Chapter 1
Introduction

1.1. Introduction

Molecular medicine has discovered many new therapeutic modalities by using state-of-the-art techniques in molecular biology. High through-put, in-vitro assays that screen for pharmacological actions on the desired cell type are frequently used to design new drugs. Although such agents are certainly justified by their success in-vitro, they frequently perform much less effectively in-vivo where the agent must reach its target cells in a tissue in sufficient quantities to be potent while sparing bystander organs. Depending on the route of administration, the endothelium and/or epithelium form significant barriers that greatly limit the in-vivo accessibility of many drugs, antibodies, and gene vectors to their intended target sites of pharmacological action, namely, the cells inside the tissue (Jain, R. K. et al., 1998). Hence, the major challenge for formulation development scientist is to design drug delivery strategies that deliver the therapeutic agents to the desired intracellular targets based on ability to understand, utilize, modify and exploit membrane trafficking pathways (Miller, N. et al., 1995).

Intracellular drug delivery refers to the delivery of therapeutic agents to specific compartments or organelles within the cell. The therapeutic agent could be a low molecular weight drug or a macromolecule like protein or DNA. Thus targeted intracellular drug delivery results in higher bioavailability of a therapeutic agent at its site of action (i.e. distinct intracellular compartments or microenvironments), potentiates the pharmacologic effect of the drug, and at the same time, reduces the side effects. (Jayanth Panyam et al., 2004). There are a number of pathways through which a therapeutic agent or a carrier can enter a cell. Such as Endocytosis, Caveolar endocytosis, Pinocytosis, Phagocytosis, In receptor-mediated endocytosis, Folate Receptor-Mediated Endocytosis, Transferrin Receptor-Mediated Endocytosis, Biotin Receptor-Mediated Endocytosis, Wheat Germ Agglutinin-Mediated Endocytosis, ICAM-1-Mediated Endocytosis, Antibody-Mediated Endocytosis, Physical or mechanical methods use force to traverse the cell membrane, e.g. Particle bombardment accelerates ,Microinjection and Electroporation. The various Intracellular Targets are Endo-Lysosomal Targeting, Cytoplasmic Delivery, Nuclear Targeting, and Mitochondrial Targeting.

A number of different carriers have been used for targeting different organelles including viral vectors, cationic lipids, polymer conjugates, micelles, protein transduction domains, nanoparticles and liposomes.

Nanoparticles are defined as particulate dispersions or solid particles with a size in the range of 10-1000nm. The drug is dissolved, entrapped, encapsulated or attached to a nanoparticles matrix. Depending upon the method of preparation, nanoparticles, nanospheres or nanocapsules can be obtained. Nanocapsules are systems in which the drug is confined to a cavity surrounded by a unique polymer membrane, while nanospheres are matrix systems in which the drug is physically and uniformly dispersed. In recent years, biodegradable polymeric nanoparticles, particularly those coated with hydrophilic polymer such as poly(ethylene glycol) (PEG) known as long-circulating particles, have been used as potential drug delivery devices because of their ability to circulate for a prolonged period time target a particular organ, as carriers of DNA in gene therapy, and their ability to deliver proteins, peptides and genes. (Mohanraj, V.J. et al., 2006) Biodegradable nanoparticles for pharmaceutical use are prepared from a variety of synthetic and natural polymers. Synthetic polymers such as polyacrylates, polycaprolactones, polylactides and its copolymers with polyglycolides are widely used.

A wide spectrum of synthetic and natural polymers is available for nanoparticle formation, but their biocompatibility and biodegradability are the major limiting factors for their use in the drug delivery area. Natural polymers are more restricted due to variation in their purity. Also, some natural polymers require crosslinking, which can inactivate the entrapped drug (Hans, M.L. et al., 2002). Synthetic polymers, on the other hand, offer better reproducibility of the chemical characteristics of the synthesized nanoparticles as compared to the natural polymers. Synthetic polymers from the ester family, such as poly(lactic acid), poly(hydroxybutyrate), poly(caprolactone), poly(dioxanone), or other families such as poly(cyanoacrylates), poly(acrylic acid), poly(anhydrides), poly(amides), poly(ortho esters), poly(ethylene glycol), and poly(vinyl alcohol) are suitable for drug delivery due to their biodegradability, special release profiles and biocompatibility (Ghosh, S. et al., 2004). Poly(lactide-co-glycolide acid) (PLGA), from the ester family, has been widely used in the biomedical industry as a major components in biodegradable sutures, bone fixation nails and screw. It is a well-characterized polymer, its degradation sub products are non toxic, it provides controlled drug release profiles by changing the PLGA copolymer ratio which affects the crystallinity (low crystallinity, more amorphous polymer means more fast degradation) of PLGA (Moghimi, S.M. et al 2001). FDA approved biodegradable and biocompatible polymers, poly (D,L-lactide-*co*-glycolide) (PLGA) in the with a therapeutic agent

encapsulated into the polymer is widely used for localized and sustained drug and macro-molecular delivery (Jayanth Panyama et al., 2003)

Two main procedures can be followed to form polymeric nanoparticles, namely top-down and bottom-up techniques. The top-down methods use size reduction to obtain controlled-size nanoparticles. This size reduction is based on the application of strong shear stress by wave sound emission (sonication), high pressure (microfluidization), and high speed agitation (homogenization). The bottom-up methods start from individual molecules to form nanoparticles, by polymerization. Liposomes are vesicles in which an aqueous volume is entirely enclosed by a membrane composed of lipid molecules, usually phospholipids. Spontaneously formed upon dispersion in aqueous media, the size of such vesicles can range from tens of nanometres to tens of microns in diameter. In pharmaceutical sciences, liposomes have been used traditionally as formulation ingredients to assist in formulation of poorly soluble therapeutic agents for oral or parenteral administration. Liposomes can be made of natural constituents. Their membrane is very similar to natural cell membranes and provides great convenience as models for membrane systems. Such naturally occurring constituents are cholesterol, phospholipids or fatty acids that make them a biocompatible and safe vehicle for medical *in vivo* applications. Those favourable properties can be adjusted by chemical modifications of the phospholipid-bilayer membrane of the liposome. Chemical modifications, such as saturation or pegylation of phospholipids are well established and numerous possibilities are described, which results in a vast versatility and flexibility of such phospholipid-bilayer membrane liposomes.

Conventional preparation of liposomes unfortunately involves evaporation of organic solvent, preparation of vesicles by sonication, and dialysis to provide Unilamellar vesicles. Use of SCF CO_2 to provide a "clean" and effective alternative to traditional methods of drug processing, related to micronization, encapsulation, and impregnation of molecules of interest to pharmaceutical industry; formation of micron sized particles with SCF CO2 by rapid expansion of supercritical solutions (RESS) or phase separation by gas saturated solutions (PGSS), gas antisolvent (GAS) techniques by solubilizing the drug in an organic solvent and introducing SCF to solubilize the solvent and allow the drug to precipitate and also incorporating drugs into controlled matrices; based on these techniques, nanoparticles of cyclosporine A in phospholipid and ibuprofen nanoparticles in aqueous solution were made. In the gas antisolvent (GAS) technique, in which the gas

is used as an antisolvent to reduce the solvating power of the organic solvent, the ability of the SCFs to dissolve and expand the organic solvents for precipitation of solids from organic solution is exploited (Udaya Sankar Kadimi et al., 2007). Conventional drug particle precipitation uses organic solvents as antisolvents for precipitation or as emulsifiers for emulsion process. Traces of residual organic solvents, such as methylene chloride, that may still be in the drug particles have motivated researchers to find alternative methods for particle formation. Organic solvent use should be limited for pharmaceutical manufacturing operations, based on United States Pharmacopeia (USP) standards. An alternative to reduce this problem is to use supercritical carbon dioxide (CO_2) as an antisolvent to precipitate drug particles from solution since CO_2 is nonflammable, non-toxic, inexpensive, renewable, and environmentally benign (McHugh, M.A. et al., 1994).

Polymeric conjugates have been used for targeted delivery of drugs to tumor sites because the attachment of drugs to water soluble polymer increases their solubility, reduces the side effect, and overcomes multi-drug resistance; the large size of conjugates increases blood half life and significantly alters the drug properties and pharmacokinetics; the conjugates can be tailor-made (i.e., side-chain content, molecular weight, charge etc.) for specific targeting and delivery needs; they can be designed to passively (EPR) or actively target tumor sites; and site specific drug release can be achieved by designing biodegradable spacers that can be enzymatically cleaved or that are pH sensitive. The advantages have led to the development of a wide range of plolymer-anti-cancer drug conjugates, some of which are currently in clinical trials. One of the most promising systems is based on water soluble N-(2-hydroxypropyl) methacrylamide (HPMA). HPMA homopolymer was originally developed by Kopecek and colleagues as a plasma expander (Ruth Duncan et al., 2003) HPMA copolymers has been employed to modify the in vivo biodistribution of chemotherapeutics agents and enzymes. The advantage of HPMA copolymer over other water soluble polymers is that they can be tailor-made with simple chemical modification to regulate drug and targeting moiety content for biorecognization, internalization, or subcellular trafficking depending on specific therapeutics needs. The over all molecular weight of HPMA copolymers is determined by the polymerization conditions, particularly the concentration of initiator and chain transfer agents. Various side chain moieties (isotope chelator, targeting moieties and drug) may be directly linked to the polymer chain via a biodegradable or non-biodegradable spacer.

Polymeric based delivery systems have been used as carriers for passive and active targeting of drugs in the treatment of various diseases and as novel imagine agents. Without a specific targeting ligand moderate-size (> 30 kD) polymer can passively (*via* EPR) accumulate in tumor tissues. The EPR .effect has been used to deliver macromolecular bioactive agents to solid tumors including anti-angiogenic drugs. There are number of important differences between small molecular weight drugs and polymeric conjugates of these small molecules. The advantages of polymer-based delivery system stem decreased extravasation in normal tissues because of the large molecular weight of the conjugates. Low extravasation of polymer conjugates in normal tissues generally results in reduced systemic toxicity. In this regard, predominant liver and kidney uptake of small RGD peptides has been identified as a significant disadvantage of targeting tumor angiogenesis with small peptides.

Peptide-targeted delivery has a basis in nature as many peptides are used as attachment ligands by bacteria and viruses. A major objective in the design of functionalized polymer surfaces for tissue engineering applications has been the covalent attachment of peptides that regulate cell adhesion: specifically those that promote integrin-mediated cell attachment (Shakesheff, K.M. et al., 1998 and Rezania, A. et al., 1999). Since the vast majority of mammalian cells are anchorage-dependent, they must attach and spread on a substrate to proliferate and function normally (Madri, J.A. et al., 1988 and Baldwin, S.P. et al., 1998). By controlling the surface properties of the substrate, cell attachment and growth *in vitro* can be altered (Kim, B.S. et al., 1999). A great deal of work has focused on RGD containing peptides to regulate cell adhesion (Shakesheff, K.M. et al., 1998, Besselink, G.A.J, et al., 1998 and Chinn, J.A. et al., 1998). Peptides containing the RGD (R=arginine, G=glycine, D=aspartic acid) sequence are considered important small cell-adhesive ligands (Shakesheff, K.M. et al., 1998, Besselink, G.A.J, et al., 1998). This cell-binding sequence is present in adhesive proteins like fibrinogen, vitronectin, collagens, and fibronectin (Ruoslahti, E. et al., 1987). Membrane proteins of blood platelets, endothelial cells, and several other cell types can bind RGD-containing peptides whether the peptide is in solution or immobilized onto a solid surface (Hynes, R.O. et al., 1992 and Plow, E.F et al., 1986).

RGD-based therapeutics functions as agonists to promote the interaction of cells and tissues with artificial matrices, or as antagonists to control the nature of cell-cell and cell-ECM interactions (Craig, W. S. et al., 1995). At this state of the art many different

RGD peptides have been developed. Linear and cyclic RGD peptides, and chemically designed peptidomimeticsare currently being tested by researchers.

Breast cancers are potentially life-threatening malignancies that develop in one or both breasts. Breast cancer is either noninvasive (referred to as *in situ*, confined to the site of origin) or invasive (spreading).

Noninvasive breast cancers include

- Ductal carcinoma in situ (also called intraductal carcinoma or DCIS). DCIS consist of cancer cells in the lining of the duct. DCIS is a non-invasive, early cancer, but if left untreated, it may sometimes progress to an invasive, infiltrating ductal breast cancer. DCIS is the most common type of noninvasive breast cancer.
- Lobular carcinoma in situ, or LCIS. Although it is technically not a cancer, lobular carcinoma in situ is a marker for an increased risk of invasive cancer the same or both breasts.

A diagnosis of these early cancers (DCIS and LCIS) is made when there is no evidence of invasion.

Invasive Breast Cancer

Invasive cancer occurs when cancer cells spread beyond the basement membrane, which covers the underlying connective tissue in the breast. This tissue is rich in blood vessels and lymphatic channels that are capable of carrying cancer cells beyond the breast. Invasive breast cancers include the following:

- Invasive (also called infiltrating) ductal carcinoma. This is invasive breast cancer that penetrates the wall of a milk-passage duct. It comprises between 70 - 80% of all breast cancer cases.
- Invasive (also called infiltrating) lobular carcinoma. This invasive cancer has spread through the wall of a milk-producing lobule. It accounts for 10 - 15% of all breast cancers. It may sometimes appear in both breasts, sometimes in several separate locations.

Docetaxel is a clinically well established anti-neoplastic medication used mainly for the treatment of breast, ovarian and non-small cell lung cancer. Docetaxel has an approved claim for treatment of patients who have locally advanced, or metastatic breast or non small-cell lung cancer who have undergone anthracycline-based chemotherapy and failed to stop cancer progression or relapsed. The cytotoxic activity of docetaxel is exerted by promoting and stabilising microtubule assembly, while preventing physiological microtubule depolymerisation/disassembly in the absence of guanosine triphosphate (GTP). This leads to a significant decrease in free tubulin, needed for microtubule formation and results in inhibition of mitotic cell division between metaphase and anaphase, preventing further cancer cell progeny.

The main mode of therapeutic action of docetaxel is the suppression of microtubule dynamic assembly and disassembly, rather than microtubule bundling leading to apoptosis, or the blocking of bcl-2. Intravenous administration of docetaxel results in 100% bioavailability and absorption is immediate. Oral bioavailability has been found to be 8% ± 6% on its own and when co-administered with cyclosporine, bioavailability increased to 90% ± 44%. In practice, docetaxel is administered intravenously only to increase dose precession. Docetaxel is used for the treatment of Breast cancer, Nonsmall cell lung cancer Prostate cancer Gastric adenocarcinoma Head and neck cancer.

It was hypothesized that RGD conjugated PLGA nanoparticles and liposomes loaded with docetaxel would result in higher cellular uptake and hence higher and sustained intracellular levels of drugs compared to unconjugated nanoparticles and liposomes or plain drugs by both passive and active targeting. Active targeting will be achieved by specific receptor binding of peptide and passive targeting will be achieved by EPR effect and hence present as a potential delivery system to treat breast cancer.

1.2. Research envisaged

The objectives of present investigation were development of three types nanoconstructs of docetaxel (PLGA nanoparticles, liposomes and HPMA copolymer drug conjugates), RGD-conjugation onto the nanoconstructs systems, characterization, and evaluation of these nanoconstructs on breast cancer cell lines to understand their role in breast cancer chemotherapy.

The proposed plan of work may be divided into following specific aims:

I. Literature reviews covering mechanisms, barriers and various approaches to intracellular drug delivery.

II. To prepare nanoconstructs systems of the drug docetaxel, to conjugate RGD onto the surface of these nanoconstructs and to characterize them for particle size, zeta potential, entrapment efficiency, surface morphology and *in-vitro* drug release.

III. To study the stability of unconjugated and conjugated systems with respect to appearance, particle size, zeta potential and drug content.

IV. To study the cellular uptake of unconjugated and conjugated of 6-coumarin loaded nanoconstructs using confocal microscopy.

V. To evaluate the unconjugated and conjugated nanoconstructs for their *in-vitro* antiproliferative activity using breast cell line.

VI. To study the effect of unconjugated and conjugated nanoconstructs on cell cycle and mode of cell death on breast cancer cells.

1.3. References

Baldwin, S.P. et al, 1998, Materials for protein delivery in tissue engineering, Advanced Drug Delivery Reviews, **33**, 71-86.

Besselink, G.A.J. et al, Affinity-binding of cells to sephadex derivatized with peptides and proteins, in Frontiers in biomedical polymer applications, Technomic: Lancaster, 201-213.

Chinn, J.A. et al, 1998, Blood and tissue compatibility of modified polyester: Thrombosis, inflammation, and healing, J. Biomed. Mater. Res., **39**, 130-140.

Craig, W. S. et al, 1995, Concept and progress in the development of RGD-containing peptide pharmaceuticals, Biopolymers, **37**, 157–175.

Ghosh, S. et al, 2004, Recent research and development in synthetic polymer-based drug delivery systems. Journal of Chemical Reaserach, 241-246.

Hans, M.L. et al, 2002, Biodegradable nanoparticles for drug delivery and targeting, Current Opinion Solid State Matter Science, **6**, 319-327.

Hynes, R.O. et al, Integrins: Versatility, modulation, and signaling in cell adhesion, Cell, **69, 53**, 11-25.

Jain, R. K. et al, 1998, Nat. Med., **4**, 655–657.

Jayanth Panyama et al, 2003, Biodegradable nanoparticles for drug and gene delivery to cells and tissue, Advanced Drug Delivery Reviews, **55**,329–347.

Kadimi, U.S. et al, 2007, In vitro studies on liposomal amphotericin B obtained by supercritical carbon dioxide–mediated process, Nanomedicine: Nanotechnology, Biology, and Medicine, **3**, 273–280.

Kim, B.S. et al, 1999, Engineered smooth muscle tissues: Regulating cell phenotype with the scaffold. Experimental Cell Research, **251**, 318-328.

Madri, J.A. et al, 1988, Endothelial cell-extracellular matrix interactions: Matrix as a modulator of cell functions, in Endothelial cell biology in health and disease, Plenum Press: New York, 167-188.

McHugh, M.A. et al, 1994, Supercritical Fluid Extraction, 2nd ed., Newton, MA: Butterworth-Heinemann.

Miller, N. et al, 1995, FASEB J., **9**, 190–199.

Moghimi, S.M. et al, 2001, Long-circulating and target-specific nanoparticles: Theory to practice. Pharmacological Reviews, **53**, 283-318.

Mohanraj, V.J. et al, 2006, Nanoparticles – A Review, Trop. J. Pharm. Res, **5 (1)**, 561-573.

Rezania, A. et al, 1999, Biomimetic peptide surfaces that regulate adhesion, spreading, cytoskeletal organization, and mineralization of the matrix deposited by osteoblast-like cells, Biotechnol. Prog., **15**, 19-32.

Ruoslahti, E. et al, 1987, New perspectives in cell adhesion: RGD and integrins, Science, **238**, 491-497.

Ruth Duncan et al, 2003, The Dawning Era of Polymer Therapeutics, Nature Reviews Drug Discovery, **2**, 347-360.

Shakesheff, K.M. et al, 1998, Creating biomimetic microenvironments with synthetic polymer-peptide hybrid molecules, in Polymers for tissue engineering, VSP: Utrecht., 113-124.