes of adult deaths worldwide. In India, the International Agency for Research on Cancer estimated indirectly that about 6,35,000 people died from cancer in 2008, representing about 8% of all estimated global cancer deaths and about 6% of all deaths in India [Ferlay et al. 2010]. 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 [Causes of death in India 2009; Jemal 2011]. The absolute number of cancer deaths in India is projected to increase because of population growth and increasing life expectancy [Causes of death in India 2009]. Rates of cancer deaths are expected to rise, particularly, from increases in the age-specific cancer risks of tobacco smoking, which increase the incidence of several types of cancer [Jha 2009].

In 2010, more than 5,56,000 cancer deaths were estimated in India for people of all ages, and 71.1% occurred in people aged 30–69 years. Cancer deaths accounted for 8.0% of the 2.5 million total male deaths and 12.3% of the 1.6 million total female deaths at age 30–69 years. In 2010, at all ages, the rates of cancer deaths were about 59 per 100000 for men and about 52 per 1,00,000 for women. However, the rates of cancer deaths per 1,00,000 rose sharply with age and at age 30–69 years were about 98 for men and 95 for women. Based on the actual death rates and the hypothetical absence of other causes of death, a 30 year old man in India had a 4.7% chance of dying from

cancer before the age of 70 years. The respective risk for a 30-year-old woman was 4.4% [Dikshit et al. 2012].

The latest WHO statistics suggests about 45% increase in the global cancer deaths by 2030, of which 70% would be contributed from developing countries like India [Thun 2010]. With continuous up-gradation in the field of science and technology, the need for addressing the practical problems associated with the drug therapies increased proportionately. The major portion of cancer therapy till the last couple of decades was based on parenteral route of administration [O'Neill 2002; Ruddy 2009]. However, looking at the quality of life and need of follow-up therapy after the diagnosis of the disease, oral route has gained major focus as compared to the parenteral route [Jeanneret 2011; O'Neill 2002; Partridge 2002].

2.2 Tumor physiology

Tumor biology plays an important role in drug delivery. The growth, structure, and physiology of a tumor all impact the ability of nanoparticle (NP) drug carriers to be delivered successfully. Understanding which aspects of tumor biology are beneficial and which are detrimental to delivery leads to the development of more effective and efficient drug carriers.

2.2.1 Tumor growth

A tumor grows from a single cell that undergoes some mutation that blocks its apoptotic signaling pathway causing it to uncontrollably proliferate. The rapidly replicating cells displace their healthy counterparts due to an increased demand for nutrients and subsequent waste product elimination [Brannon-Peppas 2004]. During the initial stages of tumor growth the cells rely solely on diffusion to obtain nutrients limiting their size to approximately 2 mm^3 [Jones 1998]. To bypass their diffusion-limited size the tumor cells must begin to recruit new blood vessels in a process called angiogenesis [Brannon-Peppas 2004; Brown 1998].

2.2.2 Tumor vasculature and lymphatic system

Once a tumor mass is able to initiate angiogenesis, the blood vessels continue to rapidly grow producing an unorganized and aberrant vasculature [Haley and Frenkel 2008]. Consequently, the tumor contains regions with extensive vasculature and rich blood supply and regions with poor vasculature and little blood supply. The variance in level of vasculature and the tendency of the vessels to have dead-ends and little-to-no smooth

muscle or nerve innervation results in significantly heterogeneous blood flow through the tumor tissue [Brown 1998]. Tumor vessels are also inherently leaky due to abnormal basement membranes and incomplete endothelial linings caused by the inability of pericytes to fully line the quickly proliferating cells forming the vessel walls [Baban and Seymour 1998; Haley and Frenkel 2008].

Tumors also have a reduced ability to drain fluid and waste from the interstitial space [Brannon-Peppas 2004]. The reduction in drainage is due to a poorly-defined lymphatic system caused by the demand of the quickly proliferating tumor cells [Haley and Frenkel 2008]. Unlike healthy tissue which can rapidly remove macromolecules and lipids from its interstitium, a tumor will accumulate these molecules and retain them for extended periods of time [Maeda 2001].

Additional factors present at high levels in tumor cells contribute notably to angiogenesis and vessel permeability. These factors include vascular endothelial growth factor [Roberts and Palade 1995], basic fibroblast growth factor [Dellian 1996], bradykinin [Matsumura 1988], and nitric oxide [Wu 1998]. Vascular endothelial growth factor (VEGF) increases the permeability of blood vessels by increasing both the size and quantity of fenestrations between cells [Roberts and Palade 1995]. Elevated concentrations of bradykinin and depletion of nitric oxide both result in increased extravasation of macromolecules through the tumor vasculature [Matsumura 1988; Wu 1998]. Basic fibroblast growth factor (bFGF) is active in angiogenesis as it recruits endothelial cells and increases cellular proliferation [Roberts and Palade 1995].

Combined, the highly permeable vasculature, poorly-defined lymphatic system, and elevated levels of the aforementioned factors, result in a phenomenon called the enhanced permeability and retention effect (EPR). This effect was first defined by Maeda and colleagues and explains the observed accumulation of drugs, lipids and other macromolecules (MW > 50 kDa) at the tumor site [Maeda 2001]. The EPR has been the focus of much research due to its ability to passively target macromolecules including NPs.

2.2.3 Barriers to drug delivery in tumors

Most chemotherapeutic drugs are given via a systemic injection and circulate in the bloodstream prior to reaching the tumor site. A disadvantage of this type of delivery scheme is that the agent is allowed to come into contact with both healthy tissue and the tumor. This interaction between healthy tissue and the chemotherapeutic agent is what

often leads to the debilitating side effects that accompany treatment. Another detriment to systemic delivery is that the agent will encounter numerous extra-and intracellular barriers prior to reaching the tumor site. Furthermore, the drug must retain its biological activity and reach the target site at high enough concentrations to have therapeutic efficacy. In this section we will examine the significant systemic, extra- and intracellular barriers therapeutic agents encounter.

2.2.3.1 Reticuloendothelial system and mononuclear phagocytic system

The reticuloendothelial system (RES) also known as the mononuclear phagocytic system [Kurokawa] are a group of organs and circulating macrophages whose primary function is to rid the body of foreign objects, such as bacteria [Owens 2006a]. NPs that enter the bloodstream are also subject to rapid clearance by the RES/MPS. These foreign bodies are not directly recognized by the macrophages, typically liver macrophages or Kupffer cells, and must first be coated by a layer of proteins in a process called opsonization. The proteins involved in this process are termed opsonins, a class of proteins available in the circulation. Opsonins include immunoglobulins, components of the complement system (C3, C4, and C5), fibronectin, type I collagen, and many others. These proteins, when encountering a foreign particle, adhere by a variety of interactions such as ionic, electrostatic, hydrophobic, hydrophilic and van der Waals forces [Owens 2006a]. The macrophages then identify the surface layer of bound opsonin proteins coating the foreign body and proceed to engulf the particle by phagocytosis, "cell-eating", then degrading it within an intracellular vesicle such as the lysosome [Jones and Harris 1998].

2.2.3.2 First pass renal filtering

The human body is a carefully designed system that is particularly adept at recognizing and removing foreign particles from circulation. The renal system is an essential component in the purification of the blood and is an important consideration when designing carriers for drug delivery. The kidneys filter blood through a structure known as the glomerular capillary wall. Particles with a diameter of less than 10 nm are subject to first pass renal filtration through this structure [Davis 2008; Venturoli 2005].

2.2.3.3 Heterogeneous blood flow

As mentioned previously, due to the rapid proliferation of tumor tissue, tumor vasculature is highly aberrant and unorganized. In conjunction with irregular vasculature structure is a lack of nerve enervation and smooth muscle which leads to a

heterogeneous and variable blood flow. This becomes a barrier to systemic drug delivery as the macromolecular therapeutic agent will not be evenly dispersed throughout the tumor tissue [Jang 2003]. It has been shown that areas of tumor tissue with poor blood flow are often resistant to treatment [Hori 1991].

2.2.3.4 High tumor interstitial pressure

The tumor interstitium comprises the bulk of tumor mass and consists of a collagen network and highly viscous fluid [Haley and Frenkel 2008]. The fluid within the interstitium has some quantifiable pressure that increases with tumor size and proximity to the tumor center. This pressure increase is due to a combination of factors such as rapid cellular proliferation in a confined area, high vascular permeability into the interstitium, and lack of lymphatic drainage from the interstitium [Jain 1987; Jain 1998]. Drug diffusion into the interstitium is depleted as the pressure increases. For this reason, there tends to be a lack of drug accumulation in the center of the tumor mass where the interstitial pressure is the highest [Haley and Frenkel 2008; Jain 1998].

2.2.3.5 Extracellular matrix (ECM)

The extracellular matrix is composed of fibrous proteins such as collagen and elastin, as well as a highly viscous polysaccharide-containing fluid. Its primary functions are to maintain cellular structure and integrity, modulate cellular interaction with the external milieu – including neighboring cells, regulate macromolecular transport and serve as a barrier to bacterial infiltration. In the context of drug transport, and, more specifically chemotherapeutic agent delivery, the ECM poses a formidable physical barrier. The tightly woven fibrous proteins and highly viscous ECM fluid, containing both hyaluronan and proteoglycans, each serve to reduce the diffusivity and spatial distribution of drug molecules within the tumor interstitium [Jain 1987; Jang 2003].

2.2.3.6 Intracellular transport

Once the drug component reaches the cell it must be internalized. This internalization process is termed phagocytosis, or cell eating, and consists of actin protrusions of the cellular membrane surrounding and engulfing a particle [Jones and Harris 1998]. The particle is now contained within an intracellular vesicle for transport through the cytoplasm. The particle is shuttled from the early endosome to the late endosome and finally the lysosome for degradation. Throughout this pathway the pH decreases from 7.4 to approximately 5.0. Additionally, contained within the intracellular components

are enzymes that aid in foreign body degradation. The drug must maintain its activity through both decreased pH and rampant enzymatic activity [Jones and Harris 1998].

2.3 Breast Cancer

Breast cancer is a disease in which malignant (cancer) cells form in the tissues of the breast. The breast is made up of lobes and ducts. Each breast has 15 to 20 sections called lobes, which have many smaller sections called lobules. Lobules end in dozens of tiny bulbs that can make milk. The lobes, lobules, and bulbs are linked by thin tubes called ducts. Each breast also has blood vessels and lymph vessels. The lymph vessels carry almost colorless fluid called lymph. Lymph vessels lead to organs called lymph nodes. Lymph nodes are small bean-shaped structures that are found throughout the body. They filter substances in fluid called lymph and help fight infection and disease. Clusters of lymph nodes are found near the breast in the axilla (under the arm), above the collarbone, and in the chest [Sariego 2010].

The most common type of breast cancer is ductal carcinoma, which begins in the cells of the ducts. Cancer that begins in the lobes or lobules is called lobular carcinoma and is more often found in both breasts than are other types of breast cancer. Inflammatory breast cancer is an uncommon type of breast cancer in which the breast is warm, red, and swollen. Breast cancer is sometimes caused by inherited gene mutations. The genes in cells carry the hereditary information that is received from a person's parents. Hereditary breast cancer makes up about 5 to 10% of all breast cancer. Some mutated genes related to breast cancer are more common in certain ethnic groups. Women who have certain gene mutations, such as a *BRCA1* or *BRCA2* mutation, have an increased risk of breast cancer. Also, women who had breast cancer in one breast have an increased risk of developing breast cancer in the other breast. These women also have an increased risk of ovarian cancer, and may have an increased risk of other cancers.

Breast cancer cells have receptors on their surface and in their cytoplasm and nucleus. Chemical messengers such as hormones bind to receptors, and this causes changes in the cell. Breast cancer cells may or may not have three important receptors: estrogen receptor (ER), progesterone receptor (PR), and HER2 [Petit 2011]. ER+ cancer cells depend on estrogen for their growth, so they can be treated with drugs to block estrogen effects (e.g. tamoxifen), and generally have a better prognosis. HER2+ breast

cancer has a worse prognosis, but HER2+ cancer cells respond to drugs such as the monoclonal antibody trastuzumab (in combination with conventional chemotherapy), and this has improved the prognosis significantly [Gabriel 2005; Spigel 2002]. Cells with none of these receptors are called triple-negative although they frequently express receptors for other hormones such as androgen receptor and prolactin receptor.

Discovery of ER is critical for the development of endocrine therapy in breast cancer. Expression of ERα, the predominant isoform, in breast tumors of both premenopausal and postmenopausal women is a highly predictive marker for response to antiestrogen treatment in women with ERα-positive breast cancer [Nahta 2003]. Today, endocrine therapies include the use of antiestrogens such as tamoxifen and aromatase inhibitors such as anastrozole, exemestane, and letrozole. Tamoxifen, the first antiestrogen to be used for the treatment of ERα-positive breast cancer, competitively blocks the actions of 17β-estradiol (E_2), the female hormone that binds and activates ERα in tumors. In postmenopausal women, peripheral aromatization of androgens to estrogens is the major source of plasma estrogen. Aromatase inhibitors inhibit this reaction and consequently suppress the production of circulating estrogen in postmenopausal women [Fisher et al. 1986]. Endocrine therapies are effective at 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 [Bonneterre 2001]. 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.

2.3.1 Antiestrogens

Nonsteroidal antiestrogens were initially developed as contraceptives in the 1960s. Walpole and colleagues synthesized tamoxifen (termed ICI 46, 474), a potent antiestrogen with antifertility properties in rats. However, in humans, tamoxifen induced ovulation in subfertile women. Therefore, the development of tamoxifen as a contraceptive was terminated. However, Walpole also patented the application of tamoxifen as a drug treatment for hormone dependent cancers. Thus, clinical trials were

started to evaluate tamoxifen against the standard endocrine treatment at the time, diethylstilbestrol, for the treatment of advanced breast cancer in postmenopausal women [Cole et al. 1971]. Tamoxifen not only was as effective as diethylstilbestrol for the treatment of advanced breast cancer but also had fewer side effects. Therefore, the advantage of tamoxifen over diethylstilbestrol was crucial for its subsequent evaluation as a treatment for all stages of breast cancer.

In 1962, Jensen and colleagues discovered ERα. Jensen demonstrated that E_2, the circulating female hormone that promoted breast cancer growth, binds to diverse tissue sites around a woman's body but is retained in estrogen target tissues, for example, the uterus and vagina [Jensen EV 1962]. The identification of ERα as the target of E2 action in the breast and antiestrogens blocking the binding of E_2 to ERα provided a therapeutic target and an approach for the treatment of breast cancer. Although many antiestrogens were discovered and tested during the 1960s and 1970s, only tamoxifen was considered safe enough for extensive clinical evaluation [Jordan and Dowse 1976]. Clinical trials ultimately demonstrated that patients with ERα-positive breast cancer benefited the most from tamoxifen therapy, whereas women with ERα-negative breast cancer were found to be unaffected. During subsequent clinical trials, five years of adjuvant tamoxifen treatment was found to be more effective than less than five years of treatment in improving time to tumor recurrence and overall survival [Fisher et al. 1986]. In contrast to the beneficial effects of tamoxifen as a treatment for breast cancer, both laboratory and clinical results showed that tamoxifen increased the risk of endometrial cancer in the uterus four fold in postmenopausal women compared with untreated women [Fisher et al. 1994; Gottardis et al. 1988]. These results strongly indicated that tamoxifen was not a pure antiestrogen but had selective functions depending on the target tissue.

2.3.2 EGFR and HER2/ Neu and Antiestrogen resistance

Numerous laboratory and clinical studies indicate that overexpression and/or aberrant activity of the HER2/neu (erbB2) signalling pathway is associated with antiestrogen resistance in breast cancer [Kurokawa and Arteaga 2003]. The HER2/neu receptor is a member of the EGFR family of receptor tyrosine kinases, which include HER3 (erbB3) and HER4 (erbB4) [Yarden 2001a; Yarden 2001b; Yarden and Sliwkowski 2001]. Upon dimerization, the tyrosine kinase domains located within the COOH-terminal regions of receptors are activated by an autophosphorylation cascade on specific tyrosine

residues, which activate downstream effectors, such as MAPK and Akt, which promote cellular proliferation, survival, anti-apoptosis, and transformation. HER2/neu is overexpressed and/or amplified in 25 to 30% of breast tumors and is associated with a more aggressive phenotype and poor prognosis [Spigel DR 2002]. Patients with breast tumors overexpressing HER2/neu exhibit much lower response rates to antiestrogen therapy [Spigel DR 2002]. Thus, it is suggested that one possible mechanism of resistance to tamoxifen is overexpression of HER2/neu in ERα-positive breast cancers. In addition, overexpression of EGFR and its ligands are observed in several human cancers including breast cancer [Klijn JG 1994; Tang et al. 2000]. The increased expression of EGFR is frequently associated with tumor progress ion and resistance to antiestrogens. These data suggest that EGFR and/or HER2/neu are possible targets for preventing or treating antiestrogen resistance in breast cancer. Strategies to target EGFR and HER2/neu include the use of humanized monoclonal antibodies to the receptors [Slamon DJ 2001], tyrosine kinase inhibitors that block reduction of adenosine triphosphate (ATP) to adenosine diphosphate (ADP) + Pi, and receptor antisense molecules [Nahta R 2003]. Gefitinib, (ZD 1839, Iressa®), an EGFR-specific tyrosine kinase inhibitor, has been shown to inhibit growth of breast cancer cell lines in vitro that are resistant to tamoxifen. More importantly, the combination of gefitinib and tamoxifen was shown to prevent resistance [Johnston et al. 2003], demonstrating that EGFR might be a key player in the development of antiestrogen resistance and inhibiting the activity of this receptor might be therapeutically beneficial in preventing resistance to antiestrogens. In addition to gefitinib, trastuzumab (Herceptin®) is a humanized monoclonal antibody directed against the ectodomain of the HER2/neu receptor. It has been shown to restore breast cancer cell sensitivity to tamoxifen in HER2/neu overexpressing cells [Kurokawa 2000].

2.3.3 Aromatase inhibitors

An alternate strategy to endocrine therapy, which specifically inhibits binding of E_2 to the ER, is to inhibit the production of E_2 by blocking the cytochrome p450 aromatase enzyme, the rate-limiting enzyme that converts androgens (i.e., testosterone and androstenedione) to estrogens (i.e., E_2 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 E_2 and thus better efficacy for ERα-positive breast cancer [Johnston 2003]. Recent clinical data have clearly demonstrated that anastrozole [Bonneterre 2001], letrozole [Mouridsen 2001], and exemestane [Paridaens 2003] are more effective than tamoxifen as first-line treatments in patients with metastatic breast cancer. On the basis of clinical results [Bonneterre 2001; Mouridsen 2001], currently both anastrozole and letrozole are approved by the Food and Drug Administration for first-line treatment of postmenopausal, ERα-positive advanced breast cancer. The data from advanced breast cancer trials provided the rationale to perform large scale clinical trials to determine whether there is an advantage of using aromatase inhibitors over tamoxifen in the adjuvant setting. The anastrozole, tamoxifen, and the combination of anastrozole and tamoxifen (ATAC) trial has sufficient follow-up data to confirm that anastrozole is superior to tamoxifen as a first-line adjuvant therapy in ERα-positive breast cancer with regard to disease-free survival and incidence of contralateral breast cancer [Baum 2002]. The Breast International Group [Paridaens] 1-98 study demonstrated a similar improvement in event -free survival to confirm that letrozole is superior to tamoxifen as first-line adjuvant hormonal therapy [Thürlimann 2005]. Other trials have demonstrated an improved outcome for post menopausal patient s with early- stage breast cancer treated with two to three years of tamoxifen, followed by an aromatase inhibitor, compared with five years of tamoxifen [Coombes 2007; Kaufmann 2007].

2.3.4 Mechanisms of resistance

Clinically, patients that relapse after a previous response to tamoxifen usually have a clinical response to aromatase inhibitors [Buzdar et al. 1996; Dombernowsky et al. 1998]. These results strongly indicate that the ERα continues to be expressed and is functional in breast tumors that are resistant to antiestrogens. However, although estrogen deprivation treatment might be more effective than tamoxifen in delaying resistance, eventually resistance to aromatase inhibitors will also develop. To date, it is

unclear whether similar mechanisms of actions that have been identified for tamoxifen resistance are also involved in resistance to aromatase inhibitors. The exact mechanisms contributing to aromatase inhibitor resistance has yet to be fully elucidated. However, in vitro studies have identified mutations within the aromatase gene that confers resistance to aromatase inhibitors [Kao 1996]. These mutations have not yet been identified in human breast carcinomas [Sourdaine 1994]. Other studies have demonstrated that estrogen deprivation super sensitizes the breast cancer cell to low levels of estrogen, thus creating a hypersensitive environment to overcome estrogen deprivation resulting in resistance [Chan 2002; Masamura 1995; Santen 2001]. In addition, results suggest that there is increased cross talk between growth factor receptor signalling pathways and ERα. ERα has been shown to become activated and supersensitized by several different intracellular kinases, including MAPKs, insulin-like growth factors, and the PI3-K/Akt pathway [Campbell et al. 2001; Jeng 2000; Martin et al. 2003]. Therefore, the data suggest that ERα continues to be an integral part of the breast cancer cell signalling pathway even after resistance to aromatase inhibitors has developed.

2.3.5 Role of progesterone receptor and HER2/Neu

There is emerging evidence to suggest that ER-positive cancers that do not express the progesterone receptor (PR) and/or HER2/neu are somewhat intrinsically resist ant to tamoxifen and perhaps hormonal therapy in general. Arpino et al. have demonstrated an increased relapse rate in patients with ER-positive, PR negative cancers compared with ER-positive, PR-positive cancers, treated with tamoxifen [Arpino 2005]. Patients treated with tamoxifen with ER-positive, HER2- positive metastatic breast cancers have a shorter time to treatment failure compared with ER-positive, HER2-negative cancers [De Laurentiis 2005]. In fact, Arpino et al. have demonstrated an increase in both HER1 and HER2 in ER-positive, PR-negative cancers compared to ER-positive, PR-positive cancers, suggesting an interplay between the ER and epidermal growth factor pathways [Arpino 2005].

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 [Ellis 2006; Smith 2005]. This led to a widely accepted hypothesis that aromatase inhibitors were a better choice than tamoxifen in patients with ER-positive, HER2-positive cancers. However, an analysis of

the BIG-1-98 trial demonstrates that letrozole improves outcome compared to tamoxifen in both ER-positive, HER2-positive cancers (HR 0.68) and in ER-positive, HER2-negative cancers (HR 0.72) [Viale 2007]. A recent subanalysis of the ATAC trial demonstrated a significantly improved outcome in ER-positive, HER2-negative cancers (HR 0.66) but not in ER-positive, HER2 positive cancers (HR 0.92), but this may have been due to the small number of patients in the HER positive group [Dowsett 2008]. Are HER2- positive cancers somewhat resistant to not just tamoxifen but also to aromatase inhibitors? As outlined above, a recent trial randomized patients with HR-positive, HER2 positive metastatic breast cancers to anastrozole alone or to anastrozole plus trastuzumab . Although there was no significant difference in overall survival, possibly because patients randomized to anastrozole alone could receive trastuzumab at disease progression, the time to progression was doubled from 2.4 months in the anastrozole-alone arm to 4.8 months in the combined arm (p ¼ 0.0016). The clinical benefit rate in the combination arm was 42% significantly higher than in the anastrozole alone arm. A trial that evaluated single-agent trastuzumab as first-line therapy for patients with HER2-positive cancers demonstrated a clinical benefit rate of 48% [Vogel 2002]. This suggests the intriguing possibility that HR positive, HER2-positive cancers are driven by the HER2 pathway, which renders the cancers partly resistant to hormonal therapies.

An initial evaluation of the ATAC trial using case report forms revealed that TTR was longer for anastrozole in both ER-positive/PR-positive and ER positive/ PR-negative subgroups, but the benefit was more pronounced in the ER positive/ PR-negative subgroup [HR 0.84, 95% confidence interval 0.69-1.02 vs. 0.43, 95% CI 0.31-0.61] [Dowsett 2005]. Importantly, the ER and PR analyses were not performed cent rally. More recently , a central analysis of about 2000 patients on the ATAC trial demonstrated similar improvements with the use of anastrozole compared to tamoxifen, regardless of PR status (HR anastrozole vs. tamoxifen 0.72 for ER-positive, PR-positive subgroups and 0.66 for ER-positive, PR-negative subgroups) [Dowsett 2008]. In the BIG-1-98 trial, similar benefits for letrozole compared to tamoxifen were seen in the ER-positive/PR-positive and ER-positive/ PR-negative subgroups [Viale 2007]. On the basis of this data, decisions regarding whether to start a patient on tamoxifen or an aromatase inhibitor should not be based on PR or HER2 status. Further molecular profiling may help in the future in making decisions regarding optimal hormonal therapies.

2.4 Treatment of breast cancer

2.4.1 Treatment options by stage

Early Stage Breast Cancer (Stage I and Stage II): Treatment of early stage breast cancer (Stage I and Stage II) may be surgery followed by adjuvant therapy as follows:

- Modified radical mastectomy.
- Breast-conserving surgery: Lumpectomy, partial mastectomy or segmental mastectomy.
- Breast-conserving surgery during pregnancy followed by radiation therapy after the baby is born.
- Surgery during pregnancy followed by chemotherapy after the first t.
- Clinical trials of surgery followed by hormone therapy with or without chemotherapy.

Late Stage Breast Cancer (Stage III and Stage IV): Treatment of late stage breast cancer (Stage III and Stage IV) may include the following:

- Radiation therapy.
- Chemotherapy.

The mainstay of breast cancer treatment is surgery when the tumor is localized, followed by chemotherapy (when indicated), radiotherapy and adjuvant hormonal therapy for ER positive tumors (with tamoxifen or an aromatase inhibitor). Management of breast cancer is undertaken by a multidisciplinary team based on national and international guidelines. Depending on clinical criteria (age, type of cancer, size, metastasis) patients are roughly divided to high risk and low risk cases, with each risk category following different rules for therapy. Treatment possibilities include radiation therapy, chemotherapy, hormone therapy and immune therapy.

2.4.2 Surgery

Depending on the stage and type of the tumor, just a lumpectomy (removal of the lump only) may be all that is necessary, or removal of larger amounts of breast tissue may be necessary. Surgical removal of the entire breast is called mastectomy. Lumpectomy techniques are increasingly utilized for breast-conservation cancer surgery. Studies indicate that for patients with a single tumor smaller than 4 cm, lumpectomy may be as effective as a mastectomy.

However, mastectomy may be the preferred treatment in certain instances:

- Two or more tumors exist in different areas of the breast (a "multifocal" cancer).

- The patient has previously received radiotherapy.
- The tumor is large, relative to the size of the breast.
- The patient has had scleroderma or another disease of the connective tissue, which can complicate radiotherapy.
- The patient lives in an area where radiotherapy is inaccessible.
- The patient is apprehensive about the risk of local recurrence after lumpectomy.

2.4.3 Radiation therapy

Radiation therapy is an adjuvant treatment for most women who have undergone lumpectomy and for some women who have mastectomy surgery. In these cases the purpose of radiation is to reduce the chance that the cancer will recur. Radiation therapy involves using high-energy X-rays or gamma rays that target a tumor or post surgery tumor site. This radiation is very effective in killing cancer cells that may remain after surgery or recur where the tumor was removed [Buchholz 2009].

Radiation therapy eliminates the microscopic cancer cells that may remain near the area where the tumor was surgically removed. The dose of radiation must be strong enough to ensure the elimination of cancer cells. However, radiation affects normal cells and cancer cells alike, causing some damage to the normal tissue around where the tumor was. Healthy tissue can repair itself, while cancer cells do not repair themselves as well as normal cells. For this reason, radiation treatments are given over an extended period, enabling the healthy tissue to heal. Treatments using external beam radiotherapy are typically given over a period of five to seven weeks, performed five days a week. Each treatment takes about 15 minutes. A newer approach, called 'accelerated partial breast irradiation' (APBI), uses brachytherapy to deliver the radiation in a much shorter period of time. APBI delivers radiation to only the immediate region surrounding the original tumor and can typically be completed over the course of one week [Hendrick 2010].

Side effects of radiation therapy

External beam radiation therapy is a non-invasive treatment with some short term and some longer-term side effects. Patients undergoing some weeks of treatment usually experience fatigue caused by the healthy tissue repairing itself and aside from this there can be no side effects at all. However many breast cancer patients develop a suntan-like change in skin color in the exact area being treated. As with a suntan, this darkening of the skin usually returns to normal in the one to two months after treatment. In some

cases permanent changes in color and texture of the skin is experienced. Other side effects sometimes experienced with radiation can include:

- muscle stiffness
- mild swelling
- tenderness in the area
- lymphedema

2.4.4 Chemotherapy

Chemotherapeutic agents are, in the broadest sense, small drug-like molecules that disrupt the normal functioning of a cell by inhibiting replication or inducing apoptosis [Feng 2003]. Due to their proficiency at provoking cytotoxic effects, chemotherapeutic agents have been almost exclusively utilized in the treatment of cancer, where they exhibit the most deleterious effects to rapidly proliferating cells [Feng 2003]. Prominent chemotherapeutic agents include paclitaxel, doxorubicin, daunorubicin, cisplatin, and docetaxel. Paclitaxel and docetaxel are both taxanes, components that function by stabilizing the microtubules and preventing mitosis from progressing from metaphase to anaphase [Rowinsky 1997]. Doxorubicin and daunorubicin belong to a class of chemotherapeutics known as the anthracyclines. These molecules are among the most effective drugs available, inducing the greatest degree of cytotoxicity and used to treat the widest variety of tumor types including aggressive lymphoma, breast cancer, and myeloblastic leukemia [Minotti 2004; Weiss 1992]. Doxorubicin has been shown to target the topoisomerase-II-DNA complex, disrupting the DNA and preventing cellular replication [Hurley 2002]. Similarly, cisplatin, a platinum-compound, modifies cellular DNA which activates signaling pathways that triggers apoptosis [Boulikas 2003].

The primary concern with utilizing the aforementioned chemotherapeutic agents is their inability to differentiate between healthy and tumor tissue [Maeda 2001]. The drugs will attack all cells without discrimination, being particularly harmful to any rapidly proliferating cells in the body such as hair, intestinal epithelial cells, and bone marrow [Feng 2003]. The most cytotoxic agents are the most effective but often result in severe side effects. Doxorubicin is widely considered to be best anti-cancer drug available today but results in side effects such as, nausea, fatigue, and extensive and often fatal cardiotoxicity [Minotti 2004]. Oncologists must, therefore, optimize the balance between the effectiveness of the drug and a patient's ability to tolerate the accompanying side effects [Feng 2003]. Nanoscale carrier systems designed to target

specific disease conditions could be utilized to alleviate some, if not all, of these cytotoxic effects to healthy cells.

However, oral delivery of anticancer drugs is a great challenge owing to their peculiar physicochemical properties, and physiological barriers such as pre-systemic metabolism and gastrointestinal instability. Upon oral administration of such drugs, only a fraction of dose is available to systemic circulation for execution of therapeutic response *e.g.* oral bioavailability of paclitaxel, docetaxel, doxorubicin, tamoxifen, etc. is in the range of 5–20% [Kuppens 2005; Peltier 2006; Troutman 2003]. Broadly, this could be attributed to low aqueous solubility, poor intestinal permeability, high level of P-glycoprotein (P-gp) efflux and pre-systemic metabolism. The P-gp efflux also has a key role in the execution of multidrug resistance in the tumor cells and thereby needs special consideration while designing the formulation of poor biopharmaceutical properties, as the amount which is required to achieve the therapeutic response might be very high ultimately leading to multidrug resistance.

The therapeutic efficacy of the formulation depends upon its capability to deliver the drug at the right place and at the right time in amount adequate enough to yield a therapeutic response. Comparative therapeutic equivalence of oral and intravenous routes has been studied for wide variety of drugs and promising results were observed in most of the cases. Cyclophosphamide yields no statistical significant difference in the area under the plasma disappearance curve (AUC) and generated similar cytotoxic metabolic products upon administration through oral and parenteral routes thereby suggesting the therapeutic equivalence, irrespective of the route of delivery [Struck 1987]. Paclitaxel in nanoparticulate dosage form administered by oral route had shown promising tumor reduction in animals compared to commercially available intravenous formulation at 50% reduced dose [Bhardwaj 2009]. Co-administration of cyclosporin A further potentiated its oral bioavailability, due to inhibition of the P-gp efflux pump and CYP 3A4, both being limitations for oral bioavailability [Asperen 1998]. Similarly, topotecan has also been found to be equally effective irrespective of the route of administration with upper hand in reduced toxicity *via* oral route [Gore 2002; von Pawel 2001]. The other drugs which have been evaluated include docetaxel, paclitaxel, doxorubicin, cisplatin [Urien 2005], ifosfamide/mesna combination [Manegold 1996] and melphalan [Bosanquet 1982] to name a few. The studies suggest that by virtue of appropriate pharmaceutical/pharmacological interventions, the inherent

problems associated with oral route (owing to various physiological barriers) can be overcome in comparison to intravenous route of administration.

2.5 Targeted therapy

In patients whose cancer expresses an over-abundance of the HER2 protein, a monoclonal antibody known as trastuzumab (Herceptin) is used to block the activity of the HER2 protein in breast cancer cells, slowing their growth. In the advanced cancer setting, trastuzumab use in combination with chemotherapy can both delay cancer growth as well as improve the recipient's survival [22]. More recently, several clinical trials have also confirmed that in the adjuvant setting, i.e. postoperative following breast cancer surgery, the use of trastuzumab for up to one year also delays the recurrence of breast cancer and improves survival[23][24][25].

Other types of targeted therapies that are being researched to fight cancer include:

- Angiogenesis inhibitors. These antibodies prevent the growth of new blood vessels, cutting off the supply of oxygen and nutrients to cancer cells.
- Signal transduction inhibitors. These antibodies block signals inside the cancer cell that helps the cells divide, stopping the cancer from growing.
- Antibodies/antagonists for other hormones/receptors such as androgen receptors and prolactin receptors, which are present in a high proportion of breast cancers

Targeted cancer therapies are drugs or other substances that block the growth and spread of cancer by interfering with specific molecules involved in tumor growth and progression. Because scientists often call these molecules "molecular targets," targeted cancer therapies are sometimes called "molecularly targeted drugs," "molecularly targeted therapies," or other similar names. By focusing on molecular and cellular changes that are specific to cancer, targeted cancer therapies may be more effective than other types of treatment, including chemotherapy and radiotherapy, and less harmful to normal cells. Many targeted cancer therapies have been approved by the U. S. Food and Drug Administration (FDA) for the treatment of specific types of cancer. Others are being studied in clinical trials (research studies with people), and many more are in preclinical testing (research studies with animals). Targeted cancer therapies are being studied for use alone, in combination with other targeted therapies, and in combination with other cancer treatments, such as chemotherapy.

Targeted cancer therapies interfere with cancer cell division (proliferation) and spread in different ways. Many of these therapies focus on proteins that are involved in cell signaling pathways, which form a complex communication system that governs basic cellular functions and activities, such as cell division, cell movement, cell responses to specific external stimuli, and even cell death. By blocking signals that tell cancer cells to grow and divide uncontrollably, targeted cancer therapies can help stop cancer progression and may induce cancer cell death through a process known as apoptosis. Other targeted therapies can cause cancer cell death directly, by specifically inducing apoptosis, or indirectly, by stimulating the immune system to recognize and destroy cancer cells and/or by delivering toxic substances directly to the cancer cells.

The development of targeted therapies, therefore, requires the identification of good targets – that is, targets that are known to play a key role in cancer cell growth and survival. For example, most cases of chronic myeloid leukemia (CML) are caused by the formation of a gene called *BCR-ABL*. This gene is formed when pieces of chromosome 9 and chromosome 22 break off and trade places. One of the changed chromosomes resulting from this switch contains part of the *ABL* gene from chromosome 9 fused to part of the *BCR* gene from chromosome 22. The protein normally produced by the *ABL* gene [Astete] is a signaling molecule that plays an important role in controlling cell proliferation and usually must interact with other signaling molecules to be active. However, Abl signaling is always active in the protein (Bcr-Abl) produced by the *BCR-ABL* fusion gene. This activity promotes the continuous proliferation of CML cells. Therefore, Bcr-Abl represents a good molecule to target.

Once a target has been identified, a therapy must be developed. Most targeted therapies are either small-molecule drugs or monoclonal antibodies. Small-molecule drugs are typically able to diffuse into cells and can act on targets that are found inside the cell. Most monoclonal antibodies cannot penetrate the cell's plasma membrane and are directed against targets that are outside cells or on the cell surface. Candidates for small-molecule drugs are usually identified in studies known as drug screens – laboratory tests that look at the effects of thousands of test compounds on a specific target, such as Bcr-Abl. The best candidates are then chemically modified to produce numerous closely related versions, and these are tested to identify the most effective and specific drugs. Monoclonal antibodies, by contrast, are prepared first by immunizing animals (typically mice) with purified target molecules. The immunized

animals will make many different types of antibodies against the target. Next, spleen cells, each of which makes only one type of antibody, are collected from the immunized animals and fused with myeloma cells. Cloning of these fused cells generates cultures of cells that produce large amounts of a single type of antibody, known as a monoclonal antibody. These antibodies are then tested to find the ones that react best with the target.

Before they can be used in humans, monoclonal antibodies are "humanized" by replacing as much of the animal portion of the antibody as possible with human portions. This is done through genetic engineering. Humanizing is necessary to prevent the human immune system from recognizing the monoclonal antibody as "foreign" and destroying it before it has a chance to interact with and inactivate its target molecule. The first molecular target for targeted cancer therapy was the cellular receptor for the female sex hormone estrogen, which many breast cancers require for growth. When estrogen binds to the estrogen receptor (ER) inside cells, the resulting hormone-receptor complex activates the expression of specific genes, including genes involved in cell growth and proliferation. Research has shown that interfering with estrogen's ability to stimulate the growth of breast cancer cells that have these receptors (ER-positive breast cancer cells) is an effective treatment approach. Several drugs that interfere with estrogen binding to the ER have been approved by the FDA for the treatment of ER-positive breast cancer. Drugs called selective estrogen receptor modulators (SERMs), including tamoxifen and toremifene (Fareston®), bind to the ER and prevent estrogen binding. Another drug, fulvestrant (Faslodex®), binds to the ER and promotes its destruction, thereby reducing ER levels inside cells.

Aromatase inhibitors (AIs) are another class of targeted drugs that interfere with estrogen's ability to promote the growth of ER-positive breast cancers. The enzyme aromatase is necessary to produce estrogen in the body. Blocking the activity of aromatase lowers estrogen levels and inhibits the growth of cancers that need estrogen to grow. AIs are used mostly in women who have reached menopause because the ovaries of premenopausal women can produce enough aromatase to override the inhibition. Three AIs have been approved by the FDA for the treatment of ER-positive breast cancer: Anastrozole (Arimidex®), exemestane (Aromasin®), and letrozole (Femara®). Targeted cancer therapies have been developed that interfere with a variety of other cellular processes.

2.5.1 Nanoparticulate drug delivery system

Therapeutic NP technologies have revolutionized the drug development process and changed the landscape of the pharmaceutical industry [Allen 2004; Farokhzad 2009; Petros 2010; Wagner 2006]. By virtue of their unique physicochemical properties, NPs have shown promise in delivering a range of molecules at a desired site in the body. Nanoparticulate technologies may improve the therapeutic index of drugs by enhancing their efficacy and/or increasing their tolerability in the body. NPs have also been reported to improve the bioavailability of hydrophobic drugs, protect the active pharmaceutical ingredient from physiological barriers, as well as enable the development of novel classes of bioactive macromolecules like proteins, DNA and siRNA [Shi 2010]. Additionally, the incorporation of imaging contrast agents within NPs allowed us to visualize the site of drug delivery or monitor the in vivo efficacy of the therapeutic agent [Cai 2007]. So far, US Food and Drug Administration (FDA) have approved over two-dozen nanotechnology products for clinical use, and many are under clinical and preclinical development [Davis 2008; Wagner 2006]. Interestingly, the majority of these clinically approved, first-generation nanotechnology products are comprised of liposomal drugs and polymer–drug conjugates, which are relatively simple and generally lack active targeting or controlled drug release components. To develop safer and more effective therapeutic NPs, researchers have designed novel multifunctional NP platforms for cell/tissue-specific targeting, sustained or triggered drug delivery, co-delivery of synergistic drug combinations, etc. Among these functions, targeted delivery is critical for the successful development of next-generation nanotechnology products [Shi 2010].

Targeted drug delivery system can be formulated by conjugating drug-encapsulated NPs with targeting ligands, which could facilitate the preferential delivery of NPs to the sites of interest while reducing undesired side effects elsewhere. Since the first description of cell-specific targeted liposomes in 1980 [Heath 1980; Leserman 1980], targeted NPs have shown some promising clinical and preclinical results in the treatment of different diseases. For tumor cell targeting, the presence of targeting ligands could enhance cellular uptake and retention of drugs via receptor-mediated endocytosis, although tumor accumulation through the enhanced permeability and retention (EPR) effect [Maeda 2001] is largely determined by the physicochemical properties of NPs and long circulation half-life [Farokhzad 2009]. Active NP targeting is also essential for the

delivery of biomacromolecules (e.g., DNA and siRNA) that require intracellular delivery for bioactivity [Farokhzad 2009]. In the case of vascular endothelial targeting for oncology or cardiovascular indications, ligand-mediated targeting may be critically important as NPs localization is not a function of EPR [Dhar 2011; Rothenfluh 2008]. In addition, efforts have been made to transport drugs across tight epithelial and endothelial barriers with nanotherapeutics (e.g., the blood–brain barrier) via ligand-mediated transcytosis. More recently, targeted NPs have been employed in solving the complex problems of multidrug resistance [Davis 2008].

Controlled release polymer technology, resulting in the control of drug exposure, has benefited virtually every branch of medicine over the past 4 decades. Many products utilizing this technology are now in clinical use, including Atridox®, Lupron Depot®, Gliadel®, Zoladex®, Trelstar® Depot, and Sandostatin® LAR® [Farokhzad 2006]. Polymeric NPs can encapsulate drugs and release them at sustained rates in the optimal range of drug concentration, thus enhancing the in vivo therapeutic efficacy, maximizing patient compliance, and facilitating the use of highly toxic, poorly soluble, or relatively unstable drugs [Brigger 2002a; Farokhzad 2006]. In general, drug release can be regulated by diffusion of the drug molecules through the polymer matrix or by differential surface and bulk erosion of the polymer. Alternatively, drug release can be triggered by specific microenvironments in the body (e.g., changes in pH, temperature, and enzymatic activities) or manipulated by external events (e.g., electric field, magnetic field, and ultrasound) [Ganta 2008; Kale 2010; Oh 2007]. By further functionalization with targeting ligands, controlled release polymeric NPs could deliver therapeutic agents in a spatiotemporally regulated fashion, which may be essential to many medical applications.

Over the past few decades, different nanotechnology platforms were studied for their use in therapeutic applications [Davis 2008]. These NPs platforms have been developed to enhance the pharmacological properties and therapeutic index of a myriad of drugs [Allen 2004; Moghimi 2005]. Four major classes of nanoparticulate delivery systems, includes liposomes, polymeric NPs, lipid–polymer hybrid NPs, and dendrimers (figure 2.2).

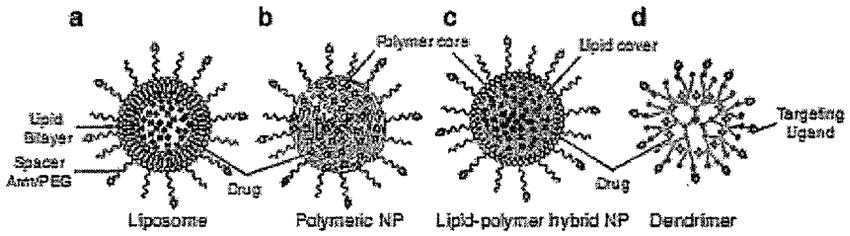


Figure 2.2 Nanoparticulate platforms for the targeted and controlled delivery of drugs, (a) liposome, (b) polymeric nanoparticle, (c) lipid polymer hybrid nanoparticle and (d) dendrimer.

2.5.1.1 Polymeric nanoparticles (Nanospheres and nanocapsules)

Nanospheres consist of a spherical polymeric matrix within which a drug is encapsulated (figure 2.3A). The drug is typically distributed evenly throughout this matrix and released into the environment via diffusion. The composition of the polymer matrix and its ability to imbibe fluids will determine how rapidly the drug will be released [Brigger 2002b].

Nanocapsules are often referred to as reservoir systems as they contain the active ingredient in a core separated from the environment by a polymeric membrane (figure 2.3B) [Haley and Frenkel 2008]. By saturating the core, the active ingredient can diffuse through the membrane with an approximately constant release rate. This release behavior is attractive for drug delivery applications.

The above nanoparticulate systems have been widely explored for diffusion-driven drug release due to their large surface-to-volume ratios, which allow for drug release at feasible and clinically relevant time scales. There is a surge in the development of nanoparticulate systems that do not rely solely on diffusion mechanisms for drug release. Instead, this new class of NPs is able to respond to environmental, chemical, thermal, or biological triggers [Caldorera-Moore 2009; Liechty and Peppas 2012; Schoener 2012]. These 'smart materials' will release their therapeutic payload only when triggered. A more complete review on environmentally responsive carriers was recently published by Liechty et al. Although the diffusion-driven NPs are unable to respond directly to their environment there are means by which these systems can target and accumulate in the tumor interstitium.

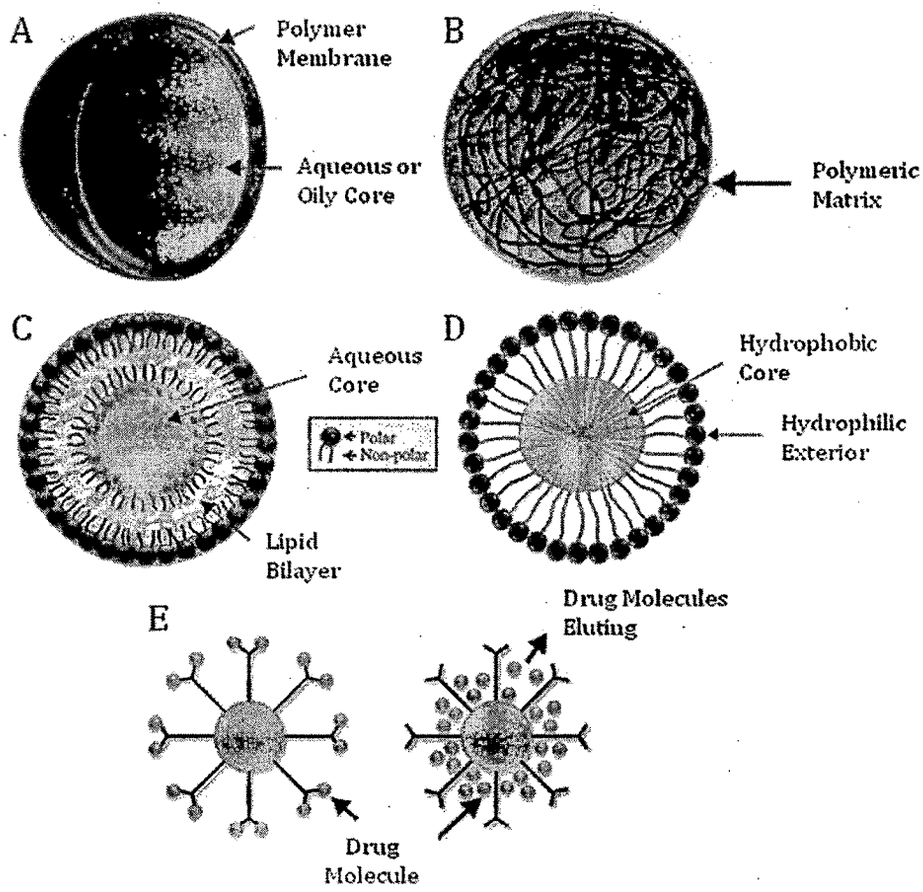


Figure 2.3 Particle schematics, (A) nanosphere, (B) nanocapsule (C) liposome, (D) micelle, (E) dendrimers functionalized with complexed (left) and encapsulated drug molecules.

Polymeric NPs have made a significant clinical impact by improving the pharmaceutical efficacy and dosing of a variety of already approved drugs [Davis 2008; Greco 2009]; however, their drug loading efficiency may be limited by the number of conjugation sites in the polymer, and most of them lack the ability of active targeting or controlling drug release. In order to further enhance the drug loading capacity and incorporate the spatial and/or temporal control over drug delivery, many biocompatible polymeric NPs platforms have been developed [Chan 2010; Napier 2007]. Different polymers like poly lactic-co-glycolic acid (PLGA), poly caprolactone (PCL), poly butyl cyanoacrylate (PBCA), etc. are already used in formulation of nanoparticulate drug delivery systems for anticancer drug delivery. Polymeric micelles have attracted substantial attention for their remarkable potential as therapeutic carriers [Matsumura 2009]. Polymeric

micelles can be formed by self assembly of amphiphilic polymers with two or more polymer chains of different hydrophobicity. In aqueous environments, these block copolymers can spontaneously self-assemble into core-shell nanostructures, with a hydrophobic core and a hydrophilic shell [Chan 2010; Matsumura 2009]. To date several polymeric micelles have reached different stages of clinical development, and these systems have demonstrated enhanced accumulation of therapeutic agents at the target site and/or reduced adverse effects of therapeutic agents [Sutton 2007]. Among them, NK911 [Matsumura 2004] and NK105 utilize PEG-poly(aspartic acid) copolymer to carry and protect the anticancer agents doxorubicin and paclitaxel, respectively. Notably, NK105 was shown to reduce the reported adverse effects of paclitaxel, which include neurotoxicity, myelosuppression, and allergic reactions. A cisplatin-incorporated polymeric micelle-based system, NC-6004, is being examined in Phase I/II clinical trials and has demonstrated several distinct features, including sustained cisplatin release, promoted accumulation of cisplatin in cancer cells, and reduced nephrotoxicity and neurotoxicity associated with cisplatin [Wilson 2008]. Another PEG-poly(glutamic acid) based polymeric micelle, NK012, loaded with 7-ethyl-10-hydroxycamptothecin (SN-38), has been shown to exert more potent antitumor activity against various human tumor xenografts than irinotecan (CPT-11), a water-soluble prodrug of SN-38 [Hamaguchi 2010; Hamaguchi 2005]. More impressively, the nontargeted polymeric micelle composed of poly(l-lactic acid)-PEG (Genexol-PM), for delivery of paclitaxel, was first approved for cancer therapy in Korea in 2007 and is currently being evaluated in a clinical Phase II trial in the United States for the treatment of metastatic pancreatic cancer [Kim 2004; Lee 2008].

The conjugation of polymeric NPs with targeting ligands could also enable drug delivery in a spatially and temporally controlled manner, which may further enhance the therapeutic efficacy of drugs and reduce their toxic side effects. Aptamer-targeted polymeric NPs have been developed and its application in cancer therapy was studied [Dhar 2011; Gao 2010]. For example, A10 RNA aptamer-conjugated PLGA–PEG NPs were developed that can recognize PSMA (prostate-specific membrane antigen), expressed on the cancer cell surface [Farokhzad 2004]. This PLGA–PEG NPs can substantially reduce tumor growth in a human prostate cancer tumor xenograft mouse model. More recently, a strategy was reported for precisely engineering PLGA–PEG NPs with different biophysicochemical properties in a reproducible manner, whereby

enabling the systematic screening of the targeted polymer NPs for optimization [Gu 2008]. Building on these efforts, BIND Biosciences has developed a selfassembled, targeted polymeric NP (BIND-014) and is currently evaluating this nanotherapeutic candidate in Phase I/II clinical trials for the treatment of solid tumors [Service 2010].

2.5.1.2 Liposomes

Liposomes are composed of amphiphilic molecules that are comprised of both polar and nonpolar components that self-assemble into colloidal particles (figure 2.3C). This self-assembly produces a spherical structure with the polar components of the molecule contacting the polar environment and the nonpolar components contacting the nonpolar environment [Lasic 1998]. The most common classification of liposomes is by the number of lipid bilayers present in the colloidal structure, with unilamellar liposomes containing one lipid bilayer and multilamellar liposomes containing multiple lipid bilayers. Due to their amphiphilic nature liposomes are capable of encapsulating both polar and nonpolar compounds for delivery [Lasic 1998].

Liposomes are attractive for drug delivery applications for numerous reasons, including their resemblance to cell membranes in both structure and composition. Additionally, liposomes can be readily formed with nontoxic, nonimmunogenic, natural and biodegradable amphiphilic molecules [Haley and Frenkel 2008; Lasic 1998]. Liposomes by themselves tend to be slightly sterically unstable and are cleared rapidly from the bloodstream. For drug delivery applications, this behavior is remedied by functionalizing the liposomal surface with poly(ethylene glycol) tethers to impart increased steric stabilization [Lasic 1998]. The surface of the liposome can also be modified with ligands for active targeting. A pegylated biodegradable liposome was used to encapsulate doxorubicin and became the first liposome-based treatment for cancer (Doxil) [Haley and Frenkel 2008].

2.5.1.3 Micelles

The micelle is composed of amphiphilic molecules that self-assemble into a structure with a hydrophobic core and a hydrophilic exterior (figure 2.3D) [Liechty 2012]. Micellar structure lends itself well to drug delivery applications for multiple reasons. Micelles typically have diameters of less than 100 nm, allowing them to participate in extravasation through the fenestrations in tumor vessels and limiting their uptake by the MPS/RES system. Their hydrophilic surface characteristics also shield them from immediate recognition and subsequently increase circulation time [Lavasanifar 2002].

Hydrophobic drugs can be loaded into the core of the micellar structure and protected by the hydrophilic corona during transport to the tumor site [Kwon 1995].

2.5.1.4 Dendrimers

Dendrimers are highly branched molecules that display a high degree of monodispersity and a well-defined structure [Hughes 2005]. They are stable and have surfaces that can be readily functionalized with targeting ligands and molecules such as folic acid [Majoros 2006]. Drug molecules can be encapsulated in the dendrimer's multifunctional core and protected by the extensive branching. Drug molecules, such as paclitaxel, can also be attached to the exterior of the dendrimer (figure 2.3E) [Majoros 2006].

2.6 Targeting

One significant challenge for the successful development of therapeutic NPs is its rapid clearance during systemic delivery. When NPs enter the bloodstream, the particle surface may experience nonspecific protein adsorption (opsonization), thereby making them more visible to phagocytic cells [Alexis 2008b; Owens 2006b]. After opsonization, NPs could be rapidly cleared from the bloodstream through phagocytosis by the mononuclear phagocyte system [Kurokawa] in the liver and by spleen filtration [Eisenstein 2006; Ostuni 2001]. Therefore, the factors that could affect the clearance and biodistribution of NPs, such as particle physicochemical properties and targeting ligand functionalization [Alexis 2008b], should be evaluated for the optimal design of therapeutic NPs.

2.6.1 Passive targeting

Passive targeting of NPs takes advantage of the abnormal tumor physiology and structure that results in the EPR effect. The permeability of the vasculature and retention by an insufficient lymphatic system can passively accumulate macromolecules and increase their tumor concentration by 70-fold [Duncan 2003]. This accumulation will only be observed if the macromolecules avoid clearance by mechanisms such as renal clearance and uptake by the MPS/RES. Two of the most important properties of effective nanocarriers are the carriers' ability to (a) remain circulating in the blood stream for a significant amount of time and (b) target specific tissues and cells [Duncan 2003]. Particle circulation time, targeting, and the ability to overcome biological barriers is also dependent on a particle's shape, size, and surface characteristics. The lifespan of a NPs within circulation is modulated by its interactions with the

environment and can be modified by changing its size, particle shape, and surface characteristics [Davis 2008].

2.6.1.1 Size

The size of NPs has an extremely important impact on its interaction with its environment. As stated previously, a particle must be at least 10 nm in diameter to avoid clearance by first pass renal filtration [Davis 2008; Venturoli 2005]. The largest size of NPs to be used for drug delivery to a tumor is determined by a multitude of factors. As passive targeting is entirely dependent on diffusion-mediated transport into the tumor, size is important. Dreher and colleagues have shown that particles on the order of hundreds of nanometers in diameter can accumulate in the tumor tissue. Using dextran as a model macromolecule they showed that increasing the molecular weight from 3.3 kDa to 2 MDa reduced permeability by two orders of magnitude. Larger molecules were able to accumulate but were primarily contained close to the vascular surface within the tumor. Conversely, smaller molecules could penetrate more deeply into the tumor interstitium and achieve a more homogenous distribution. These observed behaviors are attributed to the effective interstitial diffusion coefficient, which decreases as the molecular weight of the diffusing molecule increases [Dreher 2006]. Extrapolating from macromolecules to NPs, it has been determined that the upper bound size for NPs participating in the EPR effect is approximately 400 nm [Alexis 2008a]. Particles larger than 400 nm are simply unable to diffuse through the tumor interstitium in sufficient quantities to have any clinical or therapeutic effect.

While 400 nm is the upper bound for harnessing the effect of EPR there are other important factors that narrow the effective size range of NPs. The leaky vasculature in tumors is highly permeable due to the increased size and quantity of fenestrations as well as incomplete or abnormal basement membranes [Haley and Frenkel 2008; Roberts and Palade 1995]. These fenestrations are typically 50–100 nm in size and, although not the only mechanism of permeating into the tumor interstitium, an important pathway for NP accumulation. Looking solely at clearing mechanisms, it has been shown that particles with diameters less than 200 nm will be cleared much less rapidly than particles with diameters over 200 nm [Alexis 2008a; Matsumura 1988; Moghimi 1993]. With all of the above factors taken into consideration, an approximate upper bound of 150 nm has been determined [Liechty 2012]. Therefore, in order to be an effective drug carrier the NP should have a diameter between 10–150 nm. This size

range will ensure longer circulation time and increased accumulation in the tumor interstitium.

On the basis of physiological parameters such as hepatic filtration, tissue extravasation/diffusion, and kidney excretion, it is clear that particle size plays a key factor in the long circulation and biodistribution of NPs. NPs smaller than 10 nm can be rapidly cleared by the kidneys or through extravasation, while larger NPs may have higher tendency to be cleared by cells of the mononuclear phagocyte system (MPS, also referred to as reticuloendothelial system, RES) [Petros 2010]. For example, in vivo biodistribution results of polystyrene NPs with consistent composition and varying particle size of 50 and 500 nm showed higher level of agglomeration of the larger NPs in the liver [Nagayama 2007]. Another study compared different size ranges of PEGylated spherical NPs (<100 nm, 100–200 nm, and >200 nm) for protein absorption, NP uptake by murine macrophages, and blood clearance kinetics [Fang 2006b]. It was observed that NPs <100 nm have a higher potential to circulate in the blood for long periods of time and experience reduced hepatic filtration. NP size also plays a key role in tumor accumulation through the EPR effect. Several studies have tried to determine the gap size in the leaky vasculature. For example, sterically stabilized liposomes of 100–600 nm were used for transvascular transport, and the cutoff size of the pores was estimated to be 400–600 nm in diameter [Yuan 1995]. In another study, the pore cutoff size was estimated to be between 7 and 100 nm at 34°C and was increased to >400 nm at 42°C, allowing all NPs tested (~7 nm albumin, and 100, 200, and 400 nm liposomes) to be delivered to the tumor interstitium to some degree [Kong 2000]. Therefore, to capitalize on the EPR effect and to efficiently escape from the physiological barriers, many studies advocate the optimal NP size range of approximately 10–250 nm [Alexis 2008b].

2.6.1.2 Particle shape

Development of novel particle fabrication methods that allow for precise control over particle shape and size [Champion 2007; Glangchai 2008; Rolland 2005] has allowed for researchers to explore the effects of particle shape on particle bio-distribution and cellular internalization. The effects of particle shape and potentially the particles' curvature on cellular internalization was shown by Chan et al. [Chithrani 2007]. It was reported that 14 and 75 nm spherical NPs were up-taken by cells 3.75–5 times more than 74-by-14 nm rod-shaped particles. Gratton et al. have also demonstrated the

effects of *in vitro* cellular internalization in HeLa cells. The group reported that cylindrical NPs had the highest percentage of cellular internalization [Gratton 2008]. Specifically, NPs with 150 nm diameter and 450 nm height showed the highest internalization percentage and were taken up 4 times faster than symmetrical particles (aspect ratio of 1200 by 200 nm cubes). These findings suggest that NPs' aspect ratio also plays an important role in cellular uptake. However, in the same studies, 100 nm diameter particles with an aspect ratio of 3 had a lower degree of internalization compared with 150 nm particles with the same aspect ratio. The group also observed that cylindrical-shaped particles with 500 nm or 1 μm diameters and 1 μm height had reduced internalization in comparison with smaller particles but showed higher uptake than micrometer-sized square cross-section particles. This result suggests that the uptake kinetics is probably a function of both size and shape.

2.6.1.3 Surface characteristics

The surface of a particle is the primary medium by which it interacts with its environment. This is of even greater important with NPs because of their large surface-to-volume ratio and relatively large surface area [Storm 1995]. The surface can be modified by polymer content or functionalization which will impact how the environment "sees" the particle. When contemplating the question of drug delivery it is essential to consider how to modify the particle so it remains in circulation for the longest possible time to ensure tumor accumulation. It has been determined that modifying the surface of NPs by adding hydrophilic polymers results in decreased clearance by the MPS/RES system [Storm 1995]. One such hydrophilic polymer is poly(ethylene glycol). When attached to the surface of NPs PEG imparts stealth characteristics by shielding the NPs from opsonin adsorption and subsequent clearance by the MPS/RES [Alexis 2008a]. The shape, density and length of the PEG chains can be modified and have various effects on the rate of clearance. It has been shown that increasing the molecular weight of PEG chains above 2 kDa increases the half-life of the PEGylated particle [Owens 2006a]. A dense covering of PEG chains over the surface, particularly of negative particles easily recognized by the MPS/RES, is also necessary to prevent rapid clearance [Fang 2006a]. It has been established that the surface charge of NPs also could affect their uptake by the MPS cells. Neutrally charged particles have demonstrated much lower opsonization rates than charged particles [Roser 1998; Schwendener 1984]. It was found that positively charged NPs generate a higher

immune response (complement activation and conjugate activation) compared to neutral or negatively charged NP formulations [Salvador-Morales 2009]. For example, NPs with a primary amine at the surface promote higher rates of phagocytic uptake when compared to those having sulfate, hydroxyl, or carboxyl groups at the surface [Alexis 2008b; Salvador-Morales 2009]. In a review study, Davis *et al.* have proposed that the optimal range of NP surface charge should be between -10 and +10 mV for reduced phagocytosis and minimized nonspecific interactions of NPs [Davis 2009].

2.6.1.4 PEGylation

PEG chains as a hydrophilic polymer with a flexible nature can be selected as shell-forming segments, which assemble into dense palisades of tethered chains to achieve unique properties. The biocompatibility was guaranteed by the dense PEG shell, which endows the micelle with a stealth character in the blood compartment, achieving a long circulation [Kataoka 1993]. PEG chains attached to a surface or forming the corona of a nanosphere exhibit rapid chain motion in an aqueous medium and have a large excluded volume. The steric repulsion resulting from a loss of conformational entropy of the bound PEG chains upon the approach of a foreign substance and the low interfacial free energy of PEG in water contribute to the extraordinary physiological properties of nanospheres covered with PEG [Kataoka 1994; Yokoyama 1991].

Drug targeting for efficient accumulation in the body is often hampered by the rapid recognition of carrier system by the RES and by the subsequent kidney and/or hepatic elimination. Moreover, for modulated drug delivery to solid tumors, which locate outside the blood compartment, the carrier is required to exhibit not just a sufficient half-life in the blood compartment, but also the capability of extravasation at the tumor site. Recent developments led to the design of drug carriers with prolonged circulation in the vascular system [Kataoka 1993]. Cancer chemotherapy may cause severe side effects, leaving patients under extreme distress. To overcome this problem, an interest has been raised in the application of block copolymer as novel carrier systems for anticancer agents [Kataoka 1994; Kataoka 1993; Yokoyama 1991].

PEG grafted to surfaces of biomedical devices also proved to increase their biocompatibility and to reduce thrombogenicity [Holmberg 1993]. Surface modification of NPs with PEG, which has favorable intrinsic physicochemical properties (*e.g.*, high flexibility and hydrophilicity, and low toxicity and immunogenicity), was found to reduce NP accumulation in off-target organs such as liver and spleen [Knop 2010]. A

PEG shell on the NP surface shields hydrophobic or charged particles from attachment by blood proteins, leading to prolonged circulation half-life compared to non-PEGylated NPs [Moghimi 2003; Vonarbourg 2006]. The length, shape, and density of PEG chains on the NP surface largely affect its surface hydrophilicity and phagocytosis [Grefa 2000]. For example, at low PEG surface density, the PEG chains would be closer to the surface of the NP with a "mushroom" configuration, while as the density increases, most of the chains are extended away from the surface in a "brush" configuration, which decides the thickness of the PEG shell on the NP corona [Owens 2006b]. It has been postulated that the brush configuration would create more effective blocking or repulsion of opsonins than the mushroom one [Vonarbourg 2006]. In addition to PEG, some other promising hydrophilic polymers are under investigation for the same purpose, including natural polymers (*e.g.*, heparin, dextran, and chitosan) and synthetic polymers (*e.g.*, poly(amino acids), poly(glycerols), poly(2-oxazolines), and some vinyl polymers) [Knop 2010; Moghimi 1991]. The conjugation of targeting ligands to the surface of PEGylated NPs has also been shown to affect their biodistribution [Takae 2005]. Although targeting ligands could improve the cell- or tissue-specific delivery of NPs, they may compromise the particle surface properties by masking the PEG layer and adversely affecting the NPs' macrophage uptake in vivo. A recent study on the effect of ligand density has also revealed a relatively narrow window of ligand density that could result in favorable tumor targeting, while minimizing NP accumulation in the liver and spleen [Gu 2008]. Thus, the successful development of targeted NP technology for efficient drug delivery strongly depends on striking a balance between cellular targeting and immune evasion.

2.6.1.5 Limitations of passive targeting

Passive targeting can be achieved by modulating the size, shape, and surface characteristics of the NP drug carriers. However, there remain significant barriers to transport that often result in insufficient drug concentrations at the tumor site and, consequently, little therapeutic efficacy [Brigger 2002b; Gu 2007]. Furthermore, passive targeting suffers from some of the same limitations of traditional chemotherapy such as an inability to actively distinguish healthy tissue from tumor tissue.

2.6.2 Active targeting

Despite their enormous potential for drug delivery, the translation of targeted NP systems has faced considerable challenges, and only a handful of candidates have made it to clinical trials. The reason targeted NPs have demonstrated limited success in

clinical development is complex and could be multifaceted [Farokhzad 2009]. Among others, an essential aspect for the successful development of targeted NPs relies on the choice of targeting ligands. Several variables that could be considered include ligand biocompatibility, cell specificity, binding affinity, and purity of the ligand [Allen 2002]. Other important factors that have to be taken into account are the size and charge of the ligand molecule, and their ease of modification and conjugation to the NPs. The choice of ligand, from a practical perspective, is also dependent on production cost, scalability, and stability (*e.g*, organic solvent and high temperature stability) in mass production.

Active targeting takes advantage of ligand-receptor, antigen–antibody and other forms of molecular recognition to deliver a particle or drug to a specific location [Haley and Frenkel 2008]. For cancer therapy, active targeting moieties are particularly beneficial because they reduce or eliminate the delivery of potentially toxic drugs to healthy tissue. Targeted NPs delivering chemotherapeutics are of interest because they can increase therapeutic effectiveness and reduce potential side effects [Gu 2007]. Active targeting takes advantage of the over-expression of receptors, such as folate and transferrin, on the tumor cell surface [Liechty and Peppas 2012]. These targeted nanodelivery devices have performed significantly better than their non-targeted counterparts resulting in an increased cytotoxicity to tumor cells and reduction of side effects [Phillips 2010]. This section will focus on the most widely utilized active targeting ligands for tumor therapy including folate, transferrin, aptamers, antibodies, and peptides.

2.6.2.1 Folate

Folate has been one of the most extensively utilized ligands for targeted drug delivery devices. The folate receptor [Haley and Frenkel], or the high affinity membrane folate binding protein, binds the folate molecule with extremely high affinity (K_D ≈ 10^{-9}) [Gu 2007; Hilgenbrink 2005]. This receptor is also over-expressed in a variety of tumors such as ovarian carcinomas, choricarcinomas, meningiomas, uterine sarcomas, osteosarcomas, and non-Hodgkin's lymphomas [Sudimack 2000]. Particles conjugated with folate or folic acid and bound to a folate receptor are internalized by the cell and introduced to the cytoplasm (figure. 2.4A). The drug is then released by the NP in the cytoplasm of the tumor cell and proceed to interact with intracellular components [Haley and Frenkel 2008; Stella 2000].

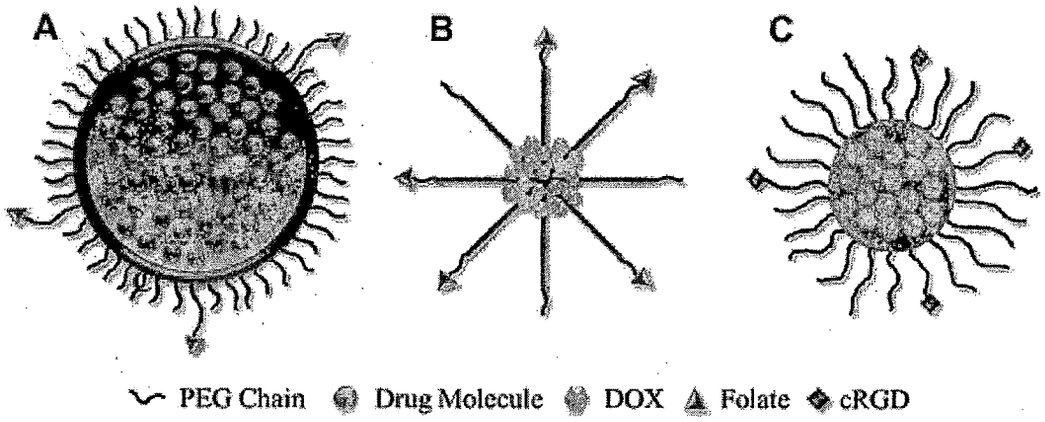


Figure 2.4 Targeted particles: (A) example of a folate receptor targeted particle. Liposome functionalized with PEG tethers to impart STEALTH characteristics and folate for tumor targeting, (B) folate-conjugated PLGA-PGA polymeric micelle loaded with encapsulated doxorubicin and (C) cRGD-functionalized PCL–PEG polymeric micelle containing encapsulated doxorubicin.

One such folate conjugated NP is a folate receptor targeted biodegradable polymeric micelle loaded with doxorubicin developed by Yoo and colleagues. Micelles were created from a copolymer of poly(D,L-lactic-co-glycolic acid) (PLGA) and poly(ethylene glycol). The PLGA allows the particle to biodegrade after delivery of its drug payload and the PEG increases the circulation time of the particles. Doxorubicin was conjugated via a chemical linkage to the PLGA while the folate was added to the PEG. The micelle (figure 2.4B and C) was tested for cytotoxicity and cardiotoxicity (a common side effect of DOX) compared to free DOX on folate-receptor-positive cell lines. It was determined that these particles exhibited increased cellular uptake, circulation time, and decreased cardiotoxicity [Yoo 2004]. The decrease of cardiotoxicity indicates that the targeting moiety was able to differentiate between healthy and tumor tissue with greater specificity than untargeted DOX. Furthermore, the increased cytotoxicity and cellular uptake shows that the folate-receptor actively internalized the conjugated particle into the cytoplasm [Yoo 2004].

2.6.2.2 Transferrin

Transferrin is another receptor-ligand pair that has been utilized for tumor targeting applications. Transferrin is a membrane glycoprotein that functions with its receptor, TfR, to aid in uptake of iron by the cell [Ponka 1999; Yoo 2004]. Much like folate, when transferrin binds to its receptor it initiates endocytosis and is internalized into the

cellular cytoplasm [Ponka 1999]. The transferrin receptor is overexpressed by as much as 10-fold on tumor cells making it an attractive option for targeted delivery of chemotherapeutics via NP carriers [Sahoo 2004]. Sahoo and colleagues have focused a great deal of attention on developing transferrin-conjugated paclitaxel-loaded NPs. The NPs were made using copolymerized PLGA and poly(vinyl alcohol) (PVA), both well-studied and defined materials for drug delivery. Transferrin was conjugated to the NP surface and loaded with paclitaxel. The conjugated and loaded NPs were introduced to a human prostate cancer cell line. These particles were compared to a simple solution of paclitaxel and loaded particles without transferrin. The transferrin-conjugated particles exhibited a sustained release profile and a cellular uptake three times greater than the unconjugated NPs. Furthermore, the conjugated NPs reduced cellular proliferation by 70%, while the unconjugated NPs only reduced it by 35%. The free paclitaxel, by comparison, only reduced proliferation by 20% [Sahoo 2004]. Transferrin-conjugated NPs have been shown to inhibit cellular proliferation and tumor growth while participating in sustained release profiles and increased cellular uptake. The effectiveness of the conjugated NPs is most likely due to their ability to be taken up by receptor-mediated endocytosis, which enhances the amount of drug delivered to tumor cells and limiting the amount delivered to healthy cells [Sahoo 2005; Sahoo 2004].

2.6.2.3 Aptamers

Aptamers are short oligonucleotides of RNA or DNA that can fold into various conformations and engage in ligand binding [Gu 2007]. However, finding such sequences is akin to finding a needle in a haystack, with only one in 10^{10} random RNA sequences folding into a configuration able to participate in ligand binding [Wilson 1999]. Systematic evolution of ligands by exponential amplification, is a process by which researchers can comb through vast populations of RNA and DNA sequences to find new aptamers to act as targeting ligands. Benefits of aptamers include their small size (≈15 kD), lack of immunogenicity, and the potential to readily penetrate and target tumor cells. It has been shown that, much like folate and transferrin, aptamers result in increased targeting specificity and more efficient drug delivery to tumor cells [Gu 2007]. An aptamer-conjugated NP has been created for the delivery of cisplatin to prostate cancer cells [Dhar 2008]. The selected target is a prostate-specific membrane antigen (PSMA) that is highly overexpressed in prostate cancer cells and can be readily targeted by a PSMA aptamer. A traditional NP carrier composed of poly(D,L-lactic-co-glycolic

acid) and poly(ethylene glycol) tethers was used to encapsulate cisplatin. Cisplatin is a platinum-based chemotherapeutic that functions by interfering with DNA transcription but is normally ineffective against prostate cancer cells when administered systemically. It is thought that targeted delivery of cisplatin could increase its therapeutic effectiveness. In fact, when compared to free cisplatin the PSMA aptamer-targeted Pt(IV)-encapsulated PLGA-b-PEG NPs are 80 times more toxic to prostate cancer cells expressing PSMA. Aptamer-conjugated NPs have significant potential as cancer-drug-delivery vehicles.

2.6.2.4 Antibodies (monoclonal antibodies)

Antibodies and antibody fragments form an important class of targeting ligands with a high degree of specificity for cellular receptors and a wide range of binding affinities and have been extensively investigated in targeted drug delivery [Torchilin 2008]. Over the past 2 decades, the feasibility of antibody-based tissue targeting has been clinically demonstrated with several different monoclonal antibodies (mAbs) approved by the FDA [Gabizon 2001]. The recent advances in hybridoma technology have led to the development of chimeric, humanized, and fully human mAbs to reduce their immunogenicity. The ability of engineered mAbs to target disease processes has been demonstrated by the success of several monoclonal antibody therapeutics, including cetuximab rituximab, trastuzumab, and bevacizumab [Wang 2008]. mAbs have been used to direct the NP carriers in a site-specific manner. For example, mAb-conjugated PLA NPs exhibited a sixfold increase in the rate of particle uptake compared with nontargeted particles [Nobs 2004]. Additionally, J591, a mAb against PSMA, was conjugated to G5-PAMAM dendrimers and showed enhanced binding affinity for LNCaP cells, as compared to nontarget PC3 cells [Patri 2004]. Nevertheless, mAb conjugated NPs encounter considerable challenges and limitations for drug delivery, since mAb are complex and large (~150 kDa) molecules and require significant engineering at the molecular level to be effective [Brennan 2004].

Compared to mAbs, antibody fragments have demonstrated higher potential for the engineering of targeted NPs as they are smaller in size and lack the complement activation region of mAbs, while retaining the antigen binding specificity [Carter 2001]. Recent advances in protein engineering have led to the development of antibody fragments such as scFv (single-chain variable fragments), Fab (fragments of antigen binding), their dimers (F(ab') and diabody), and recombinant products [Pavlinkova

2001]. Some pioneering examples of antibody fragment-targeted liposomes (immunoliposomes) in clinical trials include MCC-465 that uses F(ab')2 for the targeted delivery of doxorubicin [Sankhala 2009] and SGT-53 that uses scFv to deliver tumor suppressor gene, p53.

Like aptamers, antibodies attached to the surfaces of NPs target specific antigens present on the cell membrane. The use of antibodies as targeting moieties has been extensively investigated over the past decade and has resulted in numerous available treatments (Table 2.1) [Adams 2005; Brannon-Peppas 2004; Gu 2007; Weber 2007]. Unconjugated antibodies have been shown to have antitumor effects on lymphomas, breast cancers, non-Hodgkin's lymphomas, colorectal cancers and chronic lymphocytic leukemias [Mehren 2003; Weiner 2000]. Antibody-based treatments function by recognizing specific antigens located on the surface of cancer cells. Once an antibody–antigen interaction occurs it can induce antitumor affects by multiple mechanisms including interfering with ligand-receptor binding or suppression of protein expression [Mehren 2003].

Table 2.1 Available antibody-based cancer treatments [Adams 2005; Brannon-Peppas 2004; Weber 2007].

Drug	Antigen target	Cancer	Release date
Alemtuzumab	CD52	Chronic lymphocytic leukemia	2001
Bevacizumab	VEGF	Colorectal, lung cancer	2004
Cetuximab	EGF receptor	Colorectal cancer	2004
Gefinitib	EGFR	Advanced NSCLC	2003
Gemtuzumab	CD33	Acute meylogenous leukemia	2000
Ibritumomab tiuxetan	CD20	Non-hodgkins lymphoma	2002
Ipilimumab	CTLA-4	Advanced melanoma	2011
Ofatumumab	CD20	Chronic lymphocytic leukemia	2010
Panitumumab	EGFR	Colorectal cancer	2008
Rituximab	CD20	Lymphoma	1997
Tostiumomab	CD20	Lymphoma	2003
Trastuzumab	HER2	Breast cancer	1998

Although utilized for multiple successful treatments, antibody-based targeting had several early limitations. The antibodies for human use were often derived from mice and, in some individuals, resulted in an immune response that limited the duration and effectiveness of treatment. Another limitation was the lack of specificity and adequate targeting of the antibodies to their antigen-binding sites [Brissette 2006]. Current technology has overcome some of these early limitations. Antibodies derived from murine proteins can now be manipulated into humanized versions that will provoke little to no immune response. Furthermore, the specific binding regions can be molecularly modified to specifically target a wide variety of receptors [Brissette 2006]. The IgG molecule is extensively used for this purpose, as it contains a binding region that recognizes antigens and can be readily modified to specifically distinguish a variety of targets [Brissette 2006].

One such target is the epidermal growth factor receptor (EGFR), which is over-expressed in many cancers, and will bind to two separate ligands: epidermal growth factor and transforming growth factor-alpha [Mendelsohn 1997]. When either ligand binds to the EGFR it stimulates growth of cells and is responsible for the rapid proliferation of cells in a variety of cancers. By blocking this ligand–receptor interaction via antibody-interference, the proliferative behavior of the cell is either reduced or stopped [Mendelsohn 1997]. Hoffman and colleagues have determined that combining anti-EGFR antibodies with cisplatin and doxorubicin increases the cytotoxic effects of the drugs and, in some cancers, entirely eradicates the tumor [Hoffmann 1997]. Monoclonal antibodies have also been examined as targets for conjugated-NP drug-delivery vehicles. The Alléman group tested two different biodegradable PLA NP formulations. The first formulation was conjugated with the trastuzumab mAb (HER2 antigen) and the second with rituximab mAb (CD20 antigen). The conjugated-NPs bound to cells expressing the respective antigens at a frequency 10 times higher than non-targeted NPs [Nobs 2005].

The specificity of antibodies lends particularly well to the active targeting of a variety of tumor types due to their ability to distinguish between healthy and cancerous cells and even amongst cancer cell types. In colorectal cancers, for example, over 95% of cases express the A33 antigen which can be targeted via a humanized A33 monoclonal antibody (huA33 mAb). A number of clinical studies have shown that huA33 mAb is capable of localizing specifically to colorectal cancer cells expressing the A33 antigen

[Johnston 2012]. Recently, Johnston and colleagues, have reported on the development of a polymeric NP system composed of a silica core followed by a layer-by-layer deposition of alkyne-modified poly(*N*-vinylpyrrolidone) (PVPONAlk) and poly(methacrylic acid) [Tang et al.]. To this particle, the A33 monoclonal antibody was conjugated to the surface via click chemistry and imparted targeting characteristics to the system. Upon incubating the huA33 mAb-conjugated particles with L1M1899 colorectal cancer cells expressing the A33 antigen, it was observed that extensive internalization of the particles occurred as compared to the particles conjugated with a negative control, IgG. The antibody-conjugated particles not only preferentially interacted with the cancerous cells but were also phagocytosed, which is ideal for the delivery of chemotherapeutic agents [Johnston 2012].

While antibody-based cancer therapeutics have shown promise there are several remaining limitations that must be considered in the future. The development and modification of antibodies is a complex and expensive process that is difficult to scale-up to large-scale manufacture [Brissette 2006]. Even with fully humanized antibodies an immune response is a potential road block to treatment. Tumor penetration has also been an issue, with observed non-uniform uptake into the tumor mass [Weiner 2000]. This lack of tumor penetration has been attributed to the increased size of NPs due to the hydrodynamic radius of antibodies (≈20 nm) and an uneven distribution of antigens [Gu 2007; Weiner 2000]. Antibody fragments have been posed as a solution, as they are smaller, induce a lesser immune response, and can still selectively target antigen receptors on the surface of tumor cells [Gu 2007].

2.6.2.5 Peptides

Peptides have also been proposed as a potential targeting moiety for delivering chemotherapeutics. Peptides, much like antibodies, can be used to disrupt ligand-receptor interactions on tumor cells and lead to cessation of cellular proliferation. They have the added benefit of being much less expensive and complex to manufacture than antibodies [Brissette 2006]. Screening of potential protein ligands is typically completed using a combinatorial phage library. This technique results in ligands that range from 10–15 amino acids in length and are able to selectively bind to tumor targets with high affinity [Brissette 2006; Gu 2007; Krag 2006]. One such tumor target is the $\alpha_v\beta_3$ integrin, which is present at elevated levels on tumor cells and is an essential component of angiogenesis [Brooks 1994]. This integrin is recognized by the arginine–

glycine–aspartic acid (RGD) peptidic sequence [Byrne 2008]. The affinity of the RGD sequence to the $\alpha_v\beta_3$ integrin has potential to be exploited for drug delivery devices. Nasongkla and colleagues have functionalized the surface of polymeric micelles with a cyclic peptide containing the RGD sequence to deliver doxorubicin to Kaposi's sarcoma cells. The polymer micelle was composed of poly(ε-caprolactone)–poly(ethylene glycol) (PCL–PEG) imparting both biodegradable and long-circulating characteristics to the structure. The doxorubicin (DOX) was loaded into the polymeric micelle and preferentially located into the center of the structure. The polymer ends were then functionalized with a cyclic pentapeptide c(Arg-Gly-Asp-D-Phe-Lys) c(RGD) containing the RGD sequence to allow for selective targeting to the $\alpha_v\beta_3$ integrin. When introduced to Kaposi's sarcoma derived cells (displaying an overexpression of the $\alpha_v\beta_3$ integrin) a 30-fold increase in cellular uptake was observed between the cRGD-containing and non-functionalized micelles.

Another peptidic sequence capable of targeting behavior is Angiopep-2, the complementary ligand to the low-density lipoprotein receptor-related protein (LRP). The LRP is highly overexpressed both on the blood–brain barrier and on glioblastoma multiforme (GBM), or glioma, a tumor of the pituitary gland which is typically inoperable. The combined targeting effects of Angiopep-2 has the potential to allow a therapeutic to pass through the blood–brain barrier at sufficient concentration to then target the glioma within the brain. Xin et al., have conjugated Angiopep-2 to the surface of poly(ethylene glycol)–co-poly(-caprolactone) NPs to engage in dual-targeting of gliomas. After observing increased cellular uptake of Ang-targeted U87 MG glioma cells as compared to blank controls, the *in vivo* targeting effects were measured.

2.6.2.6 Limitations of active targeting

Active targeting moieties are capable of reducing off-target effects and improving the bioavailability of the chemotherapeutic agent. In addition, the inclusion of imaging modalities within these nanostructures yield particles that can, theoretically, be used to target and image the tumor, while simultaneously releasing a therapeutic payload. However, there are a number of limitations with active targeting that bear some discussion. The incorporation of active targeting ligands is designed to improve and enhance NP accumulation at the tumor site. What remains to be seen is whether the increased concentration of carriers and their respective payloads have any bearing upon the delivery of the therapeutic into the interior of the cell. Even if the NP carriers

are capable of preferentially collecting in the tumor site, their efficacy is wholly dependent on their ability to deliver the payload [Phillips 2010]. The harnessing of receptor-mediated endocytosis is coupled with the added challenge of encouraging endosomal-escape once the carrier or therapeutic is entrapped. Additionally, the replacement of stealth polymers, such as PEG, with the active targeting moieties can drastically affect opsonization and clearance of the carrier. In order for the active targeting ligands to perform their function they must encounter tumor cells expressing the motifs of interest. If the carriers are rapidly cleared from the bloodstream, accumulation in the liver, spleen and other RES organs will be observed, while the tumor will amass a lesser amount of the targeted carriers [Phillips 2010]. While active targeting ligands overcame a number of limitations seen with their passive targeted counterparts, additional work must be completed to enhance overall biodistribution and therapeutic efficacy of these actively targeted nanoparticulate carriers.

2.7 Poly lactic-co-glycolic acid (PLGA)

Recently, nano-sized drug delivery systems (DDS) especially biocompatible and biodegradable polymer NPs have attracted considerable interest since they can offer a suitable means of delivering small molecular weight drugs, proteins or genes to a targeted tissue or organ [Moghimi 2001; Zweers 2003]. NPs are colloidal systems that have size typically in the range of 10-1000 nm in diameter, and drug can be entrapped in, adsorbed or chemically coupled onto the polymer NP matrix [Labhasetwar 1997]. On the other hand, a number of polymers have been investigated for formulating biodegradable NPs, such as polylactide, polycaprolactone (PCL) and poly(lactide-co-glycolide) (PLGA). They are biocompatible and biodegradable polymers approved by FDA and have been studied extensively [Berton 1999; Rouzes 2000].

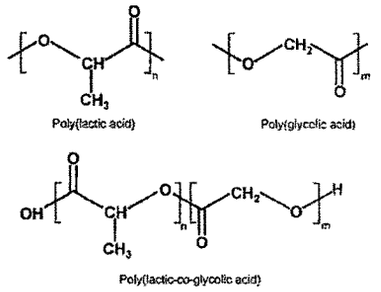


Figure 2.5 Molecular structure of lactide and glycolide based biodegradable polymer

PLGA is synthesized by means of random ring-opening co-polymerization of two different monomers, the cyclic dimers (1,4-dioxane-2,5-diones) of glycolic acid and lactic acid. Common catalysts used in the preparation of this polymer include tin(II) 2-ethylhexanoate, tin(II) alkoxides, or aluminum isopropoxide. During polymerization, successive monomeric units (of glycolic or lactic acid) are linked together in PLGA by ester linkages, thus yielding linear, aliphatic polyester as a product (figure 2.5)[Astete 2006].

Depending on the ratio of lactide to glycolide used for the polymerization, different forms of PLGA can be obtained: these are usually identified in regard to the monomers' ratio used (e.g. PLGA 75:25 identifies a copolymer whose composition is 75% lactic acid and 25% glycolic acid). All PLGAs are amorphous rather than crystalline and show a glass transition temperature in the range of 40-60 °C. Unlike the homopolymers of lactic acid (polylactide) and glycolic acid (polyglycolide) which show poor solubilities, PLGA can be dissolved by a wide range of common solvents, including chlorinated solvents, tetrahydrofuran, acetone or ethyl acetate.

PLGA degrades by hydrolysis of its ester linkages in the presence of water. It has been shown that the time required for degradation of PLGA is related to the monomers' ratio used in production: the higher the content of glycolide units, the lower the time required for degradation. An exception to this rule is the copolymer with 50:50 monomers' ratio which exhibits the faster degradation (about two months). In addition, polymers that are end-capped with esters (as opposed to the free carboxylic acid) demonstrate longer degradation half-lives..

PLGA is one of the most successfully used biodegradable nanosystem for the development of nanomedicines because it undergoes hydrolysis in the body to produce the biodegradable metabolite monomers, lactic acid and glycolic acid. Since the body effectively deals with these two monomers, there is very minimal systemic toxicity associated by using PLGA for drug delivery or biomaterial applications. PLGA NPs have been mostly prepared by emulsification–diffusion [Sahana et al. 2008], solvent emulsion–evaporation [Zambaux et al. 1999], interfacial deposition [Pinto Reis et al. 2006] and nanoprecipitation method [Barichello et al. 1999] (figure 2.6). Generally in emulsification–diffusion method, the PLGA polymers are dissolved in organic solvent, poured and separated in aqueous phase having stabilizer and subsequently emulsified by homogenizer. In solvent evaporation method, the polymers are dissolved in volatile

organic solvent and poured into continuously stirring aqueous phase with or without emulsifier/stabilizer and sonicated. Interfacial deposition methods have been used for the formation of both nanocapsule and nanospheres. The NPs are synthesized in the interfacial layer of water and organic solvent (water miscible) and finally the NPs are separated by centrifugations [Pinto Reis et al. 2006]. Most commonly used method for the preparation of PLGA NPs is nanoprecipitation. Polymer dissolved in acetone is added drop-wise into continuously stirring aqueous phase with or without emulsifier/stabilizer and consequently organic phase is evaporated under reduced pressure.

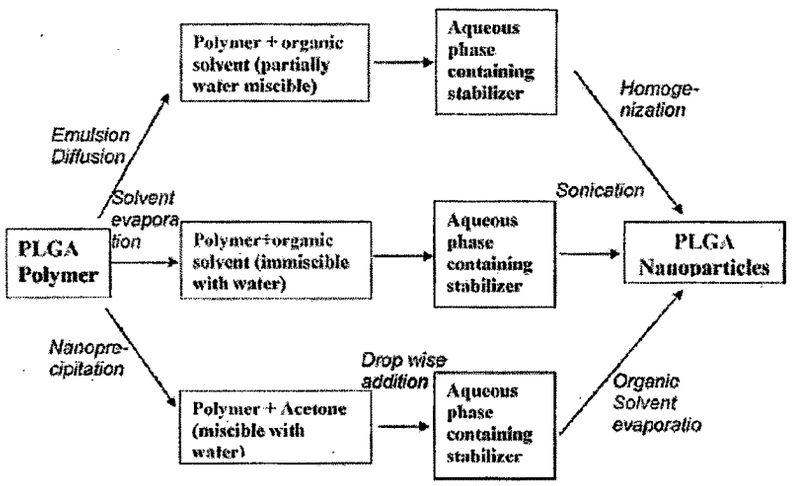


Figure 2.6 Different methods for preparation of PLGA NPs.

PLGA NPs have been used to develop the proteins and peptides nanomedicine, nano-vaccines, NPs based gene delivery system, nano-antigen and growth factor, etc. [Carrasquillo et al. 2001; Soppimath et al. 2001]. Surface modification of PLGA, drug encapsulation methods and particle size, additives added during formulation, molecular weight of drug, ratio of lactide to glycolide moieties has strong influence on the release and effective response of formulated nanomedicines [Mittal et al. 2007]. The acidic nature of PLGA monomers is not suitable for some sensitive drugs or bioactive molecules [Kumar et al. 2004]. However, the approaches to overcome these problems have been developed. PLGA nanomedicine formulations are blended with alginate, chitosan, pectin [Liu et al. 2004], poly(propylenefumarate) [Hedberg et al. 2005], polyvinylacohol [Patil et al. 2004], poly(orthoester), etc. [Wang et al. 2004]. The approval of PLGA has been granted by US Food and Drug Administration (USFDA) for human use and nanomedicines [Di Toro et al. 2004]. Various methods have been

optimized for formulations of PLGA NPs incorporating numerous anti cancer drugs [Chaudhari et al. 2012; Snehalatha et al. 2008; Yadav and Sawant 2010]. These loaded NPs protect poorly soluble and unstable payloads from the biological milieu and are small enough for capillary penetrations, cellular internalization and endosomal escape [Soppimath et al. 2001]. Furthermore, their surface is modified for targeted delivery of molecules to tumor or other tissues [Nobs et al. 2004]. The larger size of PLGA NPs is advantageous as multifunctional imaging and probes which incorporate encapsulated cancer drug, release, imaging, and targeting in a single NPs platform [Torchilin 2006].

The properties of NPs as precursor of good nanomedicine are particle size, size distribution, surface morphology, surface chemistry, surface charge, surface adhesion, surface erosion, interior porosity, drug diffusivity, drug encapsulation efficiency, drug stability and drug release kinetics. The surface charge of the NPs is important for the cellular internalization of the NPs, clustering in blood flow, adherence, and interaction with oppositely charged cells membrane [Feng 2004]. PLGA NPs are frequently used for the encapsulation of various cancer related drugs and their successful delivery in vivo. The cancer related drug paclitaxel, doxorubicin, , docetaxel, 5-fluorouracil, 9-nitrocamptothecin, cisplatin, triptorelin, dexamethasone, xanthone, etc., have been successfully encapsulated on PLGA NPs [Chaudhari et al. 2012; Derakhshandeh et al. 2010; Fonseca et al. 2002; Snehalatha et al. 2008]. The mechanism of action of these drugs, encapsulation mechanism, encapsulation efficiency, peculiar characteristic for encapsulation and drug release mechanisms are studied.

2.8 Polycaprolactone

Polycaprolactone (PCL) is one of the earliest polymers synthesized by the Carothers group in the early 1930s [Van Natta 1934]. It became commercially available following efforts to identify synthetic polymers that could be degraded by microorganisms [Huang 1985]. PCL can be prepared by either ring opening polymerization of Є-caprolactone using a variety of anionic, cationic and co-ordination catalysts or via free radical ring-opening polymerization of 2-methylene-1-3-dioxepane [Pitt 1990]. PCL is a hydrophobic, semi-crystalline polymer; its crystallinity tends to decrease with increasing molecular weight. The good solubility of PCL, its low melting point (59–64 °C) and exceptional blend-compatibility has stimulated extensive research into its potential application in the biomedical field [Chandra 1998; Nair 2007; Okada 2002].

Consequently, during the resorbable-polymer-boom of the 1970s and 1980s, PCL and its copolymers were used in a number of drug-delivery devices. Attention was drawn to these biopolymers owing to their numerous advantages over other biopolymers in use at that time. These included tailorable degradation kinetics and mechanical properties, ease of shaping and manufacture enabling appropriate pore sizes conducive to tissue in-growth, and the controlled delivery of drugs contained within their matrix. Functional groups could also be added to render the polymer more hydrophilic, adhesive, or biocompatible which enabled favourable cell responses.

Synthesis and physicochemical properties of PCL

PCL is prepared by the ring-opening polymerization of the cyclic monomer ε-caprolactone and was studied as early as the 1930s [Van Natta 1934]. Catalysts such as stannous octoate are used to catalyze the polymerization and low molecular weight alcohols can be used to control the molecular weight of the polymer [Storey 1996]. There are various mechanisms which affect the polymerization of PCL and these are anionic, cationic, co-ordination and radical. Each method affects the resulting molecular weight, molecular weight distribution, end group composition and chemical structure of the copolymers [Okada 2002]. PCL is a semi-crystalline polymer having a glass transition temperature of −60 °C and melting point ranging between 59 and 64 °C, dictated by the crystalline nature of PCL which enables easy formability at relatively low temperatures. The number average molecular weight of PCL samples may generally vary from 3000 to 80,000 g/mol and can be graded according to the molecular weight [Hayashi 1994]. PCL is soluble in chloroform, dichloromethane, carbon tetrachloride, benzene, toluene, cyclohexanone and 2-nitropropane at room temperature. It has a low solubility in acetone, 2-butanone, ethyl acetate, dimethylformamide and acetonitrile and is insoluble in alcohol, petroleum ether and diethyl ether [Coulembier 2006].

Biodegradation

Biodegradation occurs when water penetrates the entire polymer bulk, causing hydrolysis throughout the entire polymer matrix (figure 2.7a and b). Random hydrolytic chain scission would take place and produce an overall reduction in molecular weight. If water molecules can diffuse into the polymer bulk, hydrolyse the chains enabling the monomers or oligomers to diffuse out, erosion will occur gradually and equilibrium for this diffusion–reaction phenomenon would be achieved. If this equilibrium is disturbed, the degradation mechanism could provoke internal autocatalysis, via the carboxyl and

hydroxyl end group by-products. Whereas surface oligomers and carboxyl groups may freely diffuse into the surroundings (surface erosion situation), in the case of bulk degradation the internal concentration of autocatalysis products can produce an acidic gradient as the newly generated carboxyl end group formed during ester bond cleavage accumulate. This in turn accelerates the internal degradation compared to the surface, leaving an outer layer of higher molecular weight skin with a lower molecular weight, degraded, interior (figure 2.7c). The degradation mechanism thus becomes defined by bimodal molecular weight distribution. When the inner oligomers become small enough, they diffuse rapidly through the outer layer and this is accompanied by an onset of weight loss and a decrease in the rate of chain scission producing a higher molecular weight hollowed out structure. The rapid release of these oligomers and acid by-products can result in inflammatory reactions in vivo, as reported in the bioresorbable device literature [Bergsma 1995]. Furthermore, if the surrounding tissue is unable to buffer the pH change due to poor vascularization or low metabolic activity then local, temporary disturbances may arise – an example of this has been observed from fiber-reinforced PGA pins used during orthopedic surgery which led to increased osmotic pressure through local fluid accumulation at the time of rapid degradation [Bostman 1990].

PCL is suitable for controlled drug delivery due to a high permeability to many drugs excellent biocompatibility and its ability to be fully excreted from the body once bioresorbed. Biodegradation of PCL is slow in comparison to other polymers, so it is most suitable for long-term delivery extending over a period of more than 1 year. PCL also has the ability to form compatible blends with other polymers, which can affect the degradation kinetics, facilitating tailoring to fulfill desired release profiles [Freiberg 2004; Merkli 1998; Sinha 2004].

Drug release rates from PCL depends on type of formulation, method of preparation, PCL content, size and percent of drug loaded in the microcapsules. Due to a higher permeability of PCL it is blended with other polymers to improve stress, crack resistance, dyeability and control over release rate of drugs. Within the last decades, PCL polymers have been major area of interest to develop controlled delivery systems especially for peptides and proteins [Sinha 2004]. PCL nanospheres are colloidal drug-delivery systems, which act as transport carrier compartments for drugs or other active

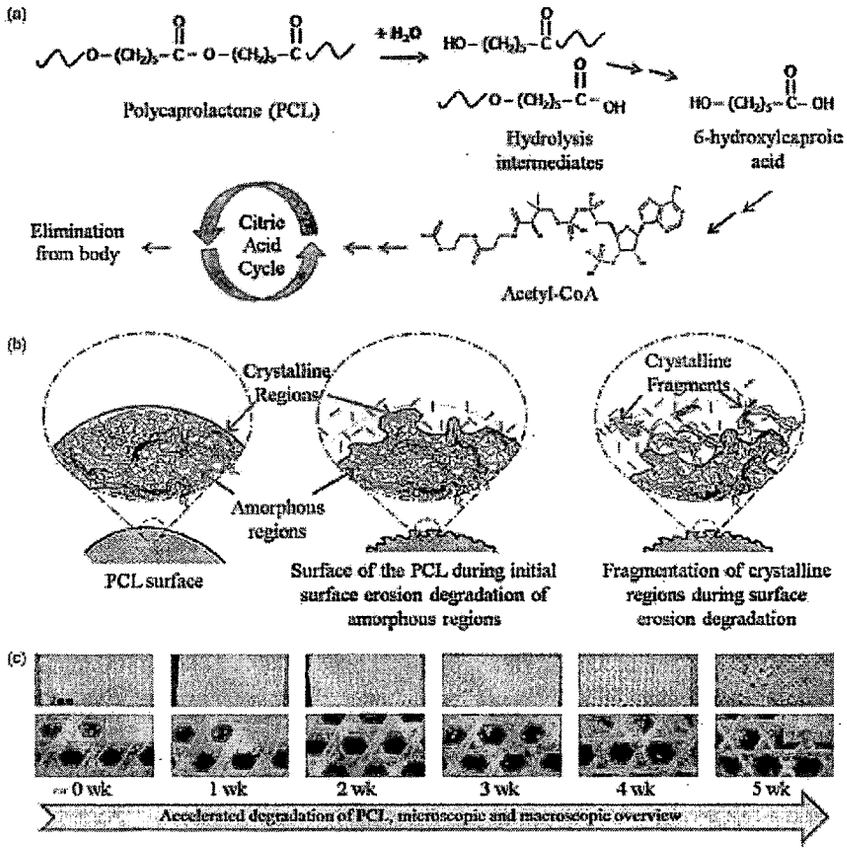


Figure 2.7 Degradation of polycaprolactone.

molecules, with a size range 10–1000 nm. Drug particles may be encapsulated, dispersed or absorbed in the nanospheres. They may also be termed as NPs or nanocapsules depending upon whether the drug is in a polymeric matrix or encapsulated in the shell. Nanospheres and nanocapsules can be prepared by the same methods as those described for microparticles, except that manufacturing parameters are adjusted to obtain nanometer size droplets. This can be obtained by using a relatively small ratio of the dispersed phase to the dispersion medium, and a substantially higher stirring speed [Zhang 2009]. Nanospheres can be used for selective targeting via the reticuloendothelial system to the liver and to cells that are phagocytically active. The size of nanospheres allows them to be administered intravenously via injection, unlike many other colloidal systems, which occlude both needles and capillaries. Injectable nanoparticulate carriers have good applicability for specific drug delivery and medical imaging, but they cannot generally be used due to their elimination by the reticuloendothelial system within seconds after intravenous injection. To overcome this limitation, monodisperse biodegradable nanospheres have

been developed from amphiphilic copolymers. These nanospheres were shown to exhibit increased blood circulation time and reduced drug accumulation in the liver of mice [Zhang 2009]. The efficacy of these colloidal particles as drug carriers is closely related to their interaction with proteins and enzymes in different body fluids. The interaction phenomenon between lysozyme, a positively charged enzyme that is highly concentrated in mucosa and two different drug carriers: nanocapsules made of an oily core coated by PCL and NPs made solely of PCL were studied. Results showed that the interaction of lysozyme with these colloidal drug carriers was highly affected by their surface charge [Calvo 1996]. Gref et al. analyzed plasma protein adsorption, zeta potential and the particle uptake by polymorphonuclear cells by biodegradable PEG-coated PLA, PLGA and PCL NPs. The influence of the PEG corona thickness and density, as well as the influence of the nature of the core was studied [Gref 2000]. The conditions to stabilize PLGA and the PCL NPs by freeze drying with several cryoprotective agents were identified. Studies indicated the necessity of adding sucrose, glucose, trehalose or gelatin to preserve the properties of NPs regardless of the freezing procedure [Saez 2000].

2.9 Anastrozole

Category: Non steroidal aromatase inhibitor

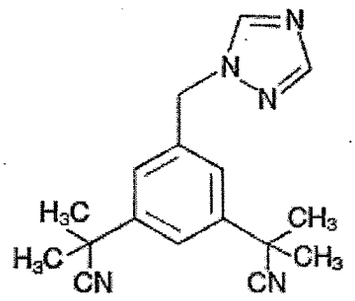
CAS Number: 120511-73-1

Proprietary name: Arimidex

Molecular formula: $C_{17}H_{19}N_5$

Molecular Weight: 293.4

Structural Formula and Chemical Name:



1,3-Benzenediacetonitrile, α, α, α', α'-tetramethyl-5-(1H-1,2,4-triazol-1-ylmethyl)

$C_{17}H_{19}N_5$

Physicochemical Properties:

Appearance and colour: An off-white powder.

Solubility: Anastrozole has moderate aqueous solubility (0.5 mg/ml at 25 °C); solubility is independent of pH in the physiological range. Anastrozole is freely soluble in methanol, acetone, ethanol, and tetrahydrofuran, and very soluble in acetonitrile.

Melting point: 81 to 82 °C.

Mechanism of action: The growth of many cancers of the breast is stimulated or maintained by estrogens. Treatment of breast cancer thought to be hormonally responsive (i.e., estrogen and/or progesterone receptor positive or receptor unknown) has included a variety of efforts to decrease estrogen levels (ovariectomy, adrenalectomy, hypophysectomy) or inhibit estrogen effects (antiestrogens and progestational agents). These interventions lead to decreased tumor mass or delayed progression of tumor growth in some women.

In postmenopausal women, estrogens are mainly derived from the action of the aromatase enzyme, which converts adrenal androgens primarily androstenedione and testosterone) to estrone and estradiol. The suppression of estrogen biosynthesis in peripheral tissues and in the cancer tissue itself can therefore be achieved by specifically inhibiting the aromatase enzyme. Anastrozole is a potent and selective non-steroidal aromatase inhibitor. It significantly lowers serum estradiol concentrations and has no detectable effect on formation of adrenal corticosteroids or aldosterone.

Pharmacokinetics

Absorption

Inhibition of aromatase activity is primarily due to anastrozole, the parent drug. Absorption of anastrozole is rapid and maximum plasma concentrations typically occur within 2 hours of dosing under fasted conditions. Studies with radiolabeled drug have demonstrated that orally administered anastrozole is well absorbed into the systemic circulation. Food reduces the rate but not the overall extent of anastrozole absorption. The mean Cmax of anastrozole decreased by 16% and the median Tmax was delayed from 2 to 5 h when anastrozole was administered 30 minutes after food. The pharmacokinetics of anastrozole are linear over the dose range of 1 to 20 mg, and do not change with repeated dosing. The pharmacokinetics of anastrozole was similar in patients and healthy volunteers.

Distribution

Steady-state plasma levels are approximately 3 to 4 fold higher than levels observed after a single dose of ARIMIDEX (anastrozole). Plasma concentrations approach steady-state levels at about 7 days of once daily dosing. Anastrozole is 40% bound to plasma proteins in the therapeutic range.

Metabolism

Metabolism of anastrozole occurs by N-dealkylation, hydroxylation and glucuronidation. Three metabolites of anastrozole (triazole, a glucuronide conjugate of hydroxy-anastrozole, and a glucuronide conjugate of anastrozole itself) have been identified in human plasma and urine. The major circulating metabolite of anastrozole, triazole, lacks pharmacologic activity.

Anastrozole inhibited reactions catalyzed by cytochrome P450 1A2, 2C8/9, and 3A4 *in vitro* with Ki values which were approximately 30 times higher than the mean steady-state Cmax values observed following a 1 mg daily dose. Anastrozole had no inhibitory effect on reactions catalyzed by cytochrome P450 2A6 or 2D6 *in vitro*. Administration of a single 30 mg/kg or multiple 10 mg/kg doses of anastrozole to healthy subjects had no effect on the clearance of antipyrine or urinary recovery of antipyrine metabolites.

Excretion

Eighty-five percent of radiolabeled anastrozole was recovered in feces and urine. Hepatic metabolism accounts for approximately 85% of anastrozole elimination. Renal elimination accounts for approximately 10% of total clearance. The mean elimination half-life of anastrozole is 50 h.

Indications: Advanced breast cancer in postmenopausal women with progression following tamoxifen therapy; first-line treatment of postmenopausal women with hormone receptor positive or hormone receptor unknown locally advanced or metastatic breast cancer.

Dosage and administration: The recommended dose of anastrozole is one tablet of 1 mg administered orally.

Adverse reactions:

Cardiovascular: Hypertension; thrombophlebitis; edema; vasodilation; chest pain.

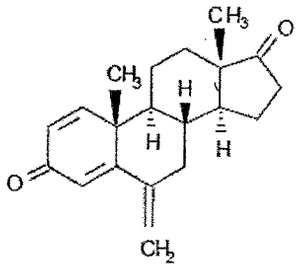
CNS: Asthenia; headache; paresthesias; somnolence; confusion; insomnia; anxiety; dizziness; depression; hypertonia; lethargy; paresthesia.

Dermatologic: Alopecia; pruritus; rash; sweating.

GI: Low to moderate potential for nausea and vomiting; diarrhea; constipation; anorexia; increased LFTs (GGT, AST, ALT); GI disturbances; abdominal pain; dry mouth.
Genitourinary: UTIs; vaginal dryness; menstrual bleeding; sexual inactivity; atrophy of the female reproductive organs; pregnancy loss; pelvic pain.
Respiratory: Dyspnea; sinusitis; bronchitis; cough increased; pharyngitis.
Miscellaneous: Myalgia; arthralgia; breast pain; hot flashes; pain; back pain; peripheral edema; bone pain; flu-syndrome; tumor flare; weight gain; leukorrhea; edema.
The risk of bone fractures is a major drawback of oral anastrozole therapy.
To overcome these side effects and targeting of anastrozole for cancer treatment, different formulations like stealth NPs [Sarkar K. 2008] and microparticles [Zidan 2006] are already studied by researchers. Zidan et al. developed sustained release PLGA based anastrozole microparticles using emulsion/extraction method for treatment of breast cancer. Sarkar et al. formulated dendrimer-based stealth NPs composed of a PAMAM dendrimers core and PEG layer encapsulating anastrozole with a target to improve its water solubility.

2.10 Exemestane

Category: Irreversible steroidal aromatase inhibitor
CAS Number: 107868-30-4
Proprietary name: Aromasin
Molecular formula: $C_{20}H_{24}O_2$
Molecular Weight: 296.41
Structural Formula and Chemical Name:



6-methylenandrosta-1,4-diene-3,17-dione

Physicochemical Properties:
Appearance and colour: A white to slightly yellow crystalline powder.

Solubility: Freely soluble in N, N-dimethylformamide, soluble in methanol, and practically insoluble in water.

Melting point: 180 to 182 °C.

Mechanism of action:

Breast cancer cell growth may be estrogen-dependent. Aromatase is the principal enzyme that converts androgens to estrogens both in pre- and postmenopausal women. While the main source of estrogen (primarily estradiol) is the ovary in premenopausal women, the principal source of circulating estrogens in postmenopausal women is from conversion of adrenal and ovarian androgens (androstenedione and testosterone) to estrogens (estrone and estradiol) by the aromatase enzyme in peripheral tissues. Estrogen deprivation through aromatase inhibition is an effective and selective treatment for some postmenopausal patients with hormone-dependent breast cancer.

Exemestane is an irreversible, steroidal aromatase inactivator, structurally related to the natural substrate androstenedione. It acts as a false substrate for the aromatase enzyme, and is processed to an intermediate that binds irreversibly to the active site of the enzyme, causing its inactivation, an effect also known as "suicide inhibition." Exemestane significantly lowers circulating estrogen concentrations in postmenopausal women, but has no detectable effect on adrenal biosynthesis of corticosteroids or aldosterone. Exemestane has no effect on other enzymes involved in the steroidogenic pathway up to a concentration at least 600 times higher than that inhibiting the aromatase enzyme.

Pharmacokinetics

Following oral administration to healthy postmenopausal women, exemestane is rapidly absorbed. After maximum plasma concentration is reached, levels decline polyexponentially with a mean terminal half-life of about 24 h. Exemestane is extensively distributed and is cleared from the systemic circulation primarily by metabolism. The pharmacokinetics of exemestane is dose proportional after single (10 to 200 mg) or repeated oral doses (0.5 to 50 mg). Following repeated daily doses of exemestane 25 mg, plasma concentrations of unchanged drug are similar to levels measured after a single dose.

Pharmacokinetic parameters in postmenopausal women with advanced breast cancer following single or repeated doses have been compared with those in healthy, postmenopausal women. Exemestane appeared to be more rapidly absorbed in the

women with breast cancer than in the healthy women, with a mean tmax of 1.2 h in the women with breast cancer and 2.9 h in the healthy women. After repeated dosing, the average oral clearance in women with advanced breast cancer was 45% lower than the oral clearance in healthy postmenopausal women, with corresponding higher systemic exposure. Mean AUC values following repeated doses in women with breast cancer (75.4 ng·h/ml) were about twice those in healthy women (41.4 ng·h/ml).

Absorption

Following oral administration of radiolabeled exemestane, at least 42% of radioactivity was absorbed from the gastrointestinal tract. Exemestane plasma levels increased by approximately 40% after a high-fat breakfast.

Distribution

Exemestane is distributed extensively into tissues. Exemestane is 90% bound to plasma proteins and the fraction bound is independent of the total concentration. Albumin and $\alpha_1$1-acid glycoprotein both contribute to the binding. The distribution of exemestane and its metabolites into blood cells is negligible.

Metabolism/Elimination

Following administration of radiolabeled exemestane to healthy postmenopausal women, the cumulative amounts of radioactivity excreted in urine and feces were similar (42 ± 3% in urine and 42 ± 6% in feces over a 1week collection period). The amount of drug excreted unchanged in urine was less than 1% of the dose. Exemestane is extensively metabolized, with levels of the unchanged drug in plasma accounting for less than 10% of the total radioactivity. The initial steps in the metabolism of exemestane are oxidation of the methylene group in position 6 and reduction of the 17-keto group with subsequent formation of many secondary metabolites. Each metabolite accounts only for a limited amount of drug-related material. The metabolites are inactive or inhibit aromatase with decreased potency compared with the parent drug. One metabolite may have androgenic activity (see). Studies using human liver preparations indicate that cytochrome P 450 3A4 (CYP 3A4) is the principal isoenzyme involved in the oxidation of exemestane. Exemestane is metabolized by cytochrome P 450 3A4 (CYP 3A4) and aldoketoreductases. It does not inhibit any of the major CYP isoenzymes, including CYP 1A2, 2C9, 2D6, 2E1, and 3A4.

Indications: Advanced breast cancer in postmenopausal women with progression following tamoxifen therapy; first-line treatment of postmenopausal women with

hormone receptor positive or hormone receptor unknown locally advanced or metastatic breast cancer.

Dosage and administration: The recommended dose of exemestane is one tablet of 25 mg administered orally.

Adverse reactions:

Cardiovascular: Chest pain, hypertension, peripheral edema.

CNS: Fatigue, depression, insomnia, anxiety, headache, dizziness.

Dermatologic: Rash, increased sweating, androgenic effects reported including hypertrichosis, hair loss and acne.

Endocrine: Hot flushes, weight gain.

GI: Low potential for nausea and vomiting, anorexia, constipation, diarrhea and increased appetite.

Hematologic: Lymphopenia.

Musculoskeletal: Musculoskeletal pain, anthralgia.

Respiratory: Dyspnea, coughing.

Miscellaneous: Flu like symptoms with fever, hoarseness.

Major drawbacks of orally delivered exemestane are musculoskeletal side effects including arthralgia (14.6 to 28.8%), pain in limb (9%), osteoarthritis (5.9%), myalgia (5.5%), back pain, pathological fracture, and skeletal pain.

To overcome these side effects and targeting of exemestane for cancer treatment, different formulations like SMEDDS [Singh 2008], powdered proliposomes [Hiremath 2009], PLGA NPs [Li 2013] and proliposomes [Jukanti 2011] are already studied by researchers. Singh et al. prepared and characterized SMEDDS containing exemestane and reported enhanced dissolution of the drug [Singh 2008]. Hiremath et al. developed proliposomal powder formulations using different ratios of drug (exemestane), distearoyl-phosphatidylcholine (DSPC), cholesterol and dimyristoyl-phosphatidylglycerol (DMPG) by solvent evaporation method to enhance the oral bioavailability of exemestane by improving solubility, dissolution and/or intestinal permeability [Hiremath 2009]. The in vitro transport studies in rat intestine, PAMPA and Caco-2 models revealed that the proliposomes were successful in enhancing the permeation of exemestane. These proliposomal formulations of exemestane could provide improved oral bioavailability due to enhanced solubility, permeability and hence absorption. Li et al. formulated PLGA and PLGA/MMT by a modified solvent

extraction/evaporation technology with vitamin E succinated polyethylene glycol 1000 (TPGS) as emulsifier for oral delivery. Exemestane formulated as NPs showed enhanced cytotoxicity than drug solution [Li 2013]. Jukanti et al. prepared proliposomes with the objective of improved and sustained transdermal delivery of exemestane. Proliposome gel showed significant improvement in bioavailability (2.4 folds) compared to oral suspension [Jukanti 2011].

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