Introduction

1. INTRODUCTION

Cancer is a generic term for a large group of diseases that can affect any part of the body. Other terms used are malignant tumors and neoplasms. One defining feature of cancer is the rapid creation of abnormal cells that grow beyond their usual boundaries, and which can then invade adjoining parts of the body and spread to other organs. This process is referred to as metastasis which is major cause of death from cancer. Cancer is a leading cause of death worldwide and accounted for 7.6 million deaths (around 13% of all deaths) in 2008. The main types of cancer and death statistics per year:

- lung (1.4 million deaths)
- stomach (740 000 deaths)
- liver (700 000 deaths)
- colorectal (610 000 deaths)
- breast (460 000 deaths).

More than 70% of all cancer deaths occurred in low- and middle-income countries. Deaths from cancer worldwide are projected to continuous rising over 11 millions in 2030 (WHO international, www.who.int). Metastasis is the fatal stage in all cancer which remains incurable in spite of lots of research in recent scenario. Bone is the most prone site for metastasis because of its physiological environment which supports tumor inoculation and progress. Some commonly occurred human cancer such as prostate cancer, breast cancer, renal carcinoma, thyroid cancer and multiple myelomas have high vulnerability (more than 50% case) of metastasized at bone site in advanced stage. Patients diagnosed with bone metastasis suffer with multiple disease complication such as bone pain, fractures, spinal cord compression, high blood calcium levels and anemia (Mundy 1997). Because of complications and incomplete understanding of disease, it still hinders development of effective drug delivery to bone metastasis.

1.1 Outline of the problem

The reason behind high rate of metastasis occurrence to bone is that bone is a particularly 'fertile soil' for the tumor cells to grow tumor by providing abundance growth factors secreted by osteoclast cell which promote tumor growth and metastasis

progress. The high rate of metastasis occurrence at bone is because of the reason that bone is a particularly 'fertile soil' for tumor cells to grow tumor by providing abundance growth factors secreted by osteoclast cell which promote tumor growth and metastasis progress. Bone is a large repository of immobilized growth factors such as transforming growth factor b (TGFb), insulin-like growth factors I and II, fibroblast growth factor, platelet-derived growth factor, and bone morphogenic proteins and parathyroid hormone related peptide (PTHrP) (Sohara Y et al, 2005). These growth factors help cancer cell to invade and growth in bone matrix.

Bone comprises more than 50% mass of body but still having only 7% cardiac output. So the conventional chemotherapy doesn't get desire concentration for tumor suppression. An ideal chemotherapeutic drug designed for the treatment of bone metastasis should specifically target the cancer cells in bone, resulting in cytotoxicity to malignant cells while sparing normal cells, particularly in the bone marrow. Research with targeted delivery seeks to construct drugs or drug conjugates that exploit this concept of tissue or cellular selectivity (Amal A et al, 2006). A number of attempts have been made to use bisphosphonates to deliver chemotherapy or radiotherapy on the basis of their known affinity for bone (Fleisch H, 1998). The proposed nano-scale targeting system can potentially be applied to a large variety of drugs for the treatment of bone diseases. It is anticipated that the bone selective delivery of the nanoscale encapsulated drug can lead to locally prolonged drug concentrations which potentially enhancing their therapeutic effect. In addition, decreased systemic toxicity is a potential major benefit that can be achieved with this targeting approach.

1.2 Nanotechnology and tumor targeting

Nanotechnology could be defined as the technology that has allowed for the control, manipulation, study, and manufacture of structures and devices in the "nanometer" size range. These nano-sized objects, e.g., "nanoparticles", take on novel properties and functions that differ markedly from those seen from items made of identical materials. The small size, customized surface, improved solubility, and multi-functionality of nanoparticles will continue to open many doors and create new biomedical applications. Indeed, the novel properties of nanoparticles offer the ability to interact with complex cellular functions in new ways. This rapidly growing field requires cross-disciplinary research and provides opportunities to design and develop

multifunctional devices that can target, diagnose, and treat devastating diseases such as cancer.

If new anticancer molecular medicines have to be translated into efficacious treatment modalities, an efficient means of systemic delivery to combat metastasis disease, as well as primary disease, is required. It is also crucial to develop ways of targeting these new therapies to tumor cells specifically, safely, efficiently and repeatedly, not only to enhance anti-tumor efficacy but also to reduce adverse effects on normal tissues. Enhance Permeation and Retention (EPR) is a phenomenon originally described by Matsumura and Maeda (1986) which associated with tumors as a result of differences in their neovasculature compared to that of normal tissues. Tumor blood vessels are described as 'leaky' because they are irregular in shape and dilated, with disorganized and poorly aligned endothelial cells (Modi, S. et al., 2006, Iyer, A.K. et al., 2006). This, along with poor lymphatic drainage, results in 'leakage' of plasma components from the circulation into the interstitial space of the tumors. There is a size relationship with this effect: larger and long-circulating macromolecules (>30-45 kDa) are retained in the tumor tissue longer, whereas smaller molecules easily diffuse back out (Modi, et al., 2006; Iyer, et al., 2006; Maeda, 2001). This form of 'passive targeting' has been shown to contribute to the increased efficacy that has been associated with large nanocarriers or polymer-conjugated drugs (Iyer, et al., 2006).

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

increased with the PEG density on the particle over a range of 6 to 15 mol %. Similarly, Fang et al. (2006) reported that the peak tumor concentration, as well as the peak accumulation time, of nanoparticle delivered ^{125}I labeled recombinant human tumor necrosis factor-a (rHuTNF-a) varied with the PEG molecular weight, surface density and the size of the nanoparticle.

Tumor specificity (active targeting) can be accomplished by including a ligand in the complex, such as the RGD peptide (Garanger, E. et al.; 2007), epidermal growth factor (EGF) (Bruin et al.; 2007), folate (Hilgenbrink and Low; 2005), transferrin (Tf) (Xu, L. et al.; 1999) or antibodies and antibody fragments, such as a single-chain variable fragment (scFv), that recognizes a cell-surface receptor (Dass, C.R. and Choong, P.F.; 2006). In most cases, these ligand-receptor interactions result in efficient uptake of the complex into the tumor cell by receptor mediated endocytosis. The prevailing theory has been the presence of the ligand in the liposome or polymeric NP and micelles also serves to direct to the tumor. However, the recent observations of Bartlett et al. (2007) and Kirpotin et al. (2006) have propose that the ligand does not increase tumor localization, but instead functions primarily in the uptake of the nanoparticle by the tumor cell. Conversely, the data of Wu et al. (Wu et al.; 2000) demonstrate that although a targeting ligand does not direct a nanocomplex to the tumor, its presence does play a significant role in tumor localization. Thus, it is important to attempt and reconcile these differing conclusions.

1.3 Proposed rationale of Project

Bone has hydroxy apatite as a major mineral component. It covers with bone line cell throughout the body. It substantially uncover at the place where osteoclast cells get activated for active remodeling of bone matrix. This site have high amount of activated growth hormone factors which favor circulating cancer cell to metastatize. After metastasis the tumor start grow and starts uncontrolled bone remodeling. Targeting the open mineral composite (apatite) of the bone presents itself as the ideal way to deal with metastasis bone cancer (Perry and Figgitt, 2004).

Bisphosphonates (BPs) are group of drugs which reduce bone erosion and restore bone density in osteoporosis and bone related diseases. Many clinical trials have proved that BPs restore bone homeostasis and reduce risk of osteoporotic

complication with good tolerance and safety (Shane, 2010). Bone undergoes regular makeover, where bone formation and erosion happen concurrently. BPs found to induce apoptosis of osteoclast cells which are responsible for bone erosion (Weinstein, 2009). Additionally, BPs found strong affinity toward bone mass. All BPs distributed in bone very fast after injection and found in 100 times high concentration in bone in comparison to C_{max} concentration after injection and concentration remain high even after 6 month of injection (Tianling Chen et al, 2002). Because of its strong selectivity and affinity, BPs are now widely used as a bone imagine agents in conjugation with radiopharmaceuticals.

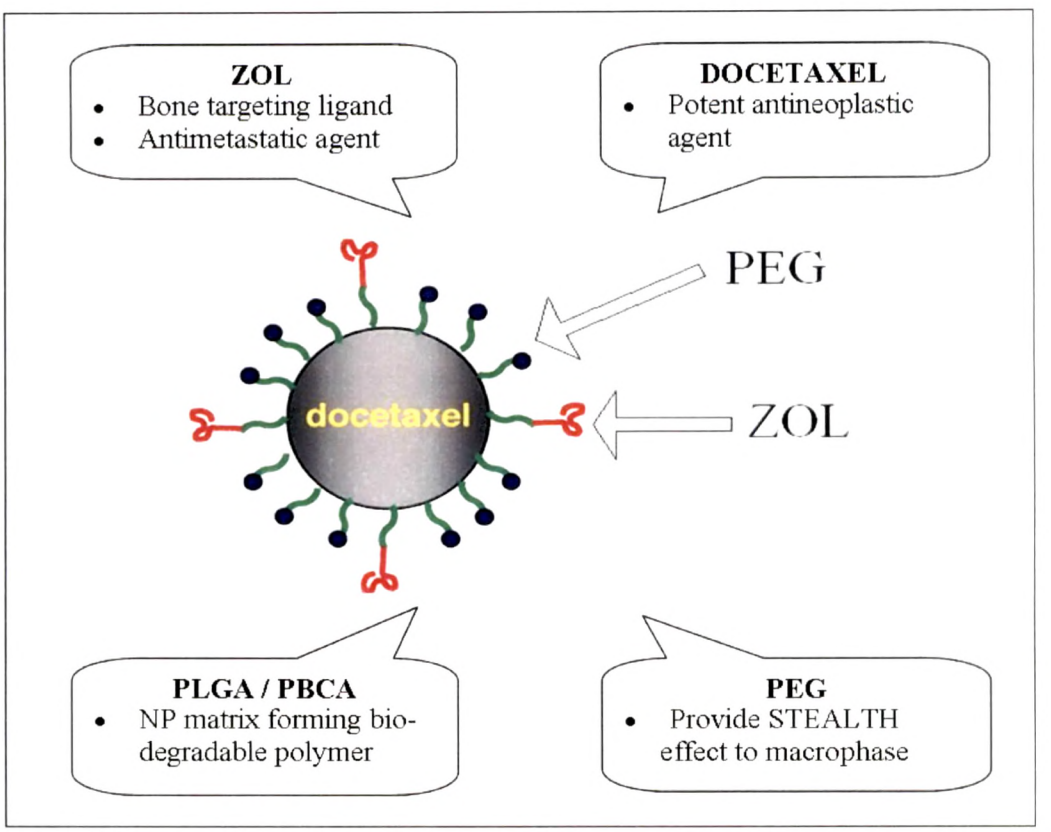

Figure 1.1: Strategy for bone metastasis targeted nanocarrier

Because of the unique features of BPs, many attempts have been made to conjugate bone therapeutic agents in order to get osteotropicity. These include estradiol (Thompson, 1989; Yokogawa, 2001), prostaglandin E2 (Gil, 1999), Src (protein tyrosine kinase pp60c-Src) homology 2 inhibitors (Shakespeare, 2003; Violette,

2001), diclofenac (Hirabayashi, 2001), fluroquinolone, cisplatin, melphalan, methotrexate (Hosain, 1996), radiopharmaceuticals like 99mTc hydroxyethylidene disphosphonate, 99mTc methylene disphosphonate, 99mTc hydroxymethylene disphosphonate (Fancis, 1987) and samarium (153Sm) lexidronam (QuadrametR) (Lamb, 1997). Peptides and proteins have also been proposed by Gittens et al. for conjugation with BPs to induce bone specificity (Gittens et al., 2005). Hengst et al. has suggested use of CHOL-TOE-BP as targeting moiety for liposomal drug delivery to bone (Hengst 2007). BP conjugates were also used as delivery anchor for treatment of osteoporosis (Gil 1999). Liu et al. 2008 demonstrated use of alendronate-β-cyclodextrin conjugate as a bone anabolic agent.

Along with osteotropicity and utility as bone homeostasis enhancer, Zoledronic acid (ZOL), a nitrogen containing BP recently found to have anticancer activity. As anticancer agent, ZOL found to have multiple targets and have activity such as apoptotic to cancer cells, antiangiogenesis, reduce Vascular endothelial growth factor (VEGF) level and circular occult tumor cells in blood, antiadhesion activity to tumor and osteoclast cells which increase its potential as an anticancer drug (Michele Caraglia et al 2006, Allan Lipton 2008). As molecular mechanism, it was found that ZOL inhibit mevalonate pathway and protein isoprenylation. The enzyme target in the mevalonate pathway is farnesyl pyrophosphate synthase (FPP synthase). Inhibition of this enzyme prevents formation of some important signaling molecules and modification of proteins which lead to a loss of osteoclast or cancer cell function and induce apoptotic cell death (Rogers, 2003). Monkonen group earlier established new mechanism which reports that ZOL was found to induce formation of a novel ATP analog, triphosphoric acid 1-adenosin-5'-yl ester 3-(3-methylbut-3-enyl) ester (ApppI) by inhibiting mevalonate pathway in cells which causes apoptotic cell death (Monkonnen 2006). The inhibition of FPP synthase also cause to the accumulation of isopentenyl pyrophosphate (IPP), which further converted to ApppI via aminoacylt RNA-synthetases. There is a clear correlation between the ability of ZOL to inhibit FPP synthase and protein prenylation *in vitro* (Dunford et al., 2001) and their abilities as antiosteoclast and anticancer in vivo by induction of ApppI and IPP formation have been established successfully in animal studies (Raikkonen et al 2009, Monkonnen 2007).

Combination therapy of ZOL and Docetaxel (DTX) are already proven to have synergistic effects. Karabulut et al. (2009) have explored the possible synergistic cytotoxic effects of combination therapy of DTX and ZOL in hormone-refractory PC-3 and DU-145 prostate cancer cell lines. The apoptotic pathways induced in DTX and ZOL was found due to down regulation of antiapoptotic protein Bcl-2 in PC-3 and DU-145 cell lines (Karbulut et al, 2009). Phase I clinical study on DTX-ZOL combination on prostate cancer cells in vitro condition shows that ZOL and DTX combination are more synergistic as cytotoxic effect when given in sequence (Lafaioli et al. 2007).

Major part of the bone remains low perfused and isolated. Thus, chemotherapeutic drug never achieve desired concentration for tumor suppression. Brubaker et al. (2006), examined effect of DTX and ZOL on LuCaP 23.1 prostate cancer xenograft model and shows that ZOL decreased proliferation of LuCaP 23.1 in the bone environment *in vivo* condition, while DTX fails to inhibit growth of tumor with the concentration which is effective for subcutaneous tumor. The contradictory *in vitro* and *in vivo* results explained the inability of DTX to gain desire concentration at tumor site. A number of attempts have been made to use BPs to deliver chemotherapy based on their known affinity for bone. The proposed nano-scale targeting system can potentially be applied to localize DTX and ZOL to obtain high concentration at bone metastasis site to enhance therapeutic outcomes. Poly(lactide-Glycolide) acid (PLGA) and PolyButylCyanoAcrylate (PBCA) have proven for human use by US FDA for its biocompatibility and biodegradability and now widely in use for drug delivery purpose (Shah N et al 2009, Chaudhari K.R. et al 2010). Here, PLGA NP and PBCA NP were used as a nanocarrier based drug delivery system.

Bisphosphonates, in addition to bone targeting ligand, work as a potent cytotoxic and anti-angiogenic in nature which give added advantage of its usage (Green J. R., 2003). Several potential mechanisms have been proposed to account for the observed *in vivo* anti-tumor effects of the anticancer drugs with bisphosphonates. Bisphosphonates have direct cytotoxic or cytostatic effects on tumor cells, inhibition of osteoclastogenesis and osteoclast mediated bone resorption, inhibition of tumor cell invasion of the bone, and anti-angiogenic effects (Green J. R., 2003). The hypothesis that inhibition of osteoclastogenesis and osteoclast mediated bone resorption can

inhibit tumor cell growth in bone is compelling and may explain much of the observed anti-tumor activity in animal models. This hypothesis is based on the theory that tumor cells colonize the bone and grow well in bone because of the abundance of local growth factors. Osteoclasts liberate or secrete these growth factors during bone resorption and make them available to tumors (Croucher et al, 2003). This theory is supported not only by the data with bisphosphonates, but also by recent data showing that specific inhibitors of osteoclastogenesis, namely RANK-Fc (Receptor Activator of Nuclear factor kappa B) and osteoprotegerin (OPG), which secretes on administration of bisphosphonates (pamidronate and zoledronic acid) can also reduce skeletal tumor burden in animal models and metastasis progress by blocking RANK-RANKL (Receptor Activator of Nuclear factor kappa B ligand) based signaling pathway (Yaccoby et al, 2001; Morony et al, 2002).

Taxols has been proved as a potent antineoplastic activity against a wide range of cancers. In combination with bisphosphonates exert synergistic action with fivefold increase in an apoptosis. Along with anticancer activity taxol with bisphosphonates effectively reduce circulated occult tumor cells (OTC) in blood which prevent mestastasis (Lipton, 2008).

1.4 Hypothesis

So, it is hypothesized that the proposed nanoparticle based anticancer drug entrapped functionalized targeted system may target bone metastasis with synergistic effect on metastatic tumor by anticancer drugs along with bisphosphonate, reduce bone complication by controlling ostreoclast activity by bisphosphonate, same time reduce further metastasis progress by reducing activation of growth factors by controlling ostreoclast activity and reducing occult tumor cell count in blood.

1.5 Aims and Objectives

The purpose of this study is formulation of an anticancer agent (Docetaxel) into novel drug delivery systems such as PLGA NP and PBCA NP in order to bone metastasis targeting using Zoledronate as a targeting ligand. The detail objective of this study is as below:

- Formulation of polymeric biodegradable poly(lactide-co-glycolide) and poly(n-butyl cyanoacrylate) nanoparticles loaded with DTX.

- Optimization of the various formulation and process parameters.
- Modification of the surface of PLGA NPs and PBCA NP by PEGylation
- Ligand attachment (ZOL) on the surface of PEGylated NP
- Study the DSC, XRD, FTIR and NMR patterns of excipients and nanoparticles.
- To characterize the prepared formulations for entrapment efficiency (%), particle size, zeta potential and its morphological properties by transmission electron microscopy.
- To carry out *In-vitro* drug release from the formulation
- To carryout stability studies at various environmental conditions.
- To carried out cell line studies using RAW264, MCF-7 and BO2 cell lines.
  - ✓ Cell cytotoxicity
  - ✓ Cell cycle ananlysis using PI staining using flowcytometry
  - ✓ Phagocytosis challenge test for PEGylation evaluation
  - ✓ Endocytosis-exocytosis to evaluated up take and residence time of nanoparticle in cell
  - ✓ Apoptosis using flowcytometry
  - ✓ Endocytosis route characterization in presence and absence of ligand
  - ✓ Confocal microscopy to conform association of nanoparticles to endosomes and lysosomes as well as early endosomal realease
  - ✓ Western blotting to evaluate expression level of key apoptosis proteins
  - ✓ IPP and AppI estimation by LCMS to estimate mavalonate pathway blockage

- Animal Studies
  - ✓ To perform the in-vivo pharmacokinetics and biodistribution studies by radio labeling in tumor bearing swiss albino mice.

1.6 Reference

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