Development of Mineralocorticoid Receptor-Mediated Anticancer Liposomal Delivery System

THESIS

Submitted in partial fulfillment

of the requirements for the degree of

DOCTOR OF PHILOSOPHY

By

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BIRLA INSTITUTE OF TECHNOLOGY & SCIENCE PILANI
HYDERABAD CAMPUS

2015

BIRLA INSTITUTE OF TECHNOLOGY AND SCIENCE, PILANI

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CERTIFICATE

This is to certify that the thesis entitled **"Development of Receptor-Mediated Anticancer Liposomal Delivery System"** submitted by **Priyanka Sharma** ID No **2010PHXF427H** for award of Ph.D. of the Institute embodies original work done by her under my supervision.

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Acknowledgement

In my research career, I gained a lot and lost a lot. I wish to acknowledge the remarkable individuals who stood with me in my gains and losses.

At an outset, I would like to express my deep sense of gratitude and thanks to **Dr. Kumar Pranav Narayan,** my thesis supervisor. I will always be indebted to him for his valuable guidance and constant encouragement.

I want to thank my DAC (doctoral advisory committee) members, **Prof. Suman Kapur** and **Prof. Vidya Rajesh** for their inspiring support and invaluable scientific suggestions from time to time. They always extended helpful hands towards me whenever I needed them.

I thank **Dr Jayati Ray Dutta,** DRC (doctoral research committee) convener, for her constant encouragement and support.

I express my sincere thanks to all the faculty members of Biological Sciences Department, BITS Pilani, Hyderabad campus for their kind help and encouragement in carrying out my research work.

It is my pleasure to acknowledge ***Dr. Rajkumar Banerjee****, Principal Scientist, Indian Institute of Chemical Technology (IICT), Hyderabad for his inspiring support and invaluable scientific suggestions from time to time. He always extended his helpful hands to me whenever I needed them.*

Constant moral support extended to me by my friends and well wishers is gratefully acknowledged. I have special word of thanks for my friends ***Apurva,*** ***Akshay*** *and* ***Daipayan*** *for their love and support.* I am really fortunate for sharing a wonderful friendship with them. Writing this thesis would be extremely difficult for me without their enormous support. I gratefully acknowledge the support of my IICT labmates during the different phases of my doctoral research work.

Financial assistance from BITS Pilani, Hyderabad is gratefully acknowledged.

I appreciate the unending love and affection from my sisters, ***Anuradha,*** ***Jyotsna,*** ***Shivani*** *and my brother,* ***Aditya****. Love you all for your everlasting supports extended towards me.*

Words are inadequate to express thanks and appreciation to my husband, **Subrata** for all his love and support when I was irritable and depressed.

Last but not the least, the undying love and sacrifice of my parents, ***Sh. Amarjeet Sharma*** *&* ***Smt. Vijay Sharma****, can never be completely expressed with words alone.* They have given up many things for me. I have no phrases to praise them. Their blessings have helped me in reaching this goal.

Priyanka Sharma

Abstract

Cancer is a disease of un-controlled cellular growth and proliferation. The aim of cancer therapy is to destroy highly proliferative cancer cells selectively without affecting the normal tissues of patients. But, most of the currently available anticancer therapies possess adverse side effects as they are not highly selective towards cancer cells. This limits the dose of drugs and prolong treatment is needed and that makes cancer cells 'un-sensitized' towards drugs.

Targeted cancer therapy is an important strategy for selective cancer extinction. Cellular receptors are lucrative targets for targeted cancer therapy. Steroid hormone receptors (SHRs) ubiquitously express in many cells and hence, targeting of this class of proteins could help to target various cancer cells using a single delivery system. SHRs maintain a cytosolic inactive state by association with heat shock proteins and/or other proteins such as co-repressors. Upon ligand binding, the SHRs undergo conformational changes that involve release from the repressor proteins, and translocation to the nucleus where it can bind to specific hormone responsive sequences in the DNA of genes regulated by steroid hormones. In recent years, the role of corticoids in cancer development and progression has been an area of extensive research. Among them, the mechanism of action of glucocorticoids has been extensively studied, but relatively little is known regarding the role of mineralocorticoids in cancer therapy. The role of mineralocorticoids in controlling sodium and potassium transport in epithelial cells and in non-epithelial tissues, such as cardiac myocytes, blood vessels, the hippocampus and adipose tissue is well known.

In the present thesis, we report that spironolactone (SP), a synthetic ligand for mineralocorticoid receptor when associated with aqueous formulation of cationic lipid or liposome; it can be used as a targeting ligand for selective delivery of anticancer gene p53, to

elicit target-specific toxicity in MR over-expressed cancer cells. Eplerenone, which is another MR ligand was also used for cationic liposome formulation and both the ligands were compared for their MR-mediated delivery of genes. MR-mediated liposomal delivery system induced apoptosis in cancer cells in a targeted manner by initiating intrinsic pathway of apoptosis as analysed by Western blots. The liposomal formulations were found to be effective in inhibiting the metastasis of cells .The results obtained in the present thesis indicate that mineralocorticoid receptor can be a good target for the delivery of anticancer agents in cancer cells and opens a new possibility for targeted anticancer therapy.

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List of Abbreviations

BSA : **Bovine serum albumin**

DMEM : **Dulbecco's modified eagle's medium**

DMSO : **Dimethyl sulphoxide**

DODEAC : ***N*-di-*n*-tetradecyl-*N*,*N*-(2-hydroxyethyl)ammonium chloride**

DTT : **Dithiothreitol**

ECM : **Extracellular matrix**

EDTA : **Ethylenediaminetetraacetic acid**

FACS : **Fluorescence activated cell sorter**

FBS : **Fetal bovine serum**

FITC : **Fluorescein isothiocyanate**

GR : **Glucocorticoid receptor**

HEPES : **(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)**

hMR : **Human mineralocorticoid receptor**

$MgCl_2$: **Magnesium chloride**

MR : **Mineralocorticoid receptor**

mRNA : **Messenger ribonucleic acid**

MTT : **3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide**

NaCl : **Sodium chloride**

PAGE :**Polyacrylamide gel electrophoresis**

PBS : **Phosphate buffer saline**

pDNA : **Plasmid deoxyribonucleic acid**

PI : **Propidium iodide**

PMSF : **Phenyl methyl sulfonyl fluoride**

RNA : **Ribonucleic acid**

SDS : **Sodium dodecyl sulphate**

siRNA : **Small interfering ribonucleic acid**

TLC : **Thin layer chromatography**

Tris-HCL : **Tris(hydroxymethyl) aminomethane hydrochloride**

β-gal : **Beta-galactosidase**

Chapter 1 Introduction

Cancer is a dreadful disease causing death of millions and number of sufferers is further increasing. Cancer is a leading cause of death worldwide and it is projected to continue to rise to over 11 million in 2030. There is huge need of developing new therapeutic approaches to treat the disease. Currently, there are several treatment strategies available including radiation therapy, chemotherapy and gene therapy. Cancer drug and/or gene delivery is no longer simply wrapping the drug in new formulations for different routes of delivery. Cancer cell specific delivery of an anticancer agent is important to avoid adverse non-specific side effects over normal tissues.

With the advent of techniques in molecular biology, abnormalities in cancer cells at a molecular level have increased dramatically. Upon identifying oncogenes, tumor suppressor genes & development of high-throughput DNA sequencing and genome-wide expression profiling, newer varieties of targeted therapies have come into being. Cancer occurs at a molecular level when multiple subsets of genes undergo genetic alterations, either activation of oncogenes or inactivation of tumor suppressor genes. Early diagnosis of cancer is difficult because of the lack of specific symptoms in early disease and the limited understanding of etiology and oncogenesis. Intensive research is going on to improve the treatment of cancer, like new chemical entity development and the development of successful ways to deliver existing drugs leading to improved therapeutic index and reduced side effect. Conventional anticancer treatments are nonspecific to target killing of tumor cells, may induce severe systemic toxicity, and produce drug resistant phenotypic growth.

Approaches in the treatment of cancer

Though cancer is often described as 'The wound that never heals', there have been countless efforts worldwide to bring a remedy that cures all these nuisances. The choice of methods depends on the location of tumor in the body, its growth rate and overall physical condition of the patient. The major limitations of these approaches are recurrence of tumor, degradation of bone and bone marrow, cardio-toxicity, adverse effects on the normal tissues, etc. Indeed, in cancer therapy, specific killing of aberrant tissues without harming the normal quiescent cells is a Himalayan task. Cancer can be treated by surgery, chemotherapy, radiation therapy, hormonal therapy and gene therapy. The choice of therapy depends upon the location and grade of the tumor and the stage of the disease, as well as the general state of the patient.

Complete removal of the cancer without damage to the rest of the body (that is, achieving cure with near-zero adverse effects) is the ideal goal of treatment and is often the goal in practice. Sometimes this can be accomplished by surgery, but the propensity of cancers to invade adjacent tissue or to spread to distant sites by microscopic metastasis often limits its effectiveness. On the other hand, chemotherapy and radiotherapy can have negative effects on normal cells. Targeted therapy, has a significant impact in the treatment of some types of cancer, and is currently a very active research area. Targeted delivery is a method of delivering medication to a patient in a manner that increases the concentration of the medication in some parts of the body relative to others. The system is based on a method that delivers a certain amount of therapeutic agents for a prolonged period of time to a targeted diseased area within the body.

Breast and lung cancer are the most frequently diagnosed malignancies and leading cause of cancer-related deaths. The local or distant metastases are very much common in most diagnosed patients of breast and lung cancer (Bendre *et al*., 2003). However, metastatic tumor cells depict the advanced diseased state. These cells are often resistant to chemotherapy due to over-expression of anti-apoptosis oncogenes, such as Bcl-2 (Olopade *et al*., 1997, Silvestrini *et al*., 1997). Despite several hundred clinical studies of both experimental and approved chemotherapeutic agents, low dose chemotherapy has limited antitumor activity, e.g. with 1-2% survival benefit for adjuvant paclitaxel (Antman, 2001). However, in high dose therapy, an overall 15% disease free survival advantage and 10% survival benefit had been demonstrated (Stemmer *et al*., 1996; Vahdat *et al*., 1998; Hudis *et al*., 1999).

Vectors are keys to success in targeted delivery system and can be viral and non-viral. Although, viral vectors are the most efficient gene transfer vehicles, there are a multitude of potentially adverse effects associated with their use. They can induce inflammatory and adverse immunogenic responses and can produce insertional mutagenesis through random integration into the host genome (Nayerosaddat *et al*., 2012). Non viral vectors offer many advantages over viral vectors including lower cyto-toxicity, immnogenicity, capability for delivery large DNA to be delivered, ease of large scale production and low frequency of integration etc. Among all non-viral vectors cationic liposomes are gaining importance. Cationic lipid/co-lipid formulations are excellent non-viral tools for the cellular delivery of DNA (Karmali & Chaudhuri, 2007).

Previously, it was shown that glucocorticoid receptor (GR) mediated liposomal delivery system can selectively deliver anticancer gene in cancer cells over normal

cells and reduce tumor growth in mouse model (Mukherjee *et al*., 2009). In recent years, the role of corticoids in cancer development and progression has been an area of extensive research. Among them, the mechanism of action of glucocorticoids has been extensively studied, but relatively little is known regarding the role of mineralocorticoids in cancer therapy.

Mineralocorticoid receptors (MRs) are nuclear hormone receptors that are ubiquitously present in all cell types and are known to mediate distinct physiological functions like regulating Na^+ and K^+ balance and water excretion (Berger *et al*., 1998). MRs are linked to cell proliferation and can be exploited for the targeted control of cell mass in cancer. The present study is aimed towards extending the concept of using mineralocorticoid receptor (MR) ligand spironolactone (SP) for selective delivery of genes in cancer cells with the following objectives:

- Design of novel cationic lipid conjugated with mineralocorticoid ligand (antagonist or agonist) and detailed structure-activity investigation.
- Testing the efficacy of the formulations in normal and cancer cells.
- Optimizing encapsulation of anticancer gene in liposomes and *in vitro* testing for its toxicity to both normal and cancer cells.

2.1 Managing cancer through targeted delivery

Targeted gene delivery or drug delivery technology represents one of the frontier areas of science, which involves multidisciplinary scientific approach, contributing to human health care. These delivery systems offer numerous advantages compared to conventional dosage forms, which include improved efficacy, reduced toxicity, and improved patient compliance and convenience. Nanocarriers with optimized physicochemical and biological properties are successfully used as delivery tools. Liposomes, solid lipids nanoparticles, dendrimers, polymers, silicon or carbon materials, and magnetic nanoparticles are the examples of nanocarriers that have been tested as drug delivery systems.

Liposomes are currently one of the well-studied delivery system for the treatment of cancer. They are being employed in the treatment of a wide variety of malignancies. Their large size relative to the vasculature of healthy tissues inhibits their uptake by certain tissues, thus avoiding non-specific toxicities. However, the leaky vasculature of solid tumors allows these large entities (liposomes) to get into the tissues and interaction with the cancer cells and release of the encapsulated drug specifically into the tumor (Tran *et al*., 2009). Liposomes have several advantages over corresponding free drugs, including favourable pharmacokinetic properties, where encapsulation of a usually rapidly cleared drug results in a considerable increase in circulation time. In addition, encapsulation of a normal thermo-labile agent such as DNA or oligonucleotides etc. protects the agent from degradation by enzymes or hydrolysis (Tardi *et al*., 2000). Since their discovery (Bangham & Horne, 1964), liposomes have attracted considerable interest owing to their organization and the versatility of their physicochemical properties. Due to their versatile nature, liposomes

can be used for diverse applications. Liposomal systems became a popular drug delivery platform for several reasons:

- They are made from naturally occurring lipids from living systems, making them nontoxic and biodegradable;
- The drug is entrapped in liposomes, so it is not exposed to the metabolic machinery of the body, preventing degradation and dilution;
- Liposomes form spontaneously in aqueous solutions and components can be added or removed in a modular way.

As a result, their properties can be tailored to the respective application. New advances in liposomal technology are being made to combine drugs or compounds in unique lipids to create stable liposomes. Recently, selective delivery of the anticancer agent doxorubicin in polyethylene glycol (PEG) liposomes for the treatment of solid tumours in patients with breast-carcinoma metastases resulted in a subsequent improvement in survival (Park, 2002).

Liposomal vesicles have drawn the attention of researchers as potential carriers of various bioactive molecules that could be used for therapeutic applications in both humans and animals. Recent work has shown that nucleic acids can be entrapped in cationic liposomes (CLs) and subsequently transfected into cultured mammalian cells, where they can express the information they carry (Kozubek *et al*., 2000; Bareholtz, 2001). CLs represent one of the most widespread non-viral transfection systems for gene delivery. CLs are usually employed as a gene delivery system because of their low toxicity, low immunogenicity, ease of preparation, size-independent delivery of nucleic

acids, and quality control and capacity for mass production at reasonable cost (Samadikhah *et al*., 2011). A solution of cationic lipids, often formed with neutral helper lipids, can be mixed with DNA to form a positively charged complex termed a lipoplex (Wasungu & Hoekstra, 2006).

Well-characterized and widely used commercial reagents for cationic lipid transfection include N-[1-(2,3-dioleyloxy) propyl]-N,N,N- trimethylammonium chloride (DOTMA) (Felgner, 1987), [1,2-bis(oleoyloxy)-3-(trimethylammonio) propane] (DOTAP) (Leventis & Silvius, 1990), 3-β[N-(N´N´-dimethylaminoethane)-carbamoyl] cholesterol (DCChol) (Gao& Huang, 1991),and dioctadecylamidoglycylspermine (DOGS) (Behr *et al.*, 1989). Table 2.1 lists various phospholipids used in liposome formation and their role in targeting particular disease.

Table 2.1 Lipid composition for liposomes and target diseases

Drug	Liposome constituents	Therapy	References
Amphotericin B	Cholesterylsulfate	Fungal Infections	Immordino *et al*., 2006
Doxorubicin	HSPC, cholesterol, and PEG 2000-DSPE, Chloesterol	Kaposi's sarcoma, Ovarian/breast cancer	Immordino *et al*., 2006; Park, 2002
Cytarabine	Triolein, DOPC, and DPPG, Chloesterol	Neoplastic meningitis and Lymphomatous meningitis	Immordino *et. al*, 2006
Inactivated hepatitis A virus (strain RG-SB)	DOPC and DOPE	Hepatitis A	Usonis *et al*., 2003; D'Acremont *et al.*, 2006
Daunorubicin	DSPC and cholesterol	Hepatitis A	Usonis *et al*., 2003; D'Acremont *et al.*, 2006
Inactivated hemaglutinine of Influenza virus strains A and B	DOPC and DOPE	Influenza	Herzog *et al*., 2009;
Doxorubicin	Palmitoleylphosphatidyl choline (POPC), DOPC-PEG	cancer	Banerjee *et al*., 2004
Chloroquine	Chloesterol	Malaria	Owais *et al*., 1995
AmphotericinB and nystatin	Egg phosphatidylcholine (egg PC), Chloesterol	Infectious diseases	Moribe & Moriyama, 2002
Linoleic acid	1,2-Distearoyl-sn-glycero-3-phosphoethanolamine(DSPE) phosphatidylcholine	Dermatology & cosmetology	Ghyczy *et al*., 1996

2.2 Cancer: The age-old immortal illness

Cancer, often described as the plague of this century, has a quite old history of 4000 long years. World-wide mortality rate of cancer is in between 100-150 of each 100,000 people. Cancer arises from uncontrolled division of a set of diseased cells those can be identified with a limitless replicative potential as well as the capacity of out-of bound migration, invasion and metastasis. There is no single definition that describes all cancers. It is a large family of diseases which show features suggestive of malignancy. They form a subset of neoplasm. A neoplasm or tumor is a group of cells that have undergone unregulated growth, and will often form a mass or lump, but may be distributed diffusely. Six characteristics of malignancies i.e. "**hallmark of cancer**" have been proposed: self-sufficiency in growth signalling; insensitivity to anti-growth signals; evasion of apoptosis; enabling of a limitless replicative potential; induction and sustainment of angiogenesis; and activation of metastasis and invasion of tissue (Douglas & Weinberg, 2000). Cells become cancer cells because of DNA (deoxyribonucleic acid) damage. DNA is in every cell and it directs all its actions. In a normal cell, when DNA is damaged the cell either repairs the damage or the cell dies. In cancer cells, the damaged DNA is not repaired, but the cell doesn't die like it should. Instead, the cell goes on making new cells that the body doesn't need. These new cells all have the same damaged DNA as the first abnormal cell does. Cancer cells often travel to other parts of the body where they can grow and form new tumors with the characteristic of their tissues of origin. This happens when the cancer cells get into the body's bloodstream or lymph vessels. Over time, the tumors replace normal tissue, crowd it, or push it aside.

Today, clinical medicine possesses an extremely long list of different pharmaceutical products and every year many new drugs are added to the list with the understanding of molecular mechanisms of diseases. Scientists and physicians are never satisfied only with a favourable drug action against the disease under treatment. The task of avoiding undesirable drug actions on normal organs and tissues and minimizing side effects of the therapy is very important. Many pharmacologically effective compounds cannot be used as drugs due to their undesirable action on normal tissues. Their specificity for the drug of choice is not based on their ability to accumulate selectively in the target organs. Normally, they are more or less evenly distributed in the whole body and to reach the target zone the drug has to cross many other organs, cells, intracellular compartments, *etc*., where it can be partially inactivated. To overcome this problem, a high concentration of drug has to be administered, which has a potential to cause undesirable complications and is sometimes expensive too. The ideal solution to such problems is the targeting of drugs using suitable carriers such as liposomes, polymers, micelles etc. Among these carriers, liposomes show great potentials of effective delivery of drugs to the site of action and of controlling the release of these drugs at a predetermined rate (Goyal *et al*., 2005).Though, the design of liposomal carriers for gene or drug delivery is an active field in current biomedical research, an important issue is the targeting of the carriers to specific cell types for which the therapeutic approach is intended.

2.3 Therapeutic strategies to fight cancer

Therapeutic selectivity for malignant cells versus normal cells is a difficult task of cancer treatment. It is not difficult to kill or inhibit the growth of cancer cells. But achieving a differential effect is much more challenging. Current therapeutic strategies to fight cancer include surgery, chemotherapy, radiation therapy, gene therapy etc. The major limitations of these approaches are reappearance of tumor, adverse effect on the normal tissues and fast dividing cells of body, degradation of bone marrow, cardio-toxicity, loss of hair, fatigue, nausea, vomiting etc. So far numerous attempts have been carried out to demystify the behaviour of this lethal disease which concludes that cancer arises from uncontrolled division of a set of diseased cells those can be identified with a limitless replicating potential as well as the capacity of out-of-bound migration, invasion and metastasis. The loss of cellular regulation leading to cancer may often arise from mutations in two broad classes of genes, namely, oncogenes and tumor suppressor genes. Some of the conventional and modern approaches of treating cancer are:

2.3.1 Chemotherapy:

Chemotherapy is the use of medication (chemicals) to treat disease. More specifically chemotherapy typically refers to the destruction of cancer cells. There are more than 100 different types of chemotherapy drugs today which can treat most cancers. Chemotherapy is often used alongside other treatments. Chemotherapy has five possible goals:

- Total remission - to cure the patient completely. In some cases chemotherapy alone can get rid of the cancer completely.

- Combination therapy - chemotherapy can help other therapies, such as radiotherapy or surgery have more effective results.
- Delay/Prevent recurrence -chemotherapy, when used to prevent the return of a cancer, is most often used after a tumor is removed surgically.
- Slow down cancer progression – used mainly when the cancer is in its advanced stages and a cure is unlikely. Chemotherapy can slow down the advancement of the cancer.
- To relieve symptoms – used for patients with advanced cancer.

The goals of treatment with chemotherapy depend on the type of cancer and how much it has spread. In some situations, the primary goal of chemotherapy is to eliminate cancer cells and prevent recurrence. If it is not possible to eliminate the cancer, chemotherapy may be used to control the cancer by slowing its growth and/or to reduce symptoms caused by the cancer. Chemotherapeutic therapy has a range of side effects that depend on the type of medications used. The most common medications affect mainly the fast dividing cells of the body, such as blood cells and the cells lining the mouth, stomach, and intestines. Chemotherapy-related toxicities can occur acutely after administration, within hours or days, or chronically, from weeks to years. While chemo drugs kill cancer cells, they also harm cells that divide rapidly under normal conditions. So, cells in the bone marrow, digestive tract and hair are greatly affected by the chemo drugs. They affect normal cells also. This results in the most common side-effects of chemotherapy (McKnight, 2003).

Several chemo drugs are in use in present cancer treatments: Dacarbazine, Temozolamide, Cisplatin, Doxorubicin, vincristine, vinblastine carboplatin,5-Fluorouracil, Mitomycin C, paclitaxel etc.

2.3.2 Cancer immunotherapy

Cancer immunotherapy refers to the activation of the immune system that will lead to the destruction of cancerous cells in the body. There are three main groups of immunotherapy used to treat cancer: cell-based therapies, antibody therapies and cytokine therapies. They all exploit the fact that cancer cells often have subtly different molecules on their surface that can be detected by the immune system. These molecules, known as cancer antigens, are most commonly proteins but also include other molecules such as carbohydrates. Immunotherapy is used to provoke the immune system into attacking the tumor cells by using these cancer antigens as targets. Because of the immune system's extraordinary power, its capacity for memory, its exquisite specificity, and its central and universal role in human biology, these treatments have the potential to achieve complete, long-lasting remissions and cancer cures, with few side effects, and for any cancer patient, regardless of their cancer. When we think of immunotherapy, we think of a "natural" treatment as our body's own defense system. Immunotherapy, regardless of the approach used, does have side effects. These side effects result from revving up the immune system. They range from relatively mild "flu-like" symptoms and fatigue to more serious problems involving the gastrointestinal system, thyroid or pituitary gland or lungs. The side effects of immunotherapy are usually different from those experienced by people receiving chemotherapy or radiation therapy (Hall, 2000).

The most common side effects of immunotherapy are:

- Flu-like symptoms
- Fatigue
- Rashes
- Fever
- Drops in blood pressure

Less common side effects are:

- Colitis or other gastrointestinal problems
- Thyroid problems
- Lung problems

2.3.3 Radiation therapy

Radiation therapy is the use of high energy radiations such as x-rays to control or kill malignant cells. Radiation therapy may be curative in a number of types of cancer if they are localized to one area of the body. It may be used to prevent the tumor recurrence after removal of primary tumor by surgery e.g. in early stage of breast cancer. Higher doses can cause varying side effects during treatment and after treatment like skin irritation, fatigue, damage to epithelial surfaces, temporary soreness and ulceration, abdomen discomfort, Swelling (edema or oedema) and infertility (Hall, 2000; Carretero *et al*., 2007; Yip *et al*., 2004; Murthy *et al*., 2007 & Arepally *et al*., 2013).

2.3.4 Hormone therapy

Since in some cases cancer results from the imbalance in the proportion of certain hormones and growth factors, an efficient anti-cancer strategy may be based on

supplying or blocking specific hormones in the body. Cancers that are hormone sensitive or dependent need hormones to grow or develop. Hormone therapy for cancer is the use of medicines to block the effects of hormones. This therapy works for only those types of cancer that are hormone sensitive or hormone dependent. Administration of antagonists for hormones like progesterone, estrogen, etc has been proved to be helpful in this respect (Vincet *et al*., 2005; Boothby *et al*., 2004).Cancers that can be hormone dependent include breast cancer, prostate cancer, ovarian cancer, womb cancer and kidney cancer. Hormone therapies can slow down or stop the growth of cancer by either:

- Stopping hormones being made or
- Preventing hormones from making cancer cells grow and divide

In hormone therapy, the principle is to manipulate endocrine systems through the delivery of steroid hormones or drugs to inhibit the production or activity of such hormones. Drugs are used as antagonists against hormone receptors.

2.3.5 Cancer gene therapy

Gene therapy is an experimental technique that uses genes to treat or prevent disease. In gene therapeutic approaches for treating genetic diseases, the correct functional copies of the malfunctioning genes are administered to our body cells either *in vivo* or *ex vivo*. Researchers are testing several approaches to gene therapy: replacing a mutated gene that causes disease with a healthy copy of the gene, inactivating, or "knocking out" a mutated gene that is functioning improperly, introducing a new gene into the body to help fight a disease, etc. Gene therapy was first conceptualized in 1972, with the authors urging caution before commencing gene therapy studies in humans.

The first FDA-approved gene therapy experiment in the United States occurred in 1990, when Ashanti DeSilva was treated for adenosine deaminase deficiency (ADA) a genetic disease causing a severe combined immunodeficiency (ADA-SCID) (Sheridan, 2011). Although, the therapy did not cure Ashanthi completely but it did help her in taking small doses of the traditional PEG-ADA drug instead of large doses which she had to take earlier. Till date, nearly 1512 gene therapy based clinical trials have been completed worldwide and an increasing number of gene therapy based clinical investigations are under progress in different parts of the world (Journal of Gene Medicine Clinical Trials Web site; wiley Database). The delivery and expression of the correct gene copy (called transfection) must be carried out by the use of appropriately designed vehicle (referred to as transfection vector). The therapeutic gene must be efficiently delivered to particular cell or tissue. It must not affect other cells. The gene should be expressed at the appropriate level for a sufficient duration. Since, cell surface is negatively charged, the entry of negatively charged plasmid DNA into the cells is not an efficient process. So, the success of this therapy largely depends on the delivery systems. Although numerous viral and nonviral gene delivery systems have been developed in the last 3 decades, no delivery system has been designed that can be applied in gene therapy of all kinds of cell types *in vitro* and *in vivo* with no limitation and side effects. Ideally, the delivery vehicle must be able to remain in the bloodstream for a long time and avoid uptake by the mononuclear phagocyte system, in order to ensure its arrival at the desired targets. Moreover, this carrier must also be able to transport the DNA efficiently into the cell cytoplasm, avoiding lysosomal degradation (Ibraheem *et al*., 2014).

2.4 Transfection Vectors: key to success in gene therapy

Transfection is a method that neutralizes the issue of introducing negatively charged molecules (e.g. phosphate backbones of DNA or RNA) into cells with a negatively charged membrane. An ideal vector should allow efficient and selective transduction of the target cell, able to maintain gene expression at levels necessary for achieving therapeutic effects and should be safe. Transfection vectors commonly used in gene therapy are mainly of two types-viral and non-viral. A variety of viral and non-viral vehicles have been conceived to effect the encapsulation, *in vivo* cell targeting and intracellular delivery of therapeutic macromolecules (Maurer *et al.*, 1999; Torchilin, 2006).

2.4.1 Viral vectors

Viruses have been used as vectors in gene therapy by replacing the genes essential for the replication phase of their life cycles with the therapeutic genes of interest. The delivery of genes by a virus is termed as transduction and the infected cells are described as transduced. Molecular biologists first harnessed this machinery in the 1970s. Goff and Berg, used a modified SV-40 virus containing DNA from the bacteriophage lambda to infect monkey kidney cells maintained in culture (Goff & Berg, 1976). Majority of the clinical trials currently underway around the world are based on the use of mainly five categories of viruses, namely, retrovirus, adenovirus, adeno-associated virus, lentivirus and herpes simplex virus (Robbins *et al*., 1998).

Although, viral vectors are the most efficient gene transfer vehicles, the choice of a viral vector to deliver genetic material to cells comes with some problems. There are limited numbers of viral vectors available for therapeutic use. Any of these few viral vectors can cause the body to develop an immune response if the vector is seen as a

foreign invader (Nayak & Herzog, 2009; Zhou *et al*., 2004). Once used, the viral vector cannot be effectively used in the patient again because it will be recognized by the body. If the vaccine or gene therapy fails in clinical trials, the virus cannot be used again in the patient for a different vaccine or gene therapy in the future. Pre-existing immunity against the viral vector could also be present in the patient rendering the therapy ineffective for that patient (Nayak & Herzog, 2009). It is possible to counteract pre-existing immunity when using a viral vector by priming with a non-viral DNA vaccine, but this method presents another expense and obstacle in the vaccine distribution process (Yang *et al*., 2003). Pre-existing immunity may also be challenged by increasing vaccine dose or changing the vaccination route (Pandey *et al*., 2012). In brief, there are multitudes of potentially adverse effects associated with viral vectors. They can induce inflammatory and adverse immunogenic responses and can produce insertional mutagenesis through random integration into the host genome. The first death (September, 1999 at Pennsylvania) occurred in gene therapy clinical trials due to severe immune response to the adenovirus vector (Hollon, 2000). In addition purification and verification of sequence of recombinant vectors are time consuming processes. Size restriction of the genetic material to be delivered is also one of the drawbacks. All these complications are increasingly making non-viral transfection vectors as the vectors of choice in gene therapy.

2.4.2 Non-viral vectors

The non-viral gene delivery methods use synthetic or natural compounds or physical forces to deliver a piece of DNA into a cell. The materials used are generally less toxic and immunogenic than the viral counterparts. In addition, cell or tissue specificity can be achieved by harnessing cell-specific functionality in the design of

chemical or biological vectors, while physical procedures can provide spatial precision. Other practical advantages of non-viral approaches include ease of production and the potential for repeat administration. Non-viral vectors offer many advantages over viral vectors including lower toxicity, immunogenicity, capability for delivering large DNA, ease of large scale production and low frequency of integration etc. Previously, low levels of transfection and expression of the gene held non-viral methods at a disadvantage; however, recent advances in vector technology have yielded molecules and techniques with transfection efficiencies similar to those of viruses (Murakami and Sunada, 2011). Non-viral methods are generally viewed as less efficacious than the viral methods, and in many cases, the gene expression is short-lived. However, recent developments suggest that gene delivery by some non-viral methods has reached the efficiency and expression duration that is clinically meaningful (Al-dosari & Gao, 2009). The non-viral methods are mainly classified into physical and chemical methods.

2.4.2.1 Physical methods

Over the past decade, many physical methods have been investigated for gene transfer. These methods facilitate the transfer of genes from extracellular to nucleus by creating transient membrane holes/defects using physical forces such as local or rapid systemic injection, particle impact, electric pulse, ultrasound, or laser irradiation (Al-Dosari & Gao, 2009). Physical methods such as microinjection, gene gun, electroporation etc. are carrier-free gene delivery techniques that employ the use of a physical force to permeate the cell membrane and facilitate intracellular transfer of naked DNA.

2.4.2.1.1 Electroporation

Electroporation is a method that uses short pulses of high voltage to carry DNA across the cell membrane. This shock is thought to cause temporary formation of pores in the cell membrane, allowing DNA molecules to pass through. Electroporation is generally efficient and works across a broad range of cell types. Factors to be considered for optimal gene transfer using electroporation are the electrical field strength and pulse duration, ionic strength of the electroporation buffer, nucleic acid concentration, cell density and viability (Tsong, 1991; Rols *et al*., 2000). However, a high rate of cell death following electroporation has limited its use.

2.4.2.1.2 Gene gun

The use of particle bombardment or the gene gun is another physical method of DNA transfection. In this technique, DNA is coated onto gold particles and loaded into a device which generates a force to achieve penetration of the DNA into the cells, leaving the gold behind on a "stopping" disk. Yang *et al*., 1990 described the design of a high-voltage electric discharge device to be used for the acceleration of DNA-coated gold particles into living cells and showed that their particle bombardment technology can efficiently deliver foreign genes into liver, skin, and muscle tissues of rat or mouse *in vivo*. This method depends on the impact of heavy metal particles on target tissues and delivery of coated DNA on particles in passing. The particles are accelerated to sufficient velocity by highly pressurized inert gas, usually helium. Macroparticles made of gold, tungsten, or silver have been used for gene delivery through gene gun. One major drawback with the gene gun technology is the lack of sustained expression of the introduced genes in the target tissue. Therefore, multiple administrations are required in many cases.

2.4.2.1.3 Microinjection

This is the simplest method of non-viral transfection. Clinical trials carried out of intramuscular injection of a naked DNA plasmid have occurred with some success; however, the expression has been very low in comparison to other methods of transfection. Capecchi, 1980 used microinjection method to introduce Herpes simplex virus thymidine kinase gene into cultured mammalian cells. With the microinjection procedure transformation frequency was relatively insensitive to DNA concentration and did not depend on co-injecting with a carrier DNA. Cellular uptake of naked DNA is generally inefficient. Research efforts focusing on improving the efficiency of naked DNA uptake have yielded several novel methods. Delivery by microinjection can be used for any type of cell that is adherent in culture, including primary cells. Because siRNA can be easily and rapidly generated for any target gene, it is relatively simple to assess many effects of the knockout of any gene in any type of adherent cell in a matter of a few days. Delivery by microinjection assures that every cell receives the siRNA at a relatively equal concentration. Many of the disadvantages of the approach relate to the challenging aspects of the technique itself: It is not trivial to learn, and it is an undertaking that requires attention to detail. It also suffers the technical disadvantage that a limited number of cells are involved, which often does not permit the subsequent analysis of effects upon some biochemical parameters. The major limitation of the approach is the small amount of material obtained, but as the sensitivity of analysis techniques increases, so does the usefulness of this method.

2.4.2.1.4 Sonoporation

Sonoporation, or cellular sonication, is the use of sound (typically ultrasonic frequencies) for modifying the permeability of the cell plasma membrane. This

technique is used in order to allow uptake of large molecules such as DNA into the cells. The process of acoustic cavitation is thought to disrupt the cell membrane and allow DNA to move into cells (Yizhi *et al*., 2007). Extended exposure to low-frequency (<MHz) ultrasound has been demonstrated to result in complete cellular death (rupturing), thus cellular viability must also be accounted for when employing this technique. The bioactivity of this technique is similar to, and in some cases found superior to electroporation.

2.4.2.1.5 Magnetofection

In magnetofection, DNA is complexed to magnetic particles, and a magnet is placed underneath the tissue culture dish to bring DNA complexes into contact with a cell monolayer. The magnetofection principle is to associate nucleic acids with cationic magnetic nanoparticles: these molecular complexes are then transported into cells supported by an appropriate magnetic field (Scherer *et al*., 2002). In this way, the magnetic force allows a very rapid concentration of the entire applied vector dose onto cells, so that 100% of the cells get in contact with a significant vector dose. Magnetic fields, generally from high-field, high-gradient and rare earth metal magnets are focused over the target site and the forces on the particles as they enter the field allow them to be captured and extravasated at the target. While this may be effective for targets close to the body's surface, as the magnetic field strength falls off rapidly with distance, sites deeper within the body become more difficult to target (McBain *et al*., 2008).

2.4.2.2 Chemical methods

Various chemical methods like oligonucleotides, dendrimers, liposomes are available to enhance delivery to the target tissues. Compared with biological and physical methods of gene delivery, the major advantages of chemical vectors are their simplicity, ease of production and relatively low toxicity. Rapid progress in developing new chemical methods has led to successful gene delivery in variety of cell types. One of the most important reasons why these materials such as lipids and polymers can be used as the vectors for gene delivery is that they can interact with plasmid DNA (pDNA) to form nano-sized complexes, which is the premise to pass through the cell membrane.

2.4.2.2.1 Oligonucleotides

There are several methods by which use of oligonucleotides to deactivate the genes in the diseased process is achieved. One strategy uses antisense specific to the target gene to disrupt the transcription of the faulty gene. Another uses small molecules of RNA called siRNA to signal the cell to cleave specific unique sequences in the mRNA transcript of the faulty gene, disrupting translation of the faulty mRNA, and therefore expression of the gene. Single stranded DNA oligonucleotides can be used to direct a single base change within a mutant gene. The oligonucleotide is designed to anneal with complementarity to the target gene with the exception of a central base, the target base, which serves as the template base for repair. This technique is referred to as oligonucleotide mediated gene repair, targeted gene repair, or targeted nucleotide alteration.

2.4.2.2.2 Dendrimers

Dendrimers are repetitively branched molecules. They are synthesized from branched monomer units in a stepwise manner and thus it is possible to control their molecular properties, such as size, shape, dimension, and polarity, which depend on the branched monomer units (Yang *et al*., 2009). For targeting drugs to tumor tissues, the multivalency of dendrimers are widely exploited for covalent attachment of special targeting moieties, such as sugar (Bhadra *et al*., 2005), folic acid (Licciardi *et al*., 2006), antibody (Patri *et al*., 2004) and biotin (Yang *et al*., 2009). Drugs can also be encapsulated or conjugated with dendrimers. Studies based on dendritic polymer open up new avenues of research into the further development of drug-dendrimer complexes specific for a cancer and/or targeted organ system. Choi *et al*., 2005 synthesized and evaluated DNA-assembled polyamidoamine dendrimer clusters for cancer cell-specific targeting. Current research is being performed to find ways to use dendrimers to traffic genes into cells without damaging or deactivating the DNA. Dendrimers have an increased ionic interaction with DNA and produce very stable and highly soluble DNA complexes . However, there exist several problems to be solved. First, although dendrimer-based gene transfection reagents such as Superfect and Priofect have already been commercially available, these products are more expensive than other cationic polymers and are based on high generation dendrimers, the synthesis of which is labor-consuming. Second, the transfection efficiency of dendrimers is generation-dependent. Low-generation show poor gene transfection efficiency and lower cytotoxicity, but high-generation exhibit slightly better gene transfection efficiency and higher cytotoxicity . But ideal gene transfection reagents should have both high transfection efficiency and low cytotoxicity.

2.4.2.2.3 Liposomes

Liposomes are vesicular structures that are formed due to accumulation of lipids interacting with one another in an energetically favourable manner. Depending upon the structure and the composition of the bulk solution, liposomes can separate hydrophobic or hydrophilic molecules from the solution (Sharma *et al*., 2013). Liposomes were discovered in 1961 (published 1964) by Alec Bangham who was studying phospholipids and blood clotting, and since then they became very versatile tools in biology, biochemistry and medicine (Bangham & Horne, 1964). A liposome is a tiny bubble (vesicle), made out of the same material as a cell membrane i.e. phospholipids having a head group and a tail group. Head is hydrophilic while tail is lipophilic. In the presence of water, the heads are attracted to water and line up to form a surface facing the water. The tails are repelled by water, and line up to form a surface away from the water. In a cell, one layer of head faces outside of the cell, attracted to the water in the environment. Another layer of head faces inside the cell, attracted by water inside the cell. The hydrocarbon tails of one layer face the hydrocarbon tails of the other layer, and the combined structure forms a bilayer (Kumar *et al*., 2012).

2.6 Strategy behind cationic liposomal delivery

Use of cationic liposome formulation to transfect the DNA in cultured cells was pioneered by Flegner *et al*.,1987. After this achievement a wide range of cationic lipid based reagents have been developed in non viral gene therapy (Kumar *et al*., 2003). Cationic liposomes are prepared from cationic lipids containing a hydrophobic domain (composed usually of two aliphatic long chains or a steroid skeleton) and positively charged functionalities in their head-group region. Cationic lipids are generally formulated in combination with neutral "helper" lipids like dioleoylphosphotidyl

ethanolamine (DOPE) or cholesterol to form cationic liposomes for use as gene transfer vectors. The positively charged cationic liposomes when mixed with negatively charged DNA spontaneously form an electrostatic complex, popularly known as "lipoplex". The lipoplexes do not experience any severe electrostatic barrier as faced by the naked DNA in entering the biological cells and get endocytosed by the cell membrane. Complexation with cationic liposome usually protects DNA from nucleases present in the serum or in the cytoplasm en route the nucleus. Targeted delivery could also be achieved by cationic liposomes through covalent attachment of receptor-specific ligands to the liposomal surface.

DNA, being polyanionic macromolecule is not expected to be incorporated inside the cell as biological cell surface is negatively charged. The idea behind cationic lipid strategy is to neutralize the negative charge of plasmids with positively charged lipids to capture plasmids more efficiently and to deliver DNA into the cells (Ropert, 1999). Cationic liposomes offer many advantages over viral vectors (Samadikhah *et al*., 2011) for delivering genetic material and some are:

- Low toxicity
- Low immunogenicity
- Ease of preparation
- Size-independent delivery of nucleic acids
- Quality control and capacity for mass production at reasonable cost

Each cationic lipid has different structural aspects, such as head group size and hydrocarbon tail length. These aspects confer distinct characteristics to the lipid/DNA complex, which in turn affect association with and uptake into the cell. The positive charge on the head group facilitates spontaneous electrostatic interaction with DNA, as

well as binding of the resulting lipoplexes to the negatively charged components of the cell membrane prior to cellular uptake (Elouahabi & Ruysschaert, 2005).

2.7 Lipofection pathway

Cationic lipid mediated delivery and expression of genes into cells is often referred to as lipofection. Currently believed lipofection pathway involves following steps (Figure 2.1):

- Construction of lipid-DNA complex (lipoplex);
- Lipoplex binding to the cell surface;
- Endocytosis of the lipoplex;
- Endosomal trafficking of the lipoplex and its escape from endosome/lysosome compartment to the cytoplasm;
- Nuclear delivery of the released DNA and subsequently its transgene expression.

Electron microscopic studies have shown that internalization of lipoplex occurs mainly through endocytosis (Zabner *et al*., 1995). Endosomal release of genes into cell cytoplasm is regarded as crucial step in achieving successful transgene expression. In normal cellular trafficking pathways, the endosome fuse with digestive vesicles known as lysosomes resulting in the degradation of endosomal contents by various enzymes present in the lysosomes. Following the endosomal release step, the complexed DNA enters the cytosol and must take its way to the nucleus without being degraded by the nucleases to access the cell's transcription machinery. The nuclear membrane serves as a critical barrier in the nuclear

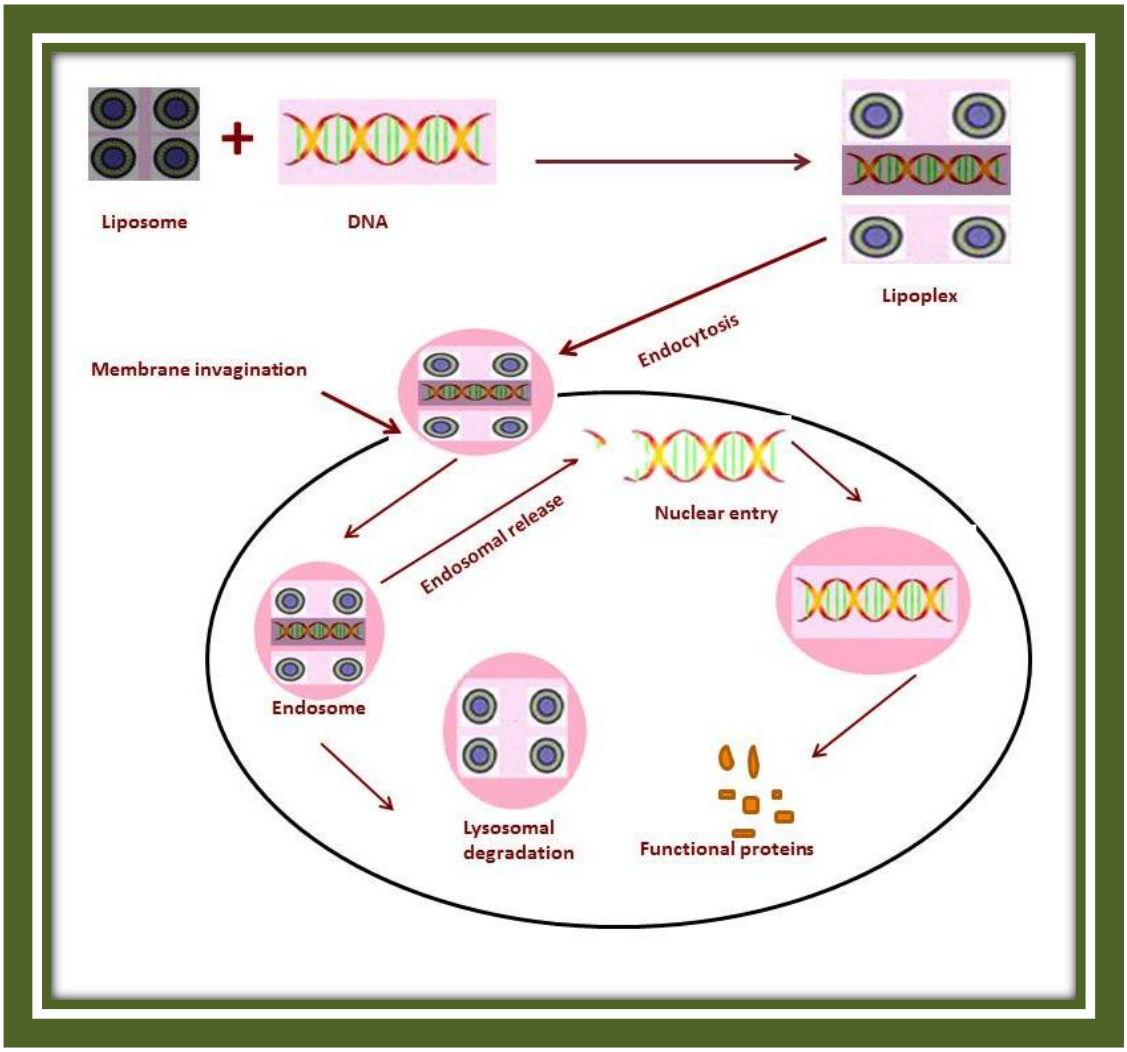


Figure 2.1: Lipofection pathway

delivery of plasmid DNA. Zanta *et al*., 1999 reported that single nuclear localization signal peptide is sufficient to carry DNA to the cell nucleus.

2.8 Structural characteristics of cationic transfection lipids

The molecular architecture of cationic transfection lipids consists of a hydrophobic domain, linker functionality and cationic head-group (Figure 2.2).

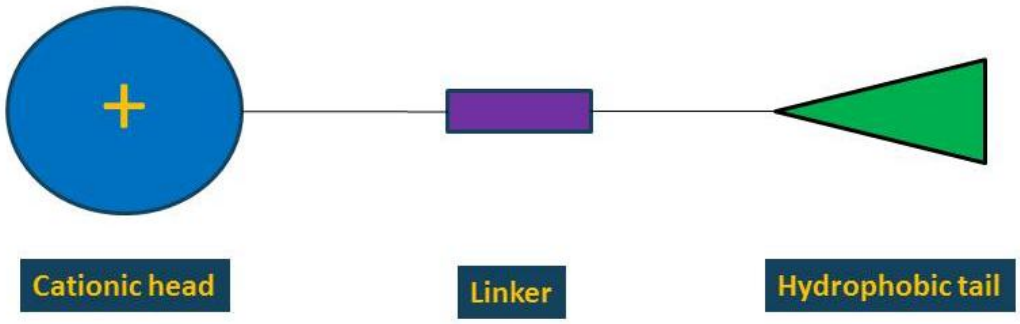


Figure 2.2: Molecular architecture for cationic lipids

Hydrophobic domain: The hydrophobic domains of cationic transfection lipids are mainly either steroid or aliphatic hydrocarbon chains. Examples of cationic amphiphiles containing the steroid hydrophobic domains are DC-Chol, BGTC (bis-guanidinium-tren- cholesterol), vitamin D, cholestane, litholic acid etc. (Fujiwara *et al*., 2000). The aliphatic hydrocarbon chains frequently contain lauryl (C12:0), myristyl (C14:0), palmityl (C16:0), stearyl (C18:0), and oleyl (C18:1) groups. Structure-activity investigations reported by various groups (Heyes *et al.*, 2002; Banerjee *et al*., 2001) suggest that lipids comprising lauryl, myristyl, and palmityl chains exhibit maximum in vitro transfection efficacies while stearyl chains are better for *in vivo* transfection (Majeti *et al*., 2004).

Head-group Domain: The hydrophilic head group domains in cationic lipid consist of quaternary ammonium group, polyamine moieties, guanidium salts, amino acids, hydroxyl groups, a heterocyclic moiety, etc. (Heyes *et al*., 2002; Niculescu-Duvaz *et al*., 2003; Mukherjee *et al*., 2008).

Linker Bonds: The relative orientation of the cationic head group and hydrocarbon anchor is governed by the nature of linker bond bridging them. The linker group can influence the gene transfer efficacy of a cationic transfection lipid. It controls the conformational flexibility, degree of stability, and biodegradability. Most commonly used linker groups include ethers, esters, carbamates, amides, carbonates, phosphonates, disulfides, etc. (Ghosh *et al*., 2002; Rajesh *et al*., 2007).

Present thesis

2.9 Steroid hormone receptors in cancer: a target for cancer therapeutics

Steroid hormone receptors are found on the plasma membrane, in the cytosol and also in the nucleus of target cells. They are generally intracellular receptors (typically cytoplasmic) and initiate signal transduction for steroid hormones which lead to changes in gene expression over a time period of hours to days. Steroid hormone receptors are members of the nuclear receptor family, a large group currently totalling approximately 150 different proteins, which function as transcription factors in many different species including both invertebrates and vertebrates. In addition to steroid hormone receptors, the nuclear receptor family consists of receptors for retinoids, thyroid hormone, fatty acids and prostaglandins and a number of so-called orphan receptors, the ligands of which have yet to be identified (White & Parker 1998). Steroid

hormones interact with receptor proteins found in the cytoplasm of target tissues. Steroid hormones (SHs) reach their target cells via the blood, where they are bound to carrier proteins. Because of their lipophilic nature it is thought that they pass the cell membrane by simple diffusion, although some evidence exists that they can also be actively taken up by endocytosis of carrier protein bound hormones (Hammes *et al*., 2005). For a long time it has been assumed that binding of the ligand resulted in a simple on/off switch of the receptor. While this is likely the case for typical agonists like estrogen and progesterone, this is not always correct for receptor antagonists. These antagonists come in two kinds; partial antagonists and full antagonists. The partial antagonist can, depending on cell type, act as a SHR agonist or antagonist. In contrast, full antagonists always inhibit the receptor, independent of cell type, in part by targeting the receptor for degradation. Binding of either type of antagonist results in major conformational changes within the ligand binding domain (LBD) and release from heat shock proteins that had protected the unliganded receptor from unfolding and aggregation (Griekspoor *et al*., 2007). So, the steroid hormone receptors (SHRs) are ligand-dependent intracellular transcription factors that are known to influence the development and growth of many human cancers. Disruptions in physiological functions of SHRs leads to several types of malignancies such as breast cancer, leukemia and lymphoma, prostate cancer, ovarian cancer, and lung cancer among others. Steroids/hormones/SHRs and their co-regulators have opened up a unique window for novel steroid-based targeted therapies for cancer. Thus, deregulation of SHR signaling in cancers compared with normal tissues can be exploited to target drugs that prevent and treat human cancers (Ahmad & Kumar, 2011).

Steroid hormone receptor (SHR) protein regulates many important functions in almost all cell types including cancerous and non-cancerous cells. In recent years, the mechanisms of action of mineralocorticoids and glucocorticoids have been an area of extensive study in the face of the apparent paradox that despite acting through very closely related receptors and a common DNA response element, these hormones exert significantly diverse physiological effects in a tissue-specific manner.

The phenomenon of Glucocorticoid receptor (GR)-mediated transactivation and repression is universal because of high sequence homology of GR among species, and, among all nuclear hormone receptor superfamily members, there is remarkably high homology in GR DNA-binding domain and Ligand-binding domains (LBD) irrespective of species (Oakley *et al*., 1999; Pratt & Toft, 1997; Ducouret *et al*., 1995). Mukherjee *et al.,* 2009 has reported that there was GR LBD in human cancer cells and normal cells exhibit differential affinity toward the same glucocorticoid ligand. Therapeutically, GR is an important target. In association with its synthetic ligand dexamethasone (Dex), it is involved in the control of metabolism. It is not only involved in regulation of development, inflammation, cell growth, proliferation, and differentiation, but also in inhibition of hypoxia-inducible factor-1, leukemia, prostate cancer, etc. (Yamamoto, 1985; Rogartsky *et al*., 1997; Leonard *et al*., 2005;Nishimura *et al*., 2001).

Similar to this recent GR-related study we naturally felt interested to utilize another cytoplasm-residing ligand-unbound Nuclear Hormone Receptor (NHR), mineralocorticoid receptor (MR). It has been shown that the HEL (human embryonic leukemia) cell proliferation is modified by the MR-specific, synthetic, spironolactones (RU 26752, ZK 91587), which clearly suggests the presence of a functional MR and

amiloride-sensitive sodium channel in leukemic cells, much as in a number of other cell types (Mirshahi *et al*., 2000). Thus, cell signalling by mineralocorticoids may be causally linked to cell proliferation and may even form an exploitable target for the control of cell mass in cancer. A number of cell signalling mechanisms may form possible targets for the mineralocorticoids. The present research is aimed towards extending this new concept of using MR ligands like spironolactone and/or eplerenone for effective delivery of genetic cargoes in cancer cell-selective manner.

2.10 Mineralocorticoid-receptor mediated targeting to human cancer

Mineralocorticoid receptors (MRs) are nuclear hormone receptors that are ubiquitously present in all cell types and are known to mediate distinct physiological functions like regulating Na^+ and K^+ balance and water excretion. The role of mineralocorticoids in controlling sodium and potassium transport in epithelial cells (Pearce *et al.*, 2003) and in non-epithelial tissues, such as cardiac myocytes, blood vessels, the hippocampus and adipose tissue (Lombes *et al*., 1992; Meijer, 2002; Caprio *et al*., 2007) is well known. MRs are linked to cell proliferation and can be exploited for the targeted control of cell mass in cancer. In recent years, the role of corticoids in cancer development and progression has been an area of extensive research. Among them, the mechanism of action of glucocorticoids has been extensively studied, but relatively little is known regarding the role of mineralocorticoids in cancer therapy.

Previously, Glucocorticoid receptor (GR) has been targeted by directly incorporating Dexamethasone (Dex) alongside the regular co-lipid cholesterol (Chol) in the cationic lipid–associated gene delivery formulation (Mukherjee *et al.*, 2009). Similarly, we incorporated Spironolactone (Spiro, the synthetic Mineralocorticoid Receptor-ligand) in cationic lipid formulation so that the cationic lipid/DNA complex

(lipoplex) would be able to target cellular MR (mineralocorticoid receptor) by its binding to MR-LBD (ligand binding domain) and safely localize the Spiro-associated–lipoplex and the genetic cargo inside the nucleus.

The human MR, of 984 amino acids, has 57% amino acid identity with GRα in the ligand binding domain (LBD), and 94% in the DNA binding domain (DBD). The MR LBD is a complex and multifunctional domain that spans 251 aa. It is relatively conserved among steroid receptors (~55% homology) and highly conserved across species (80-97% homology), and allows selective hormone binding, thus transducing endocrine messages into specific transcriptional responses. The MR LBD crystal structure has recently been solved, thus confirming the remarkable similarity in structure among all steroid receptors (SR). Finally, on the basis of the high similarity between the LBD of MR and GR, and considering the evolutionary tree of this receptor subgroup, it has been proposed that MR was closer to the primordial ancestral corticosteroid receptor (Hu & Funder, 2006) which has been proposed as having high affinity for aldosterone, well before the hormone appeared (Bridgham *et al*., 2006; Viengchareun *et al*., 2007).

MR/GR/PR/AR in the unliganded state are associated with a complex of chaperone proteins, including the heat shock protein Hsp-90 and the immunophilin Hsp-56, which maintain the receptors in an inactive form with high affinity for hormone. Upon appropriate ligand binding, the associated chaperone proteins are shed, exposing nuclear localization signals in MR and GR, which are thus enabled to access and be retained in the nucleus (Funder, 1997).

As GR antagonist, dexamethasone due to its similarity with cholesterol formstable Cationic Liposome (Mukherjee *et al*., 2009), our hypothesis was

Spironolactone's structural similarity with cholesterol (chol) can also be utilized for associating it in new CL formulation.

Mukherjee *et al*., 2009 have shown that GR ligand, dexamethasone (Dex) in association with cationic liposome (termed as *targeted lipoplex*) could selectively manipulate GR in cancer cells alone for the delivery of transgenes in the nucleus, a phenomenon that remained unobserved in normal cells. Among many new observations, the targeted lipoplex showed GR-targeted transfections in all cancer cells experimented and most importantly, elicited specific nuclear translocation of targeted lipoplex in cancer cells, followed by up-regulated transactivation of glucocorticoid response element (GRE)–promoted gene.

In the given scenario, we hence hypothesize that Similar to GR targeting this new MR-ligand-associated formulation can target cancer cells via MR and transactivate MRE-genes selectively in cancer cells. Observations made by Sonder *et al*., 2006 provide new insight into the apoptotic effect of Spironolactone. However, MR-antagonism contrastingly protects the vasculature from aldosterone-induced vascular apoptosis and structural injury via rescuing protein kinase B activation. Although the anticancer effect of spironolactone is not clear and ambiguous, but in this regard, we hypothesize that after successful gene delivery through spironolactone associated lipoplex, spironolactone-mediated apoptosis in cancer cells can be induced for tumor regression.

Similar to spironolactone, another MR antagonist, Eplerenone, which is known to have high affinity towards MR, can also be utilized for stable liposome formulation. Eplerenone has a very low affinity towards other steroid hormone receptors, especially GR, which renders it to be a suitable candidate for targeted therapeutics.

3.1 Chemicals and Reagents

Lipofectamine 2000 was obtained from Invitrogen Corporation (Carlsbad, CA). Cholesterol, RU486 (mifepristone), cell culture medium and reagents, penicillin, streptomycin, kanamycin, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] and DMSO were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Fetal bovine serum was purchased from Lonza. Spironolactone was obtained from the drug aldactone (RPG Life Sciences Ltd., Ankleshwar, India) and Eplerenone isolated from the drug Eptus 25 (Glenmark Pharmaceuticals Ltd, Mumbai, India).

3.2 Cell Culture

A549 (lung cancer metastatic), MCF-7 (breast cancer), B16 (mouse melanoma) HEK293 (human embryonic kidney) and CHO (Chinese hamster ovary) cells were purchased from National Center for Cell Sciences (Pune, India). All the cells were mycoplasma free. Cells were cultured in DMEM medium containing 10% fetal bovine serum (Sigma Chemical, St Louis, MO), penicillin 50 mg/lt, streptomycin 50 mg/lt, kanamycin acid sulphate 100 mg/lt and 3.7gm/lt sodium bi-carbonate solution. Cultures of 85–90% confluency were used for all the experiments. The cells were trypsinized, counted, subcultured in 96-well plates for transfection and viability studies. The cells were allowed to adhere overnight before they were used for experiments.

3.3 Antibodies and siRNA

Bcl-2 (MA5-14937), BAX (PA5-17806), active caspases 9 (PA1-26434), active caspases 3 (PA1-29157) and cytochrome c (PA1-9586) were purchased from Pierce

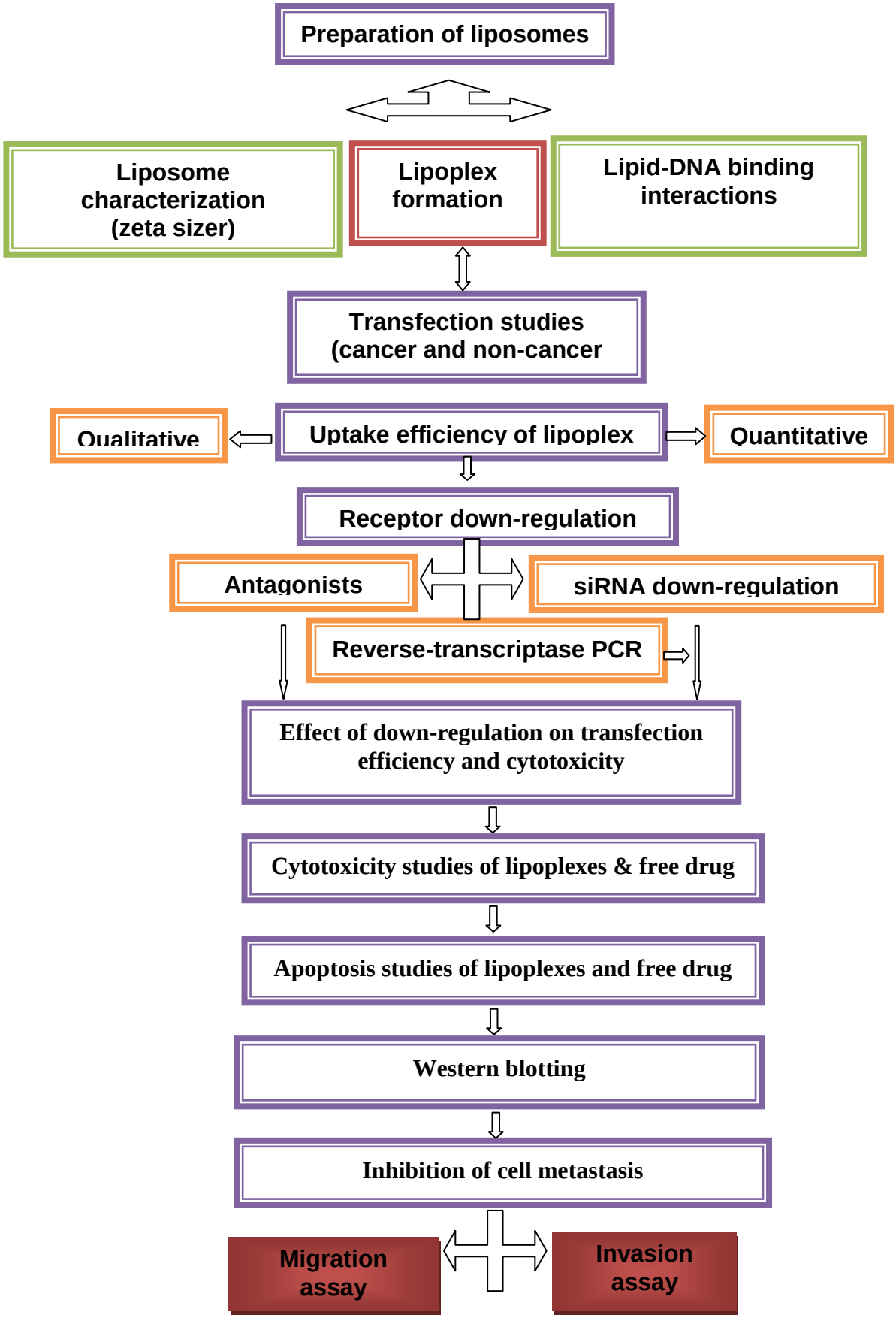
Protein Research, USA; GAPDH was purchased from Cell Signaling Technology; Goat anti-mouse IgG Alkaline Phosphatase (DC05L) was purchased from Calbiochem, Germany.MR siRNA and scrambled siRNA were purchased from Santa Cruz Biotechnology.

3.4 Transmembrane inserts & ECM gel

Transmembrane inserts for migration and invasion assay were purchased from Corning. ECM (extracellular matrix gel) for coating onto inserts was puchased from Sigma-Aldrich Inc. (St. Louis, MO, USA).

3.5 Isolation of Spironolactone and Eplerenone

- Spironolactone was isolated from Aldactone tablets (50mg by spironolactone weight). Tablets were crushed and taken in 10ml water.
- Drug was extracted by dichloromethane (2x25ml).
- Upon evaporation of non aqueous layer, free drug was crystallized out in methanol at -20ºC.
- Purity and authencity of crystallized compound (white needle, 48mg) was characterized by TLC and melting point analysis.
- Eplerenone was isolated from Eptus tablets (25mg by weight) by the same method as Spironolactone.


Preparation of liposomes
Liposome characterization (zeta sizer)
Lipoplex formation
Lipid-DNA binding interactions
Transfection studies (cancer and non-cancer
Qualitative
Uptake efficiency of lipoplex
Quantitative
Receptor down-regulation
Antagonists
siRNA down-regulation
Reverse-transcriptase PCR
Effect of down-regulation on transfection efficiency and cytotoxicity
Cytotoxicity studies of lipoplexes & free drug
Apoptosis studies of lipoplexes and free drug
Western blotting
Inhibition of cell metastasis
Migration assay
Invasion assay

3.6 Preparation of Liposomes

- Liposomes were prepared by the method described by Reddy & Banerjee, 2005. Thin lipid films were prepared by drying the chloroform solution of 1µM of DODEAC, 1µM of cholesterol and three different concentrations of spironolactone and Eplerenone i.e. 1µM, 0.75 µM and 0.50 µM for three different liposomal formulation i.e.1:1:1, 1:1:0.75, 1:1:0.50 (Table 3.1) under a gentle stream of nitrogen and dried in vacuum for at least 4 hours.
- It was hydrated with 1ml of sterile water overnight and then first vortexed to remove any adhering lipid film, then subjected to a low intensity bath sonication for 15 minutes at room temperature and finally probe sonication for 2 minutes using a constant duty cycle and output control magnitude of 2-3 Branson Sonifier 450. The liposomes are 1mM with respect to the cationic lipid.

Table 3.1 Liposomal formulations with lipid concentrations

Liposomal formulation	**Cholesterol Concentration (µM)**	**DODEAC Concentration (µM)**	**Spironolactone/Eplerenone Concentration (µM)**
SP(M1)/EP(M1)	1	1	1
SP (M2)/EP (M2)	1	1	0.75
SP (M3)/EP(M3)	1	1	0.50
DO	1	1	-

3.7 Isolation of plasmid DNA

- pCMV- SPORT-β-gal plasmid was used for transfections and was isolated by usual alkaline lysis procedure and purified by PEG-8000 precipitation as described by Majeti *et al*., 2004.
- The purity of plasmid was checked by A260/A280 and 1% gel electrophoresis.

3.8 Preparation of lipoplex and its treatment to cells

- The lipid-DNA complex or lipoplex was prepared according to the protocol described by Mukherjee *et al.,* 2009. Briefly, for in vitro studies related to toxicity and gene transfection studies,1mM liposomes were serially diluted in serum free media in final volume of 50 ml and to it fixed amounts of pDNA (0.3 µg/well of 96 well plates) diluted in 50 ml of serum free media were added.
- The charge ratios of cationic lipid to DNA were maintained as 1:1,2:1, 4:1 and 8:1.
- The mixtures were shaken in room temperature for 15 min following which 10% serum containing media (200 ml) were added to each mixture.
- Finally, 100 ml of resulting solution was then added to triplicate wells of cell containing 96-well plate.
- For RT-PCR and western blot studies, the amount of pDNA used per well in 6-well plates was 2 µg and was lipoplexed in a cationic lipid to DNA(+/-) charge ratio of 4:1.

3.9 Gel retardation of lipoplexes

- The DNA binding ability of the cationic lipids were assessed by gel retardation assay on a 1% agarose gel.
- 0.3 μg of *p*CMV- SPORT-β-gal was complexed with cationic lipids (1:1:0.75, molar ratios were mixed with DNA at final charge ratios of 8:1, 4:1, 2:1 & 1:1) in a total volume of 25μL in Hepes buffer, pH 7.4 and incubated at room temperature for 20-25 minutes.
- 4 μL of 6X loading buffer (0.25% bromo phenol blue, 40% sucrose) was added to it and 20 μL of the resultant solution was loaded in each well.
- The lipoplexes were run on 1% agarose gel, electrophoresed at 80 V for approximately 2h and the DNA bands were visualized by ultraviolet illumination.

3.10 Determination of Zeta potential, particle size, and polydispersity index of liposomes

- Values of the zeta potential of liposomes indirectly reflect vesicle surface net charge and can therefore be used to evaluate the extent of interaction of the liposomal surface cationic charges with the anionic charges of DNA.
- The average particle size and the polydispersity of the particle-size distribution of the liposomes were determined by Zeta sizer. Empty liposomes and naked plasmid were used as controls.

3.11 Gene transfections with β-gal plasmid

- Cells were seeded at a density of 10,000 cells per well in a 96-well plate usually 18-24 hours before transfection.
- Transfection of cell was performed by method described by Reddy & Banerjee, 2005. 0.3 µg of β-gal (diluted to 50µl with plain DMEM) was complexed with varying amount of cationic liposomes (diluted to 50µl with plain DMEM) for 15-20 minutes. The molar ratios (lipid:DNA) were 8:1, 4:1, 2:1, 1:1.
- After formation of lipoplexes, 200µl of DMEM containing 10% FBS (CMIX) was added to the resulting lipoplexes for triplicate experiments.
- Cells were washed with phosphate buffer saline (PBS), pH 7.4 (1×100µl) and then treated with lipoplex (100µl).
- After incubation of the cells at a humified atmosphere containing 5% CO_2 at 37ºC for 4 hours, the media was completely removed and 100 µl of DMEM containing 10%FBS (CMIX) were added to the cells.
- The reporter gene activity was assayed after 48 hrs. The media were completely removed from the wells and the cells were lysed with 50µl of 1X lysis buffer (0.1% NP-40) for 25-30 minutes. The cell lysates were assayed by taking the β-galactosidase readings and comparing the protein content of them with that of BSA.
- For RU486 and Eplerenone pretreated experiments, cells were treated with RU486 and Eplerenone (in dimethyl sulphoxide) at a final concentration of 100µmol/lt for 2 hours (Mukherjee *et al*., 2009).

- Cells were incubated at a humified atmosphere containing 5% CO_2 at 37ºC. Media were removed and the cells were washed with PBS (1×100µl). The cells were subsequently treated with the lipoplexes and reporter gene assay was performed according to the above-mentioned procedure.
- The transfection values were reported as the average values of the triplicate experiment performed in the same plate on the same day.
- To verify reproducibility, each transfection experiment was performed at least 4 times.

3.12 Cytotoxicity studies

- Cytotoxicities of the liposomes were evaluated by the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) reduction assay (Mosmann, 1983).
- Briefly, cells were seeded at a density of 10,000 cells/well in a 96-well plate usually 18–24 h before experiment. Treatments were done to triplicate wells. Cells were treated with respective liposomes for 48 hours.
- Following the termination of experiment cells were washed and promptly assayed for viability using MTT.
- Results were expressed as percent viability = [A550 (treated cells)-background/A550 (untreated cells)-background] × 100

3.13 Cellular uptake assay

- Cells were plated at a density of 10,000 cells/well. After 16-20 hours, cells were first pre-treated with RU486 for 2 hours or kept untreated.

- Then cells were treated with lipoplexes comprising red-fluorescent rhodamine-labeled lipid following the same protocol used for transfection (Reddy & Banerjee, 2005). DO and SP lipoplexes were made at lipid:DNA molar ratios of 8:1 and 4:1.
- Following the lipoplex treatment of 4 hours, cells were washed three times with PBS, then lysed with 100µl of 1× lysis buffer (0.1% NP-40) for 30 minutes, and the fluorescence of different treated groups were measured by a microplate fluorescence reader (FLX 800; Bio-Tek Instruments, Winooski, VT).
- The fluorescence of lipoplexes with the same amount of cell lysates (in a total volume of 100µl) were also measured and considered as total or 100% fluorescence.
- The percentage of uptake was calculated using the formula: percentage uptake=100× (fluorescence intensity of the fluorescence lipoplex-treated cell lysate – background)/ fluorescence intensity of lipoplex added to the cells – background).

3.14 Apoptosis study

- The annexin V-FITC- labeled apoptosis detection kit (Sigma) was used to detect and quantify apoptosis by flow cytometry as per manufacturer's protocol.
- In brief, cells (2×10^5cells/well) were seeded in 6-well plates and cultured over night in 10% fetal bovine serum media.
- After 16-18 h cells were either kept untreated or treated with targeted and non-targeted liposomes for 24 hours. Then the cells were harvested and collected by centrifugation for 5 minutes at 1000 rpm.

- Cells were then resuspended at a density of 1 x 10^6 cells/ml in 1X binding buffer and stained simultaneously with FITC- labeled Annexin V (25 ng/ml) and propidium iodide (50 ng/ml).
- Cells were analyzed using a flow cytometer (FACS Canto II) and data were analyzed with FCS Express V3 software. A minimum of 10,000 events were gated per sample.

3.15 Western blot studies

- MCF-7 cells were grown in T-25cm^2 flasks. After 16-18 h cells were either kept untreated or treated with targeted and non-targeted liposomes for 24 hours.
- Whole cell lysates from treatment groups were prepared as follows: Cells were lysed with cold buffer A (20 mM HEPES, pH 7.9, 10 mM NaCl, 3 mM $MgCl_2$, 0.1% Nonidet P-40, 10% glycerol, 0.2 mM EDTA, 1 mM DTT and 0.4 mM PMSF), which was premixed with 1× Protease inhibitor cocktail antipain and leupeptin (Calbiochem).
- Respective cell lysates were run in SDS-PAGE gel (8%–15%) and then the bands were transferred to nitrocellulose membrane and immunoblotted (Pore *et al*., 2013).
- Antibody-reactive bands were detected by BCIP/NBT substrate (Sigma–Aldrich) as alkaline phosphate conjugated secondary antibodies (Goat-anti-rabbit and Goat-anti-mouse) were used.

3.16 Mineralocorticoid Receptor Downregulation by siRNA Delivery

- siRNA based mineralocorticoid receptor down-regulation was performed following the procedure described by Pal *et al*., 2011.
- In brief, A549 and CHO cells were seeded at a density of 2×10^5 cells per well in a 6-well plate usually between 18-24 h before transfection.
- For dose optimization, 25pmol, 50pmol and 100pmol of MRsiRNA (Santa Cruz Biotech) or equal concentrations of scrambled siRNA (Santa Cruz Biotech) (diluted to 50µl with serum free DMEM) was complexed with 0.5 µl of Lipofectamine2000^{TM} (Invitrogen) (diluted to 50µl with serum free RPMI) for 15 minutes. 900µl serum free DMEM was added to the resulting complex.
- Cells were washed with phosphate-buffered saline (PBS) and then treated with respective complexes.
- After 4 h incubation cells were harvested, counted and again seeded at a density of 10,000 cells per well in 96-well plates for cytotoxicity assay.
- After 16-18h, cells were either kept untreated or treated with liposomal formulations for 4h. After 4h treatment, the cells were washed and kept in presence of fresh cell culture medium for 48 h.
- The cells were then assayed for viability using MTT as described earlier.

sc-38836: MR siRNA(h) is a pool of 3 different siRNA duplexes:

sc-38836A:

• Sense: GCAUCAGUCUGCCAUGUAUTT

• Antisense: AUACAUGGCAGACUGAUGCTT

sc-38836B:

• Sense: GAAGAACUUUGCCUUAAGUTT

• Antisense: ACUUAAGGCAAAGUUCUUCTT

sc-38836C:

• Sense: GCAAGACAGUGGCACUAAATT

• Antisense: UUUAGUGCCACUGUCUUGCTT

3.17 RT-PCR (reverse transcriptase-PCR) analysis

- Reverse transcriptase PCR (RT-PCR) was performed to measure siRNA down-regulation after 24 hours, 48 hours and 72 hours.
- A549 and CHO cells were seeded at a density of 2×10^5 cells per well in a 6-well plate usually between 18-24 h before transfection.
- Cells were treated with 25pmol, 50pmol and 100pmol of MR-siRNA or equal concentrations of scrambled siRNA (Santa Cruz Biotech) (diluted to 50µl with serum free RPMI) was complexed with 0.5 µl of Lipofectamine2000^{TM} (Invitrogen) (diluted to 50µl with serum free DMEM) for 15 minutes. 900µl serum free DMEM was added to the resulting complex.
- After the termination of treatments (24, 48 and 72 hours), treated and untreated cells were lysed directly in 6-well plates with 1 ml of TRIzol® reagent (Invitrogen) following removal of media and washing with PBS (1 ml).
- The cells were dissolved in Trizol and cell lysates were pipetted several times to clear the haziness. Cell lysates were added with 0.2 ml chloroform, shaken

vigorously for 15 seconds, incubated for 10 minutes at RT and centrifuged at 14,000 rpm for 15 minutes at 4^{o}C.

- Following centrifugation RNA was exclusively isolated in the aqueous phase and was transferred in to a fresh tube; 0.8 ml of isopropanol was added and mixed with pipette, kept for 15 minutes at –20^{o}C and centrifuged at 20,000 rpm for 20 minutes at 4^{o}C to get RNA as white pellet.
- The supernatant was removed and the RNA pellet was washed with 500 µl cold 75% ethanol. Then it was centrifuged at 20,000 rpm for 10 minutes at 4^{o}C, ethanol aspirated out and the pellet air dried.
- Finally the RNA pellet was dissolved in 15 µl of DEPC treated water, measured the OD at 260 and 280 nm and calculated the concentration and purity.
- 260/280 ratio gives the purity (pure RNA 260/280 ratio is 1.8-2.1).
- 4 µg RNA was used for first-strand cDNA synthesis using the first-strand cDNA synthesis kit (Super script III, Invitrogen) with random hexamers provided in the kit in a final volume of 20 µl.
- Briefly, in a PCR tube 4 µl (1µg/µl) of isolated RNA, 1µl of primers and 1µl of dNTP mix were mixed and heated to 65^{o}C for 5 min and cooled to 4^{o}C on PCR machine. In a separate PCR tube 2 µl of 10x RT buffer, 4µl of 25 mM $MgCl_2$, 2 µl of 0.1M DTT, 1µl of RNAse out (40 U/µl) and 1µl of superscript III are mixed.
- Solution of both the PCR tubes were mixed and performed the reverse transcription reaction and synthesized the cDNA at different temperatures 10 min at 25^{o}C, 50 min at 50^{o}C and 5 min at 85^{o}C and 30 min at 4^{o}C.

- Aliquots (2.5 µl) of the cDNA mixture were used to amplify mRNA for MR gene and 18S RNA (as a loading control) by PCR using the PCR SuperMix (Invitrogen, USA). Amplifying solution contains 2.5µl of cDNA + 2µl of mixture of Primers (Forward and Reverse, 25pmol/µl) + 25.5µl of PCR superMix.
- The temperatures cycles used to amplify above genes were as follows: initial denaturation step 94^{o}C (2min), denaturation step 94^{o}C (30 sec), primer annealing step 54^{o}C, elongation step 72^{o}C (30 sec), final elongation temperature 72^{o}C (5min).
- The amplified sequences were resolved on a 2%agarose gel electrophoresis and visualized using 0.1% ethidium bromide under UV light. Detailed sequences of the primers are provided in Table 3.2.

Table 3.2 Human mineralocorticoid receptor (hMR) primers

Primer Set	**Sequence**	**Reference**
18s RNA	Forward: 5'- GCAATTATTCCCCATGAACG -3' Reverse: 5'- GGCCTCACTAAACCATCCAA -3'	Sinha *et al*., 2011
hMR	Forward:5'- GCTTTGATGGTAACTGTGAAGG-3' Reverse: 5'- TGTGTTGCCCTTCCACTGCT-3'	Fiore *et al*., 2006

3.18 Effect of siRNA down-regulation on transfection efficiency

- The remaining cells after seeding from the above experiment were used to evaluate the extent of down-regulation of mineralocorticoid receptor on transfection efficiency. They were plated again and kept for 24 hours. Transfection of cell was performed by method described by Reddy & Banerjee, 2005.
- 0.3 µg of β-gal (diluted to 50µl with plain DMEM) was complexed with varying amount of cationic liposomes (diluted to 50µl with plain DMEM) for 15-20 minutes.
- After incubation of the cells at a humified atmosphere containing 5% CO_2 at 37ºC for 4 hours, the media was completely removed and 100 µl of DMEM containing 10%FBS (CMIX) were added to the cells.
- The reporter gene activity was assayed after 48 hrs. The media were completely removed from the wells and the cells were lysed with 50µl of 1X lysis buffer (0.1% NP-40) for 25-30 minutes.
- The cell lysates were assayed by taking the β-galactosidase readings and comparing the protein content of them with that of BSA.

3.19 Effect of siRNA down-regulation on cytotoxicity

- A549 and CHO cells were seeded at a density of 2×10^5 cells per well in a 6-well plate usually between 18-24 h before transfection.
- 50pmol of MRsiRNA and scrambled siRNA (diluted to 50µl with serum free DMEM) was complexed with 0.5 µl of Lipofectamine2000^{TM} (Invitrogen)

(diluted to 50µl with serum free DMEM) for 15 minutes. 900µl serum free DMEM was added to the resulting complex.

- Cells were washed with phosphate-buffered saline (PBS) and then treated with respective lipoplexes.
- After 4 h incubation cells were harvested, counted and again seeded at a density of 10,000 cells per well in 96-well plates for cytotoxicity assay.
- Cytotoxicity was checked using MTT assay as described in previous section.

3.20 Migration assay

- For migration study, B16F10 cells, which are known to be very aggressive in terms of invasiveness and migration, were plated on 24-well transwell inserts (Corning).
- Cells were plated at a density of 50,000/well. After 18-24 hrs of plating, cells were left untreated or treated with lipoplex (SP, EP & DO) as described earlier and incubated.
- After 48 h of treatment, cells in the lower compartment were counted.

3.21 Invasion assay

- For invasion assay, 24 well transmembrane inserts were coated with 50-100ul of ECM gel (Sigma). Gel was thawed overnight at 2-8^{o}C overnight.
- Gel was dispensed to wells of a 24 well plate using pipettes, plates, etc. pre-cooled to 2-8°C.
- Cells were then plated on top of a thin layer of ECM.

- After 24 h, cells were either left untreated or treated with lipoplex (SP, EP & DO). 48h post-treatment, cells in the lower compartment were counted.

Cancer cells grow and divide more rapidly than normal cells. Many chemotherapeutic drugs are available to kill growing cells and hence used to retard the tumor growth. But adverse and prolonged chemotherapy-induced drug resistance limits the use of chemotherapy. Chemotherapy results in various side effects including hair loss, fatigue, weight loss, mouth sores, nausea, vomiting, easy bruising, bone marrow suppression and heart muscle damage. Not all chemotherapy is the same; some types offer fewer side effects than others.

Breast and lung cancer are the most frequently diagnosed malignancies and leading cause of cancer-related deaths. The local or distant metastases are very much common in most diagnosed patients. However, metastatic tumor cells depict the advanced state of disease. Despite several hundred clinical studies of both experimental and approved chemotherapeutic agents, low dose chemotherapy has limited antitumor activity, with 1-2% survival benefit for adjuvant paclitaxel (Antman, 2001). However, high dose therapy has an overall advantage of 15% disease free survival and a 10% survival benefit (Hudis *et al*., 1999).

Design of an efficient cationic transfection lipid needs strategies to deal with multitude of cellular barriers. Currently, lipoplex (lipid:DNA complex) mediated intracellular transfection pathways involve: (a) formation of lipoplex and their initial binding to the cell surface; (b) endocytotic internalization of the lipoplex; (c) trafficking in the endosome/lysosome compartment and escape of DNA from the endosome/lysosome compartment to the cell cytoplasm; (d) transport of the endosomally released DNA to the nucleus followed by its transgene expression (Zabner, 1995; Friend, 1996; Xu & Szoka, 1996). Thus, rational design of efficient cationic transfection lipids are often based on covalent grafting of various structural

elements (into the molecular architecture of the cationic lipids) capable of favourably modulating one or more of these mechanistic steps involved in lipofection pathways (Kumar *et al*., 2003; Zhu *et al*., 2000; Budker *et al*., 1996; Reddy *et al*., 2002). A particularly elegant design of efficient cationic transfection lipid was pioneered by Vigneron, *et al.*, (1996) in which guanidinium ions were used as the polar head-group of cholesterol based cationic amphiphiles. The rationale for using cholesterol as the hydrophobic tail was its membrane-compatible characteristics and its ability to facilitate the cellular uptake of various oligonucleotides and polar drugs. Sen & Chaudhuri, (2005) developed a series of non-cytotoxic cationic transfection lipids with guanidinium head-groups and simple aliphatic hydrocarbon tails which showed high gene transfer efficiencies in multiple cultured animal cells. Immediately after demonstrating the *in vitro* gene transfer activities of these non-cholesterol based guanidinylated cationic amphiphiles, *in vivo* studies aimed at probing their systemic and therapeutic applications was launched.

Cationic lipid-based vehicles (known as lipoplexes) have long been used for the delivery of genetic material into cells (Felgner *et al*., 1987). However, it is widely recognized that the efficiency of nucleic acid delivery achieved using lipoplexes is limited, with the further problem that the cationic lipids employed are toxic to cells (Lv *et al*., 2006). In an attempt to increase the transfection efficiency, at the same time mitigating the toxicity owing to the cationic lipids, neutral 'helper' lipids such as cholesterol and dioleoylphosphoethanolamine (DOPE), are added to the lipid vesicles used in the preparation of lipoplexes (Koltover, 1998; Zuhorn *et al*., 2003). Helper lipid has great impact on the behaviour of liposome under both *in vitro* and *in vivo* condition. Nie *et al*., (2012) reported anti-cancer effect from charged cholesterol liposome with/without PEGylation for the first time.

In the present study, [N, N-di-n-tetradecyl-N, N-(2-hydroxyethyl) ammonium chloride (DODEAC)] was used as cationic lipid in association with helper lipid, cholesterol and mineralocorticoid ligand, spironolactone or eplerenone for liposome formulation. The liposomes were associated with reporter gene (β-gal) and the resulting lipoplexes were used to transfect cells. The lipoplexes were found to be stable as shown by DNA binding assay on agarose gel. Since we are trying to target cancer cells, transfection efficiency was tested in cancer cells as well as normal cells. Transfection efficiency was found to be high in cancer cells, particularly, A549 cells, as compared to normal cells. Receptor antagonists were used to ascertain the role of specific receptor in targeting cancer cells. On receptor inhibition by specific antagonists, transfection efficiency decreased considerably proving the role of receptor in transfection on cancer cells while no effect was observed on normal cells. SP and EP targeted delivery of genes resulted in apoptosis in cell-specific manner while free drug was found to be cytotoxic irrespective of the cancerous or non-cancerous nature. In the present study, mineralocorticoids were used for the formulation of efficient and targeted liposome for the delivery of anticancer gene.

4.1 LIPOSOME PREPARATION

4.1.1 Isolation of drugs & preparation of liposomes

Mineralocorticoid Receptor ligands spironolactone and eplerenone (synthetic ligands) were extracted from Aldactone and Eptus tablets respectively using dichloromethane (DCM) as described in Materials & Methods. Characterization of the extracted drugs was done by TLC analysis (Fig.4.1). The drugs were found to be pure and hence used for liposome formation as mentioned in section 3.4. The formulations were tested for their stability, size and charge before transfecting cells.

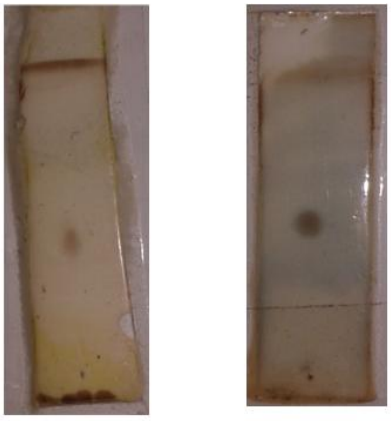


Fig. 4.1 TLC profile of isolated drugs Spironolactone & Eplerenone

4.1.2 Lipid: DNA Binding Interactions

Charge ratio i.e. number of positive charges originating from cationic lipid and number of negative charges from DNA's phosphates of lipoplexes are considered very important physicochemical characteristics for transfection efficiency (Ross & Hui, 1999; Ramezani *et al*., 2009; Liu *et al*., 1997). Toward initial characterization of the lipoplexes, the electrostatic interactions between the plasmid DNA and cationic liposomes as a function of lipid:DNA charge ratios were determined by conventional electrophoretic gel retardation assay for the lipoplexes across the lipid:DNA charge ratios i.e. 8:1 to 1:1 (Table 4.1). The lipid:DNA binding interaction studies demonstrated that liposomal formulation, SP had strong DNA binding characterization across the lipid:DNA charge ratio 8:1 to 2:1 (Fig. 4.2), however, charge ratio 1:1 had poor binding interaction with DNA.

Table 4.1 Charge ratio of different SP lipoplexes for DNA binding studies

Lipoplex	Charge ratio (Liposome:DNA)
SPD8	8:1
SPD4	4:1
SPD2	2:1
SPD1	1:1

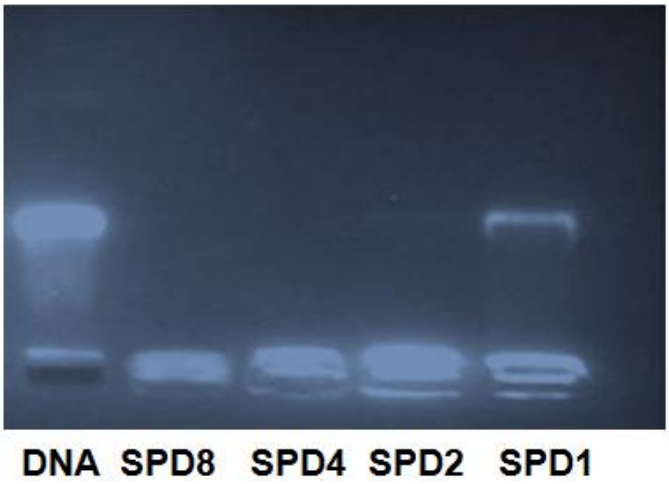


Fig. 4.2 DNA binding assay of liposome-associated plasmid DNA for SP liposomes. The lipid: DNA charge ratios are indicated at the bottom of each well. SPD8, SPD4, SPD2, SPD1 represent lipid to DNA charge ratio of 8:1, 4:1, 2:1 and 1:1 respectively. DNA represents naked plasmid DNA of β-gal.

4.1.3 Liposomes of different compositions & charge ratios have different transfection efficiencies

Cationic liposomes that contain a cationic lipid [N,N-di-n-tetradecyl-N,N-(2-hydroxyethyl) ammonium chloride (DODEAC)], cholesterol and spironolactone in a stable formulation of 1:1:1, 1:1:0.75 and 1:1:0.50 were formulated. Spironolactone is a synthetic steroidal mineralocorticoid which shows structural resemblance with cholesterol and gave a stable formulation with cationic lipid. Lipoplexes were formed with β-galactosidase (β-gal)-expressing reporter gene in varied charge ratios (+/–) and the resulting targeted lipoplexes (SP) were used in transfecting cells of both cancerous and noncancerous nature expressing MR ubiquitously. Liposomes containing lipids with a molar ratio of 1:1:0.75 and a charge ratio of 8:1 showed highest transfection efficiency and therefore selected for further studies (Fig. 4.3). Liposomes have been investigated for over 20 years as delivery systems for nucleic acids, but the process is not fully understood and depends on various physicochemical characteristics of the liposome/DNA, such as size (Ross & Hui, 1999; Almofti *et al*., 2003; Ramezani *et al*., 2009), lamellarity (Zuidam *et al*., 1999), structure (Safinya 2001), charge ratio (Almofti *et al*., 2003; Liu *et al*., 1997) and charge density (Lin *et al*., 2003).

A recent study by Brgles *et al*., 2012 has shown that the reduction of the size and increase in charge ratio of lipoplexes promotes transfection efficiency but unfortunately also induces higher cytotoxicity due to the higher amounts of cationic lipid DOTAP.

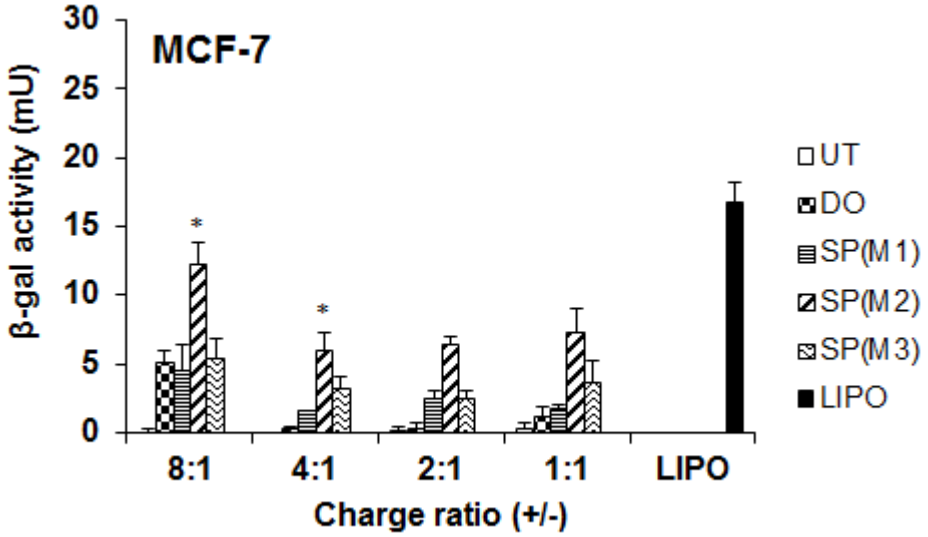


Fig. 4.3 Transfection efficiency in cancer cells with different lipid and charge ratio. Cells were transfected using 0.3µg pCMV-β-gal plasmid in SP lipoplex in different charge ratios (+/-, cationic lipid, x-axis) *$p<0.01$

4.1.4 Zeta potential, particle size, and polydispersity index of liposomes & lipoplexes

Many physicochemical factors influence CL transfection efficiency including the charge of CLs, the size of the lipoplexes, and the total lipid/pDNA ratio. These parameters can affect stability and reproducibility of used liposomes in transfection. Sizes (DO & SP) and zeta potential of liposomes (Table 4.2 & 4.3) were measured by dynamic light scattering. Zeta potential is an indirect measurement of the vesicle

Table 4.2 Size measurement for different formulations

Formulation	Angle	KCpS	Z Ave (nm)	Poly Index
DO	90	395.4	123.0	0.289
SP	90	274.4	194.7	0.524
DO-DNA	90	249.3	503.8	0.492
SP-DNA	90	132.6	436.6	0.463

Table 4.3 Measurement of Zeta potential for different formulations

Formulation	KCpS	Mobility	Zeta	Width
DO	190.64	0.173	4.8	1.6
SP	360.8	1.117	14.2	1.6
DO-DNA	240.1	1.117	2.5	1.6
SP-DNA	138.67	0.228	4.8	1.6

surface charge, and it can be used to evaluate the extent of interaction of the liposomal surface cationic charges with the anionic charges of DNA (Perrie & Gregoriadis, 2000; Eastman *et al*., 1997; Perrie *et al*., 2001).

In general, zeta potential is a function of lipid to DNA ratios, and the structure of lipoplexes with a positive zeta potential is different from lipoplexes with a negative zeta potential. Lipoplexes with positive zeta potential might correspond to the aggregated multilamellar structure (Radler *et al*., 1997). Negative zeta potential leads to free plasmids or protruding DNA-strings (Ma *et al*., 2007).

Effect of lipoplex size on transfection efficiency has not been unified so far. Some consider that there is no apparent correlation of the size of lipoplexes with transfection efficiency (Stegmann & Lagendre, 1997; Hassani *et al*., 2005), others suggest that lipoplex size influence (Rakhmanova *et al*., 2004; Rejman *et al*., 2004) transfection efficiency, even lipoplex size is a major factor (Ross & Hui, 1999; Almofti *et al*., 2003) in terms of lipofection efficiency. So, to know the size of lipoplexes, liposomes were linked with β-gal DNA, incubated and the resulting size of the lipoplexes were measured using dynamic light scattering. Complexation of liposomes with DNA resulted in an increase in the sizes (Fig. 4.4). Lipoplexes of over 700 nm mean diameter induce efficient transfection in the presence or absence of serum (Turek *et al*., 2000).The requirement for efficient transfection may be different in vivo and in vitro. Small particle size (40–80 nm) is required for high efficiency in vivo delivery owing to traversal of the capillary network (e.g. in the lungs), while 200–400 nm is the optimal size for lipoplexes in vitro (Zhadanov *et al*., 2002; Kennedy *et al*., 2000).

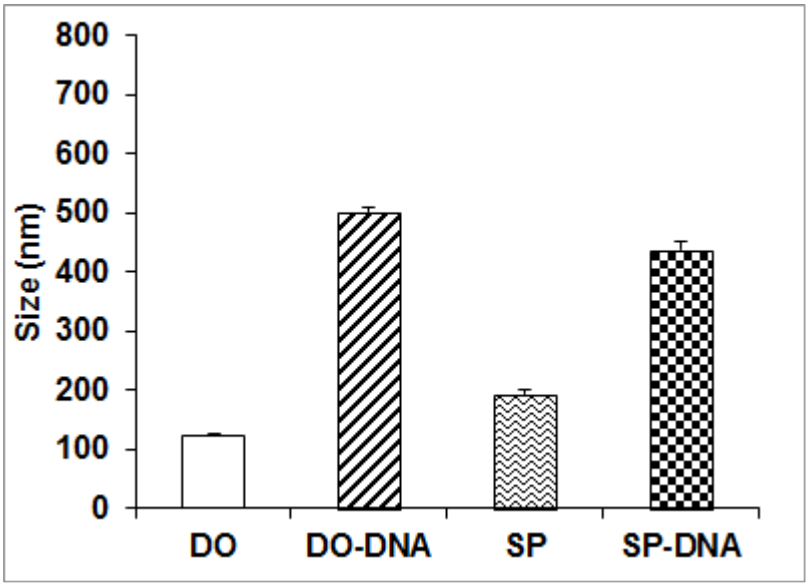


Fig. 4.4 Size measurement for liposomes and lipoplexes by dynamic light scattering

4.2 TRANSFECTION STUDIES

4.2.1 SP Liposome mediated transfections in cancer cells

Among varied formulations, liposomes (SP) containingDODEAC:Cholesterol:spironolactone with a molar ratio of 1:1:0.75 showed highest transfection efficiency and therefore we chose to use this formulation for further studies. Two cancer cells (MCF-7 and A549); and two normal cells (CHO and HEK293) were transfected with SP-lipoplex. SP lipoplex mediated transfections were found to be more efficient in cancerous cells than in non-cancerous cells. Lipoplex with 8:1 charge ratio showed higher β-galactosidase activity in MCF-7 and A549 cell lines. Compared to SP lipoplex, the non-targeted lipoplex (DO) containing equimolar amounts of cationic lipid (DODEAC) and cholesterol but without spironolactone showed much less transfection in cancer cells (Fig. 4.5).

The transfection efficiency decreased with a decrease in charge ratio. Brgles *et al*., 2012 concluded that charge ratio dictates the size of the lipoplex whereas overall size reduction and higher charge ratios promote transfection efficiency in vitro. Mukherjee *et al*., 2009 in their work on GR mediated cancer targeting showed similar kind of results; wherein targeted lipids are transfecting cancer cells in a better manner as compared to non-targeted lipids; also, the lipids have shown no effect on normal cells.

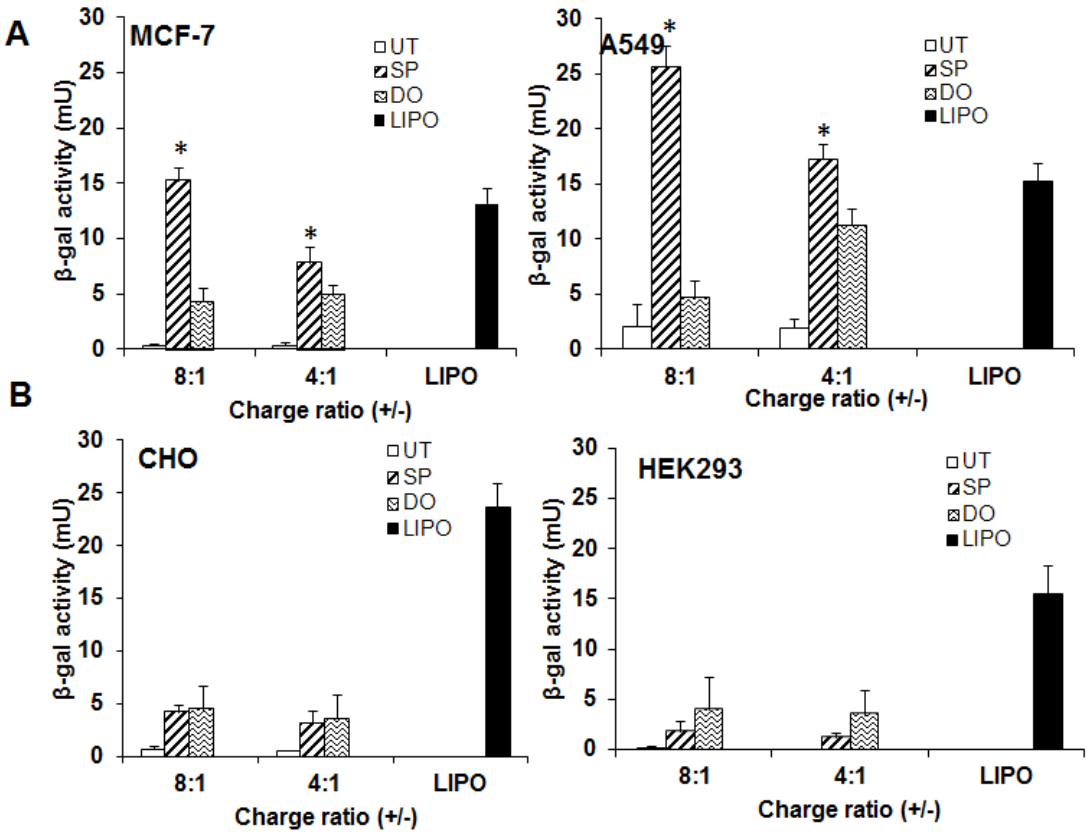


Fig. 4.5 Targeted transfection in cancer cells. Cancer (MCF-7, A549) and non cancer (CHO and HEK293) cells were transfected using 0.3µg pCMV-β-gal plasmid in SP lipoplex in different charge ratios (+/-, cationic lipid, x-axis). 48-hours post-transfection β-galactosidase enzyme in the cellular lysates was assayed (mU, y-axis). The cells were also transfected with Lipofectamine/DNA lipoplex containing same amount of plasmid as a positive control. The results are obtained from the average of triplicate experiments (*$p<0.01$)

4.3 UPTAKE EFFICIENCY OF LIPOPLEX IS EQUAL IN CANCER AND NORMAL CELLS

The difference in the transfection efficiency of cancer and normal cells by SP may be due to the difference in lipoplex uptake by these cells. To this end, SP and DO lipoplex containing rhodamine-linked "red-fluorescent" lipid was treated to both cancer (MCF-7 and B16F10) and normal CHO cells. Following treatment of lipoplex for 4 hours, respective cellular lysates were quantitatively estimated for its red-fluorescence content (Fig. 4.6). It was found that there was no significant difference in lipoplex uptake of respective cells. These findings proved that the lipoplex uptake was unrelated to transfection efficiency. Qualitative estimation of red fluorescent content of B16 cells was also done and no significant difference was found in the uptake efficiency of cells (Fig. 4.7). This data is sufficient to conclude that difference in transfection efficiency was not due to difference in uptake efficiency of the cells as uptake was found to be almost similar. Mukherjee *et al*., 2009 in their GR-mediated cancer cell targeting studies reported similar kind of results where receptor inhibition by GR antagonist RU486 did not lead to any change in uptake efficiency of liposomes.

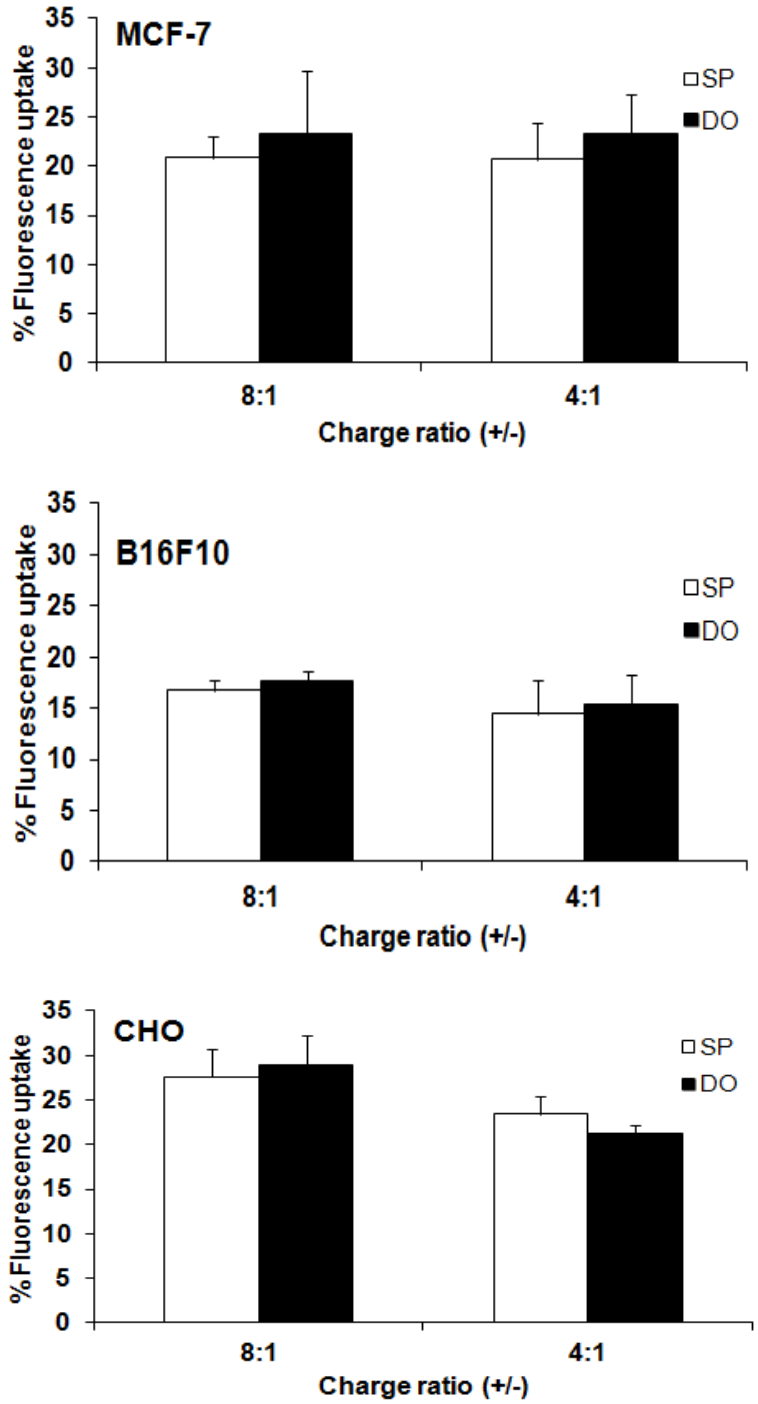


Fig. 4.6 Uptake of SP lipoplex in cells. MCF-7, B16 and CHO cells were treated with rhodamine fluorescent-labeled SP & DO lipoplex in 8:1 and 4:1 (+/-) charge ratio of cationic lipid to DNA. Cellular uptakes of the lipoplexes were measured 4 hours after the treatment. The results are obtained from the average of three experiments.

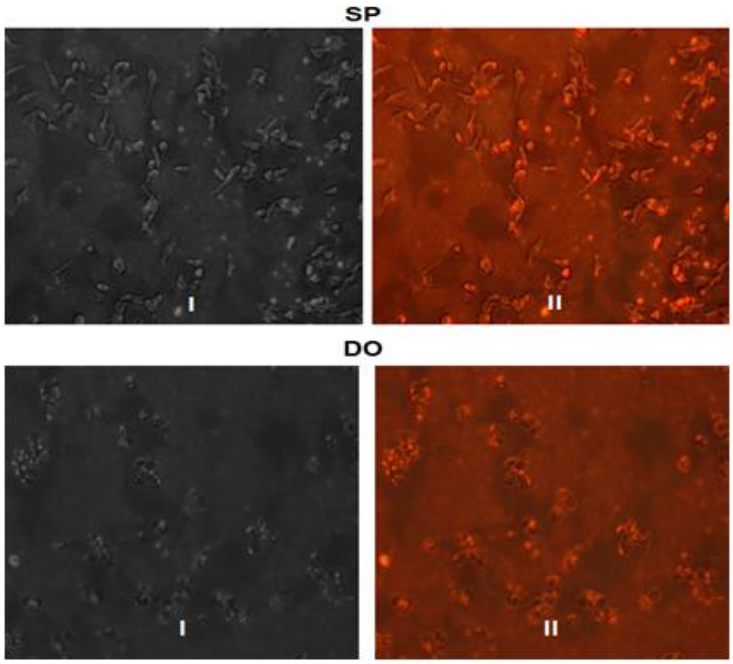
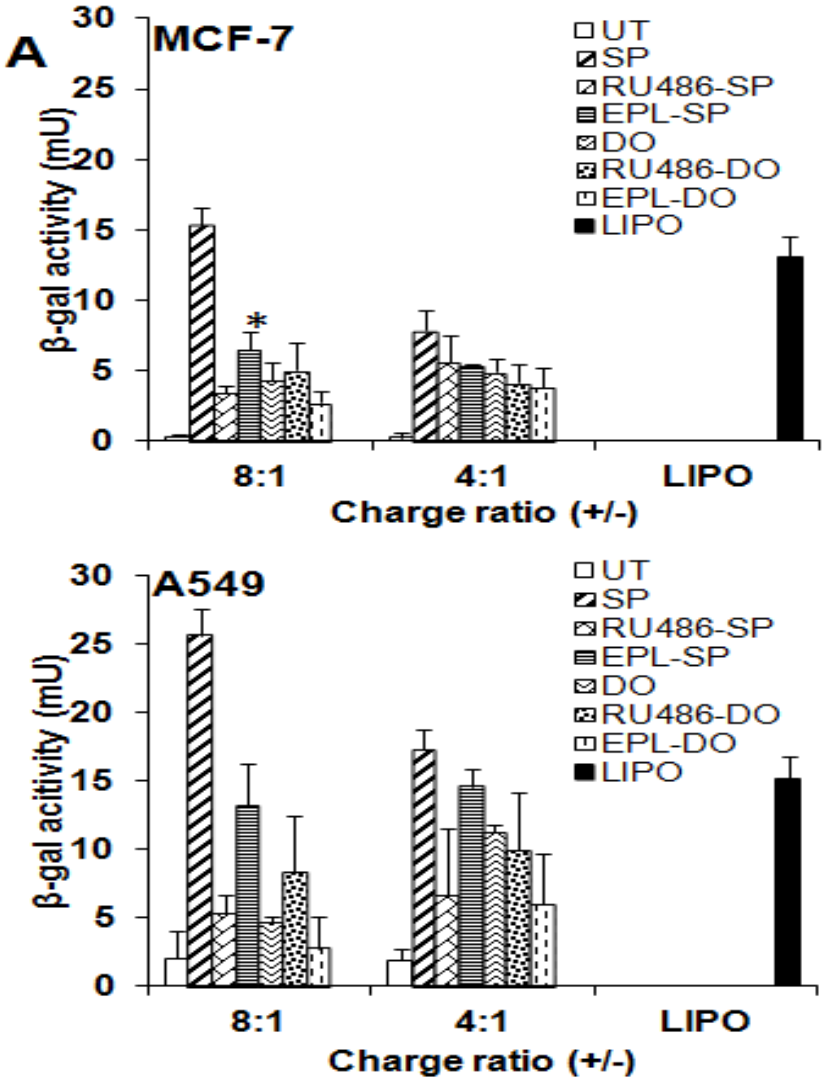


Fig. 4.7 Fluorescence microscopic images of liposomal uptake in B16F10 cells treated with SP & DO lipoplexes

4.4 MR MEDIATED TRANSFECTION IN CANCER CELLS : RECEPTOR DOWNREGULATION STUDY

4.4.1 Inhibition of MR through antagonist leads to decrease in transfection efficiency

The above transfection data indicates that SP liposome is efficiently transfecting cancer cells while there is no effect on normal cells. Here, we wanted to show if the SP lipoplex mediated gene transfection in cancer cells is MR receptor specific or not. Both cancer and normal cells were pretreated with small molecule antagonists RU486 or eplerenone (100μM) before 2h of transfection of SP lipoplex. RU486 is an antagonist for both glucocorticoid as well as mineralocorticoid receptor while eplerenone is an antagonist which is specific for mineralocorticoid receptor. Rogersen *et al*., 2004 have mentioned the importance of MR antagonism and have examined Eplerenone binding to the MR. The data indicates (Fig. 4.8) that upon pretreatment of cells with antagonist RU486 there is a decrease in transfection efficiency towards cancer cells which can be due to receptor inhibition. However, less receptor inhibition was observed for Eplerenone pretreated cells as indicated by relatively less inhibition of SP lipoplex mediated transfection. Receptor inhibition by any of the antagonists did not induce any effect on normal cells and the SP lipoplex mediated transfection remained unaltered. Mukherjee *et al*., 2009 reported that transfections in cancer cells, unlike that in non-cancer cells, were significantly decreased when cells were pretreated with antagonist RU486. Since pretreatment of cells with Eplerenone antagonist, which is specific for mineralocorticoids, also resulted in decrease in transfection efficiency, further investigation was required to know whether the delivery is mineralocorticoid mediated or glucocorticoid mediated as with RU486 (GR antagonist) transfection efficiency decreased to a significant level.


A
MCF-7
β-gal activity (mU)
30
25
20
15
10
5
0
*
8:1
4:1
LIPO
Charge ratio (+/-)
UT
SP
RU486-SP
EPL-SP
DO
RU486-DO
EPL-DO
LIPO
A549
β-gal acitivity (mU)
30
25
20
15
10
5
0
8:1
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LIPO
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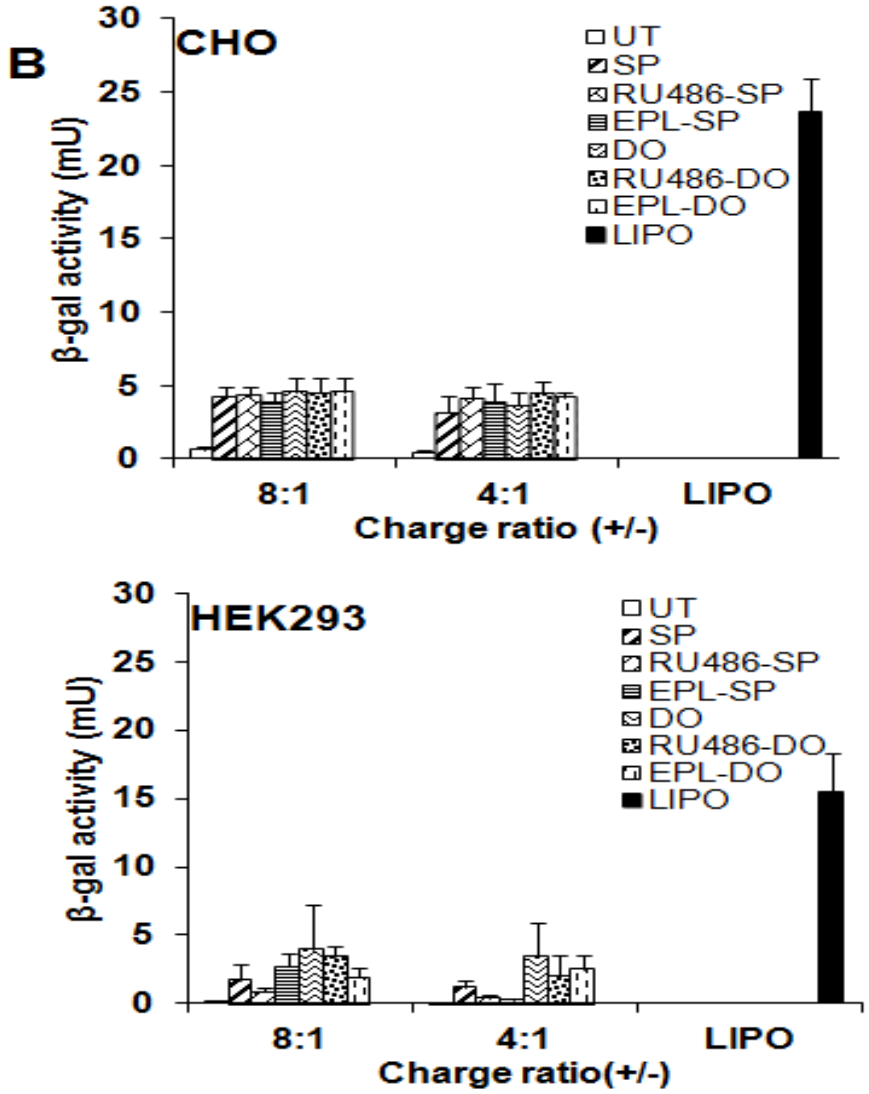

Fig. 4.8 Effect of mineralocorticoid receptor inhibition on transfection efficiency. (A) Cancer (MCF-7, A549) and (B) non cancer (CHO and HEK293) cells were transfected using 0.3µg pCMV-β-gal plasmid in SP lipoplex in different charge ratios (+/-, cationic lipid, x-axis). The cells were either untreated or pretreated with 100µmol/l RU486 and Eplerenone before the treatment of lipoplexes. 48-hours post-transfection β-galactosidase enzyme in the cellular lysates was assayed (mU, y-axis). The cells were also transfected with Lipofectamine/DNA lipoplex containing same amount of plasmid as a positive control.

4.4.2 Down-regulation of mineralocorticoid receptor by MR siRNA

The siRNA mediated selective silencing of steroid receptor was used to ascertain the role of mineralocorticoid receptor towards this selective anticancer activity of liposomes. siRNA, a modern cell based technique (Bass, 2000), is used to destroy a protein at mRNA level. At first, siRNAs (short interfering RNAs) unwind into single strand RNAs, which then assemble into endoribonuclease-containing complexes known as RNA-induced silencing complexes (RISCs). The RISC then captures a native mRNA molecule that is complement to the sequence of the siRNA. If the pairing (between mRNA and siRNA) is perfect, then the native mRNA is cut into useless RNA fragments that are not translated into proteins. If, however, the pairing is less than perfect then the RISC binds to the native mRNA and blocks the ribosome movement along the mRNA thus inhibiting the translation. In either case no protein is formed. Here, siRNA against mineralocorticoid receptor mRNA was used to destroy the production line of these receptor proteins in A549 and CHO cell lines. A scrambled siRNA was also used as a control to determine any nonspecific effect.

4.4.3 Reverse-transcriptase PCR (RT-PCR) study

In brief, A549 and CHO cells were seeded at a density of 2×10^5 cells per well in a 6-well plate usually between 18-24h before transfection. For dose optimization, 25pmol, 50 pmol and 100pmol of MR-siRNA or equal concentrations of scrambled siRNA (in 50µl serum free RPMI) was complexed with 0.5 µl of Lipofectamine2000^{TM} (Invitrogen) (in 50µl serum free RPMI) for 15 minutes. 900µl serum free DMEM was added to the resulting lipoplex. Cells were washed with PBS and treated with respective lipoplexes. After 4 h incubation cells were harvested, counted and again seeded at a density of 10,000 cells per well in 96-well plates for cytotoxicity assay and transfection

studies. After 16-18 h cells were either kept untreated or treated with liposomal formulations for 4 h. After 4h treatment, the cells were washed and kept in presence of fresh cell culture medium for 48 h. The cells were then assayed for tranfection efficiency and viability using MTT as described earlier.

The extent of down-regulation of mineralocorticoid receptor was measured by reverse transcriptase PCR studies using MR primers (Fiore *et al*., 2006) after 24 h, 48 h and 72 h. After PCR, the samples were run on 1% agarose gel. Fig. 4.9 & 4.10 depicts the reverse transcriptase- polymerase chain reaction (RT-PCR) profile of siRNA down-regulated cells after 24, 48 and 72 hours in A549 cells and CHO cells respectively. No down-regulation was observed after 24 hours while down-regulation in 50pmol treated siRNA was observed after 48 hours. No change was observed in CHO cells at 50pmol concentration as shown in Fig. 4.10. So, for further siRNA based studies 50 pmol was selected to be the optimized dose for down-regulating the receptor.

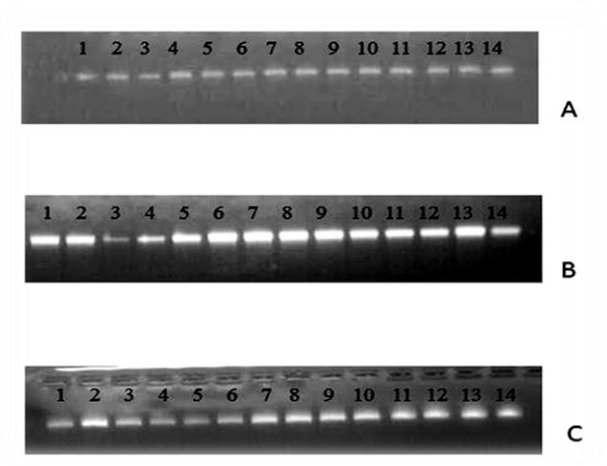

Fig. 4.9 Reverse transcriptase PCR studies of siRNA down-regulation in A549 cells. A, B & C represent 24 hours, 48 hours and 72 hours treatment. Lane wise: Lane: (1) Untreated (2) 25 pmol MR (3) 50 pmol MR (4) 100pmol MR (5) 25 pmol scrambled (6) 50 pmol scrambled (7) 100 pmol scrambled (8) 18s untreated (9) 18s 25pmol MR treated (10) 18s 50 pmol MR treated (11) 18s 100 pmol MR treated (12) 18s 25 pmol Scrambled treated (13) 18s 50 pmol Scrambled treated (14) 18s 100 pmol Scrambled treated

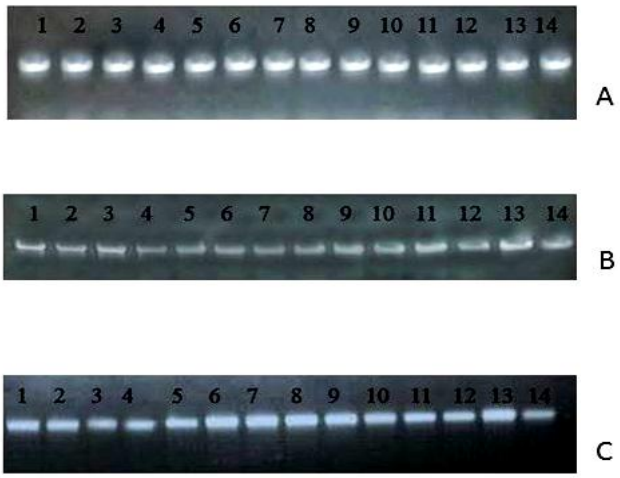

Fig. 4.10 Reverse transcriptase PCR studies of siRNA downregulation in CHO cells. A, B & C represent 24 hours, 48 hours and 72 hours treatment. Lane wise: Lane: (1) Untreated (2) 25 pmol MR (3) 50 pmol MR (4) 100pmol MR (5) 25 pmol scrambled (6) 50 pmol scrambled (7) 100 pmol scrambled (8) 18s untreated (9) 18s 25pmol MR treated (10) 18s 50 pmol MR treated (11) 18s 100 pmol MR treated (12) 18s 25 pmol Scrambled treated (13) 18s 50 pmol Scrambled treated (14) 18s 100 pmol Scrambled treated

4.4.4 Transfection efficiency of cells after MR down-regulation

siRNA pre-treated cells were treated with the lipoplexes as described earlier and kept for 48h. After completion of experiment, transfection efficiency in terms of β-gal activity was calculated using same procedure described earlier. Results clearly represent that upon siRNA down-regulation of the receptor, there was a decrease in transfection efficiency in A549 cells while no influence of siRNA down-regulation was observed in CHO cells (Fig. 4.11).

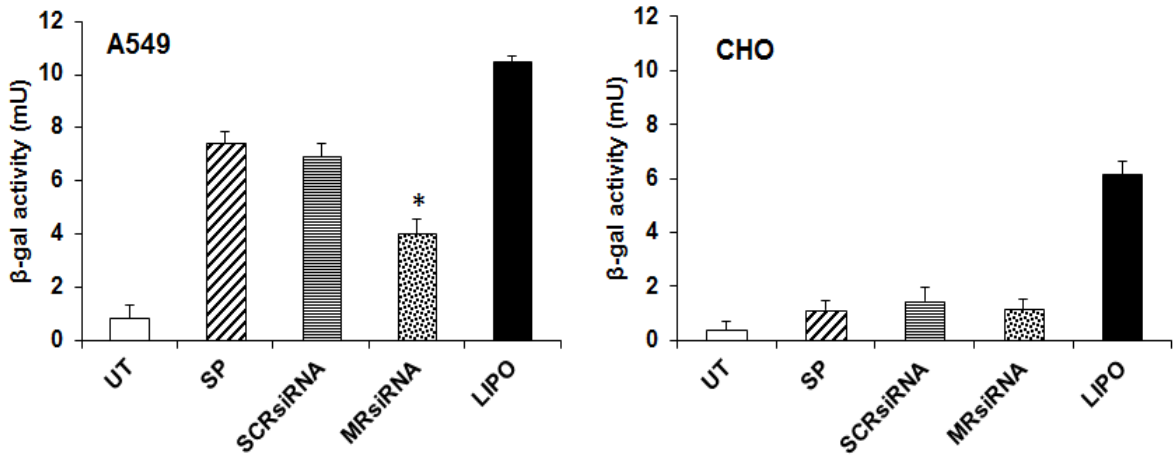

Fig. 4.11 Transfection efficiency after MR down-regulation using siRNA in A549 and CHO cells. MR down-regulation leads to decrease in β-gal transfection efficiency in A549 but not in normal CHO cell. No statistical significance was found in CHO cells. (* $p<0.01$)

4.5 CYTOTOXICITY STUDIES

4.5.1 SP lipoplex mediated cytotoxicity in cancer and normal cell lines

Cytotoxicity of SP lipoplex formulation was tested after 48h of incubation using MTT assay and results are presented in Fig.4.12. Formulation showed no toxicities in normal Chinese hamster ovary cells (CHO) and Human kidney cells (HEK293). While in MCF-7 and A549 cells, toxicity was observed when p53 plasmid DNA was used for transfection, cells transfected with control β-gal DNA showed no toxicity (Fig.4.12). This shows that liposome itself has no toxic effect towards normal cells, while the formulation having MR ligand spironolactone is toxic towards cancer cells. To ascertain that the liposomal formulation is toxic towards cancer cells and has no effect on normal cells, free drug without liposomal encapsulation was also tested in cancer and non-cancer cells. It is clear from Fig.4.13 that free drug is equally toxic to the cells irrespective of their characteristics. In other words, this shows that cancer cell-selective cytotoxicity is observed only when spironolactone is associated with the p53-lipoplex formulation. When we compared toxicity of targeted liposome i.e. SP and non-targeted liposome i.e. DO, we observed that β-gal containing DO lipoplexes and p53 containing DO lipoplexes were almost equally toxic to the cells (Fig.4.14). This proves that cell killing is because of DO liposome and not because of any anti-cancer gene like p53. This indicates that selective killing of cancer cell is achieved only when our targeted lipoplex formulation is complexed with some anticancer gene as the β-gal associated lipoplex was not found to be toxic to the cells.

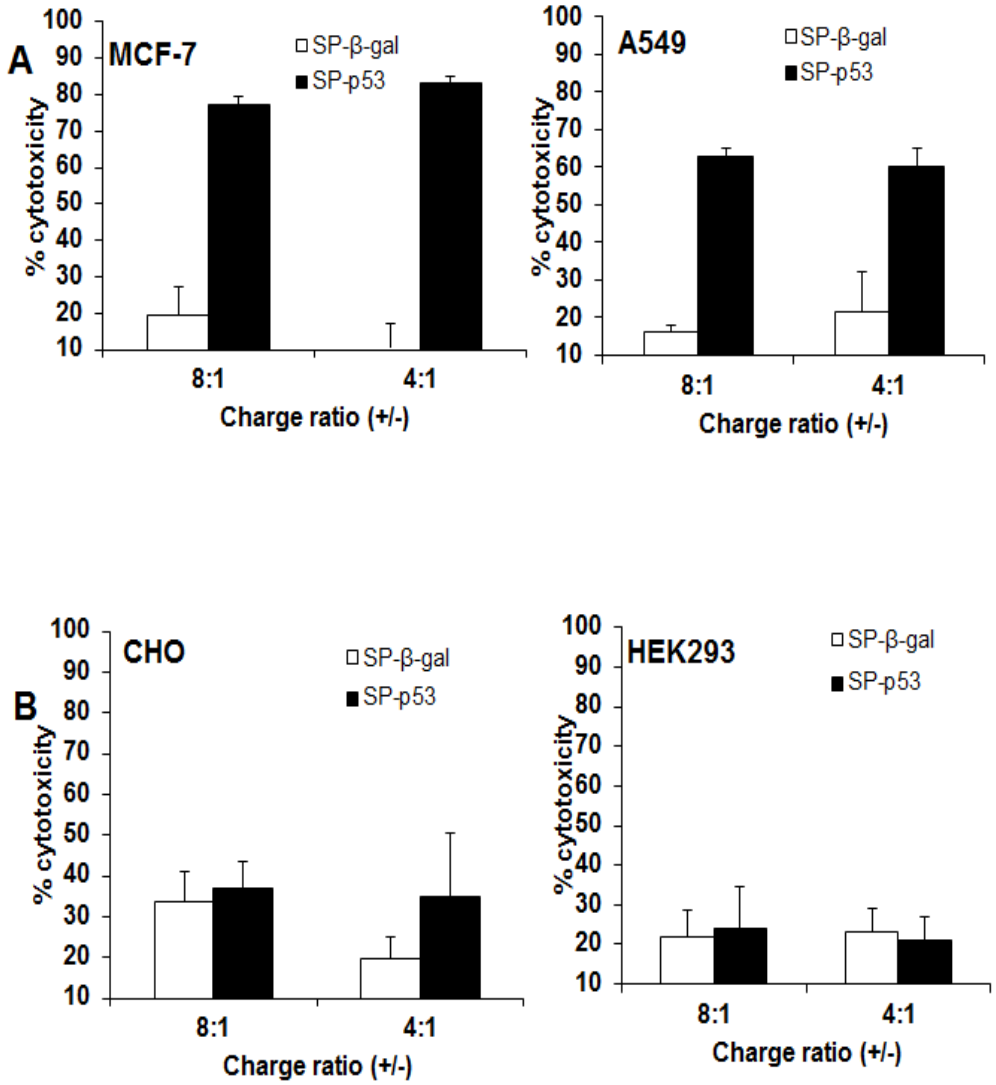

Fig. 4.12 Cytotoxic studies in cancer and non-cancer cells. MCF-7, A549, CHO and HEK-293 cells were transfected using SP lipoplexes associated with 0.3µg pCMV-β-gal plasmid and p53 for 48 hours.

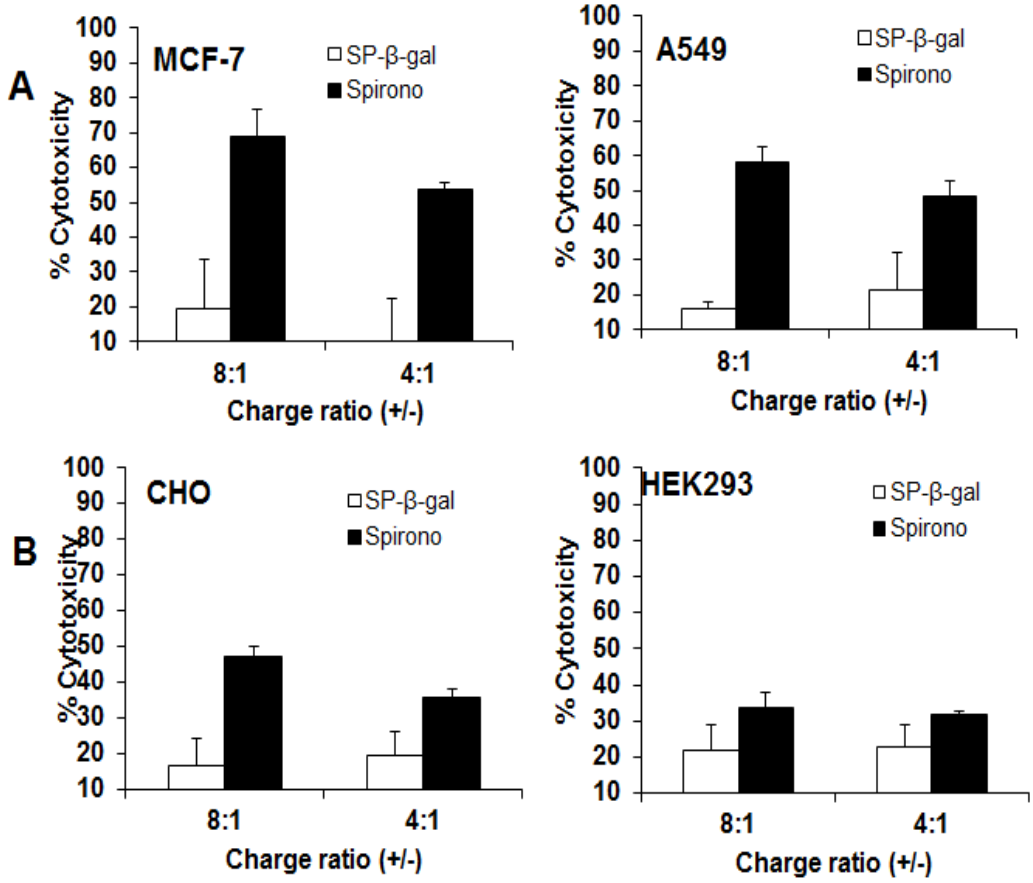

Fig. 4.13 Cytotoxicity studies of liposomes and free drug in cells. MCF-7, A549, CHO and HEK-293 cells were transfected using SP lipoplexes associated with 0.3µg pCMV-β-gal plasmid (white bar) or with equal concentration of free spironolactone (spirono) (black bar) respectively for 48 hours.

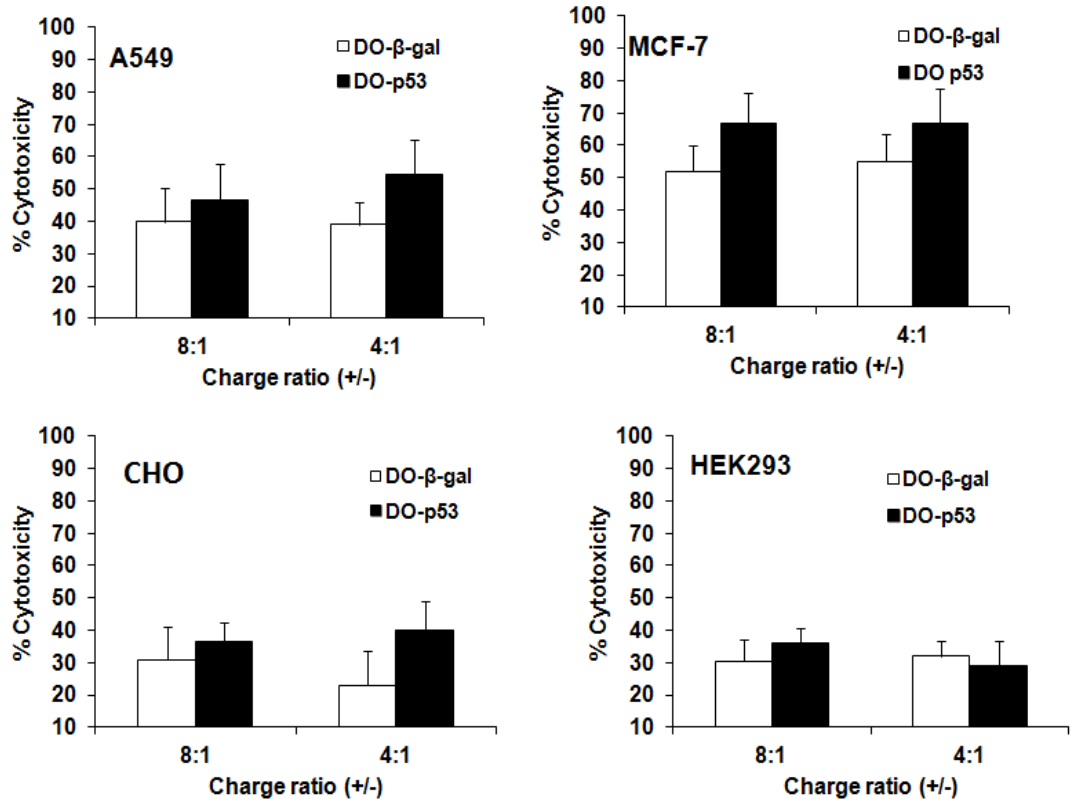

Fig. 4.14 Cytotoxic studies in cancer and non-cancer cells. MCF-7, A549, CHO and HEK-293 cells were transfected using DO lipoplexes associated with 0.3µg pCMV-β-gal plasmid and p53 for 48 hours.

4.5.2 Cytoxicity study of p53 complexed SP liposome after MR down-regulation using MR siRNA

Cells pre-treated with 50pmol of siRNA were treated with p53-complexed SP liposomes and kept for 48 h. After completion of the experiment, cytotoxicity was assessed using MTT assay as described in section 3.11. As is evident from Fig. 4.15, there was a significant decrease in cytotoxicity in A549 cells after MRsiRNA down-regulation ($p < 0.01$) compared to untreated cells or scrambled siRNA treated cells. Naturally, siRNA mediated down-regulation of MR expression caused lesser amount of MR available ultimately leading to the increase in cell viability and thus a decrease in cellular toxicity. siRNA down-regulation of MR did not influence cell toxicity in CHO cells.We observed that the liposomal formulations can be used for selective delivery of anti-cancer gene, p53 to elicit target-specific toxicity in MR over-expressed cancer cells

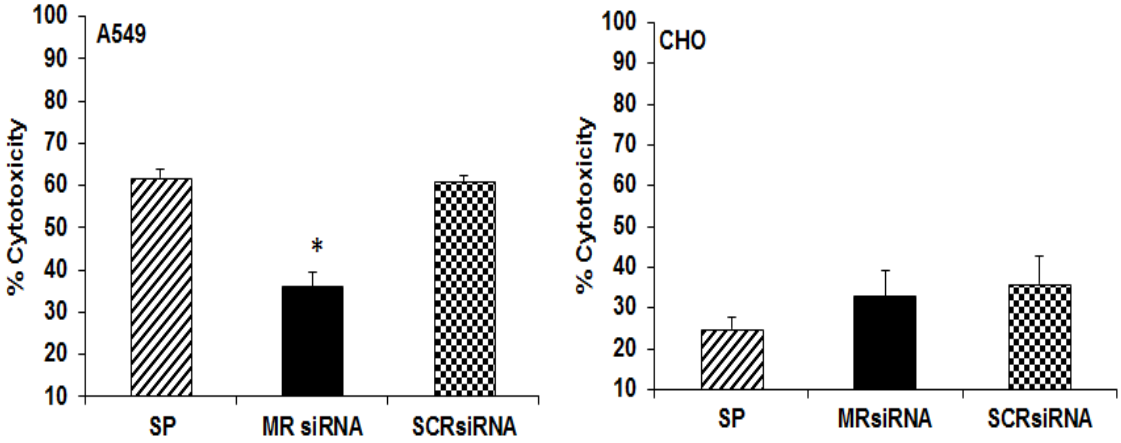

Fig. 4.15 Cytotoxicity of SP-p53 lipoplex in A549 and CHO cells upon MRsiRNA down-regulation. p53 gene was delivered using SP liposome after MR down-regulation and after 48h MTT assay was done. No statistical significance was found in CHO cells (* $p<0.01$)

4.6 EPLERENONE: SYNTHETIC MR LIGAND FOR LIPOSOME FORMULATION TO TARGET CANCER (EP LIPOSOME)

Eplerenone is an antagonist which is specific for mineralocorticoid receptor and is similar to diuretic spironolactone. Like spironolactone, it will bind to mineralocorticoid receptor and blocks the binding of MR's natural ligand, aldosterone. Eplerenone represents a molecule with improved steroid receptor selectivity and pharmacokinetic properties in man compared to spironolactone (Garthwaite & McMahon, 2004). Since after inhibiting receptor by antagonists, we could observe that RU486 is blocking the receptor but receptor inhibition by eplerenone is not much specific. Now, we know that spironolactone and eplerenone share structural similarity, eplerenone liposome may itself act as efficient transfection system. So, we tried a new liposome formulation containing eplerenone and transfected the cells. This experiment clarified the confusion created by receptor antagonism with eplerenone.

4.6.1 DNA-binding study

Stability of the formulation was tested by DNA-binding studies (Table 4.4). As expected, and shown in Fig.4.16, lipoplexes with charge ratio 8:1, 4:1 and 2:1 were found to be stable while formulations in the ratio of 1:1 did not form any complex and thus were not found to be stable. It can be because of the non-availability of cations for complexion with anionic DNA. So, charge ration is a factor which is responsible for stable complex formation and hence influences transfection efficiency.

Table 4.4 Charge ratio of different EP lipoplexes for DNA binding studies

Lipoplex	Liposome:DNA (charge ratio)
EPD8	8:1
EPD4	4:1
EPD2	2:1
EPD1	1:1

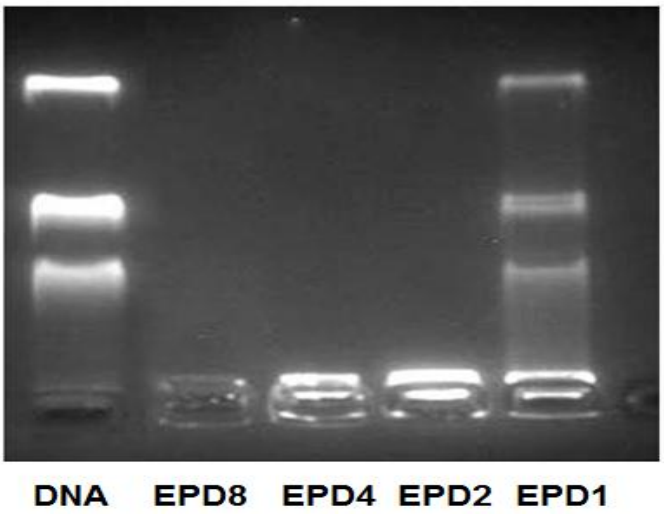

Fig. 4.16 DNA binding assay of lipoplex-associated plasmid DNA for EP liposomes. The lipid: DNA charge ratios are indicated at the bottom of each well. EPD8, EPD4, EPD2, EPD1 represent lipid to DNA charge ratio of 8:1, 4:1, 2:1 and 1:1 respectively. DNA represents naked plasmid DNA of β-gal.

4.6.2 EP liposomes have different transfection efficiencies at different molar and charge ratio

Cationic liposomes that contain a cationic lipid [N,N-di-n-tetradecyl-N,N-(2-hydroxyethyl) ammonium chloride (DODEAC)], cholesterol and eplerenone in a stable formulation of 1:1:1, 1:1:0.75 and 1:1:0.50 were formulated. Eplerenone is a synthetic steroidal mineralocorticoid which shows structural resemblance with cholesterol and gave a stable formulation with cationic lipid. β-galactosidase (β-gal)-expressing reporter gene was linked with above-mentioned EP-containing liposome in varied charge ratios (+/–) and the resulting targeted lipoplexes (SP) were used in transfecting cells of both cancerous and noncancerous nature expressing MR ubiquitously. Liposomes containing lipids with a molar ratio of 1:1:0.75 and a charge ratio of 8:1 showed highest transfection efficiency and therefore selected for further studies (Fig. 4.17).

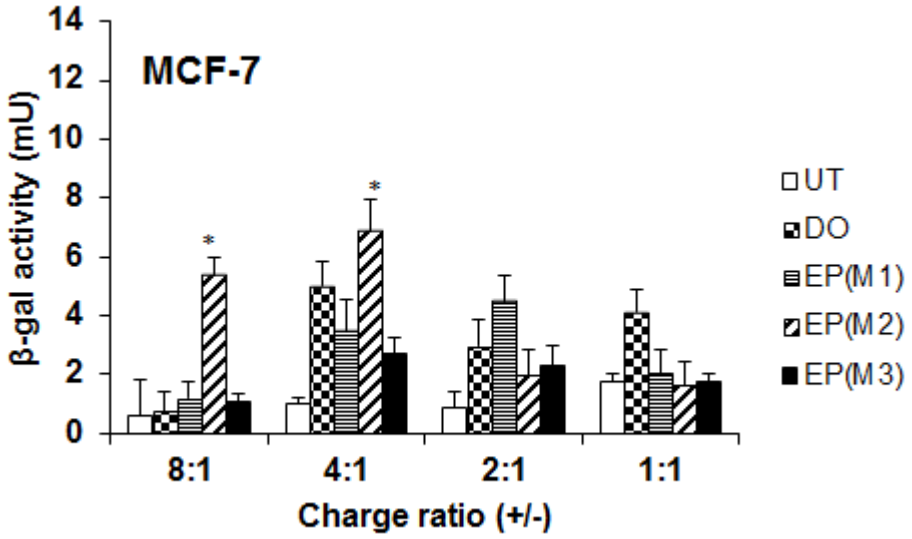

Fig. 4.17 Transfection efficiency in cancer cells with different lipid and charge ratio. Cells were transfected using 0.3µg pCMV-β-gal plasmid in EP lipoplex in different charge ratios (+/-, cationic lipid, x-axis) *p<0.01

4.6.3 Zeta size and potential

Size and zeta potential of the liposomes were measured using dynamic light scattering and results are shown in Table 4.5 & 4.6. The size of the liposome was found to be in the range of 150-160nm with a zeta potential of 14.0. Liposome size is one of the important parameter determining the nature of the entry pathway by endocytosis (Wasungu & Hoekstra, 2006). To know the size of lipoplex, which is an important parameter in transfection, EP liposomes were complexed with DNA and incubated for 20 minutes. Similar to the results obtained with SP lipoplexes, there was an increase in size of the lipoplex (Table 4.5). Although conflicting reports exist upon the optimal size of lipoplexes for lipofection, there is no doubt that high lipofection would be gained from large lipoplexes when endocytosis is dominant, because large particles facilitate membrane contact and fusion (Ma *et al*.,2007) .

Table 4.5 Size measurement for EP liposomes & lipoplexes

Formulation	Angle	KCpS	Z Ave (nm)	Poly Index
EP	**90**	**251.3**	**153.9**	**0.552**
EP-DNA	**90**	**59.63**	**613.3**	**0.492**

Table 4.6 Zeta potential for EP liposomes & lipoplexes

Formulation	KCpS	Mobility	Zeta	Width
EP	**248.0**	**1.101**	**14.0**	**1.6**
EP-DNA	**242.6**	**0.815**	**4.1**	**1.6**

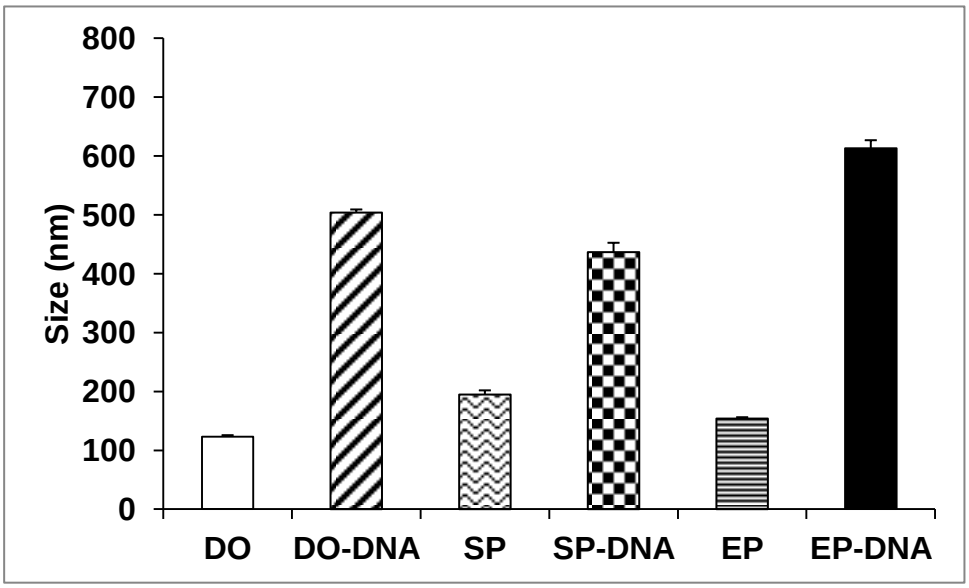

Fig. 4.18 Size measurement for different liposomes and lipoplexes by dynamic light scattering

4.6.4 EP liposome mediates transfection in cell-specific manner

Similar to the SP lipoplex mediated transfection study as described in previous section, EP-β-gal DNA lipoplexes were used for transfecting cells of cancer and non-cancer nature. After 48 h of treatment, cells were assayed for β-galactosidase activity. As shown in Fig. 4.19, EP liposome mediated transfections were found to be more efficient in cancer cells (A549 and CHO), while no effect of EP liposome treatment was observed in normal cells. In CHO, DO was found to be more transfection efficient as compared to EP & SP and this can be because of non-targeted nature of DO. DO liposomes have been designed in a way such that it will not act in a targeted manner and may or may not affect transfection efficiency.

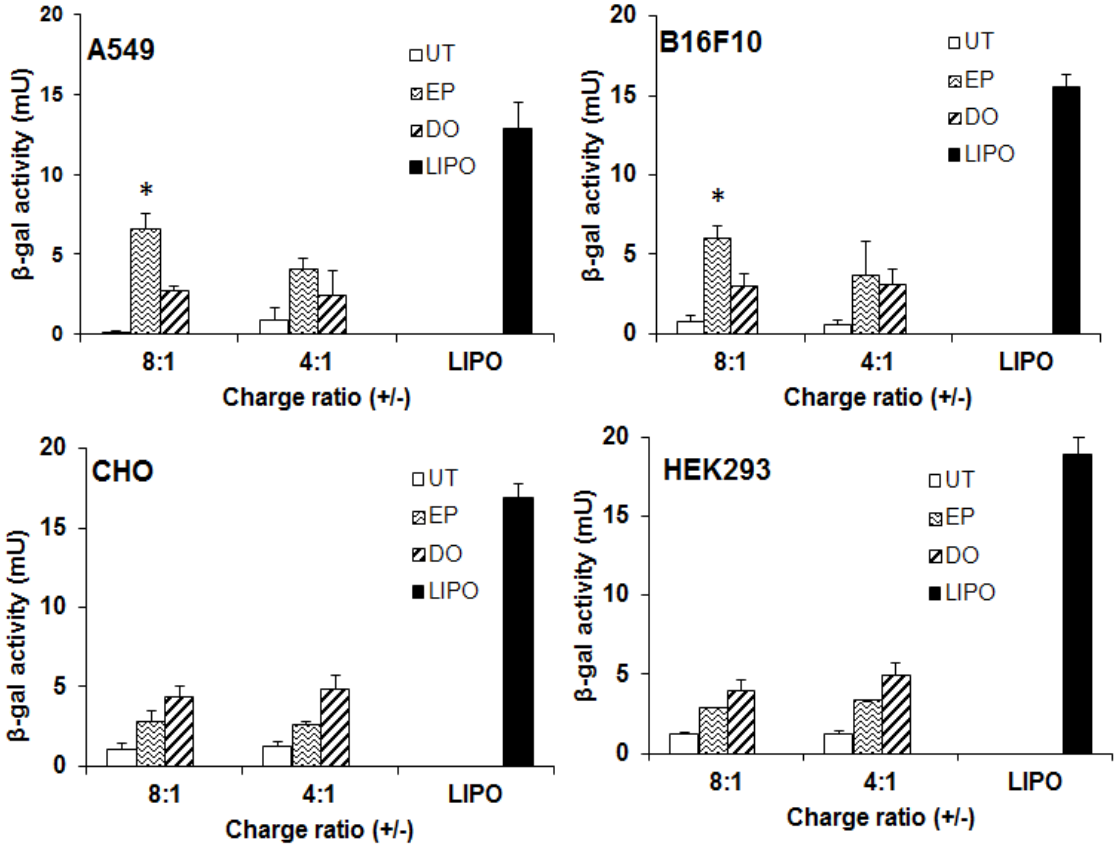

Fig. 4.19 EP liposome mediated targeted transfection in cancer cells. Cancer (MCF-7, A549) and non cancer (CHO, HEK293) cells were transfected using 0.3µg pCMV-β-gal plasmid in EP lipoplex in different charge ratios (+/-, cationic lipid, x-axis). 48-hours post-transfection β-galactosidase enzyme in the cellular lysates was assayed (mU, y-axis). The cells were also transfected with Lipofectamine/DNA lipoplex containing same amount of plasmid as a positive control. The results are obtained from the average of triplicate experiments (*$p<0.01$)

4.6.5 Uptake efficiency of EP liposome is equal in cancer and non-cancer cells

Similar to the uptake studies carried out for SP liposome to ascertain that the difference in transfection efficiency is not due to difference in lipoplex uptake, EP lipoplex containing rhodamine-linked "red-fluorescent" lipid was treated to both cancer (MCF-7 and B16F10) and normal CHO cells. Following treatment of lipoplex for 4 hours, respective cellular lysates were quantitatively estimated for its red-fluorescence content (Fig. 4.20). No significant difference was found in lipoplex uptake of respective cells. These findings proved that the lipoplex uptake was unrelated to transfection efficiency. Qualitative estimation of red fluorescent content of B16 cells was also done and no difference was found in the uptake efficiency of cells.

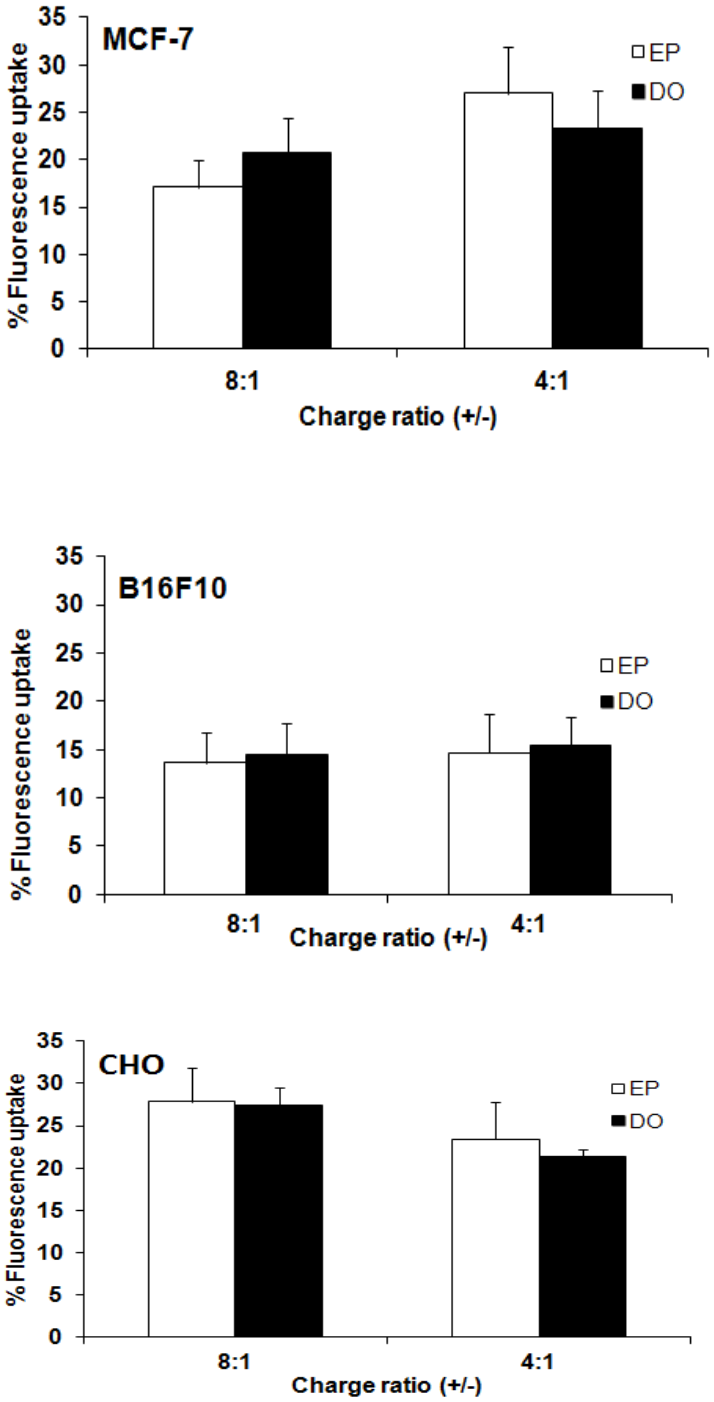

Fig. 4.20 Uptake of EP & DO lipoplex in cells. MCF-7, B16 and CHO cells were treated with rhodamine fluorescent-labeled EP & DO lipoplex in 8:1 and 4:1 (+/-) charge ratio of cationic lipid to DNA. Cellular uptakes of the lipoplexes were measured 4 hours after the treatment. The results are obtained from the average of three experiments.

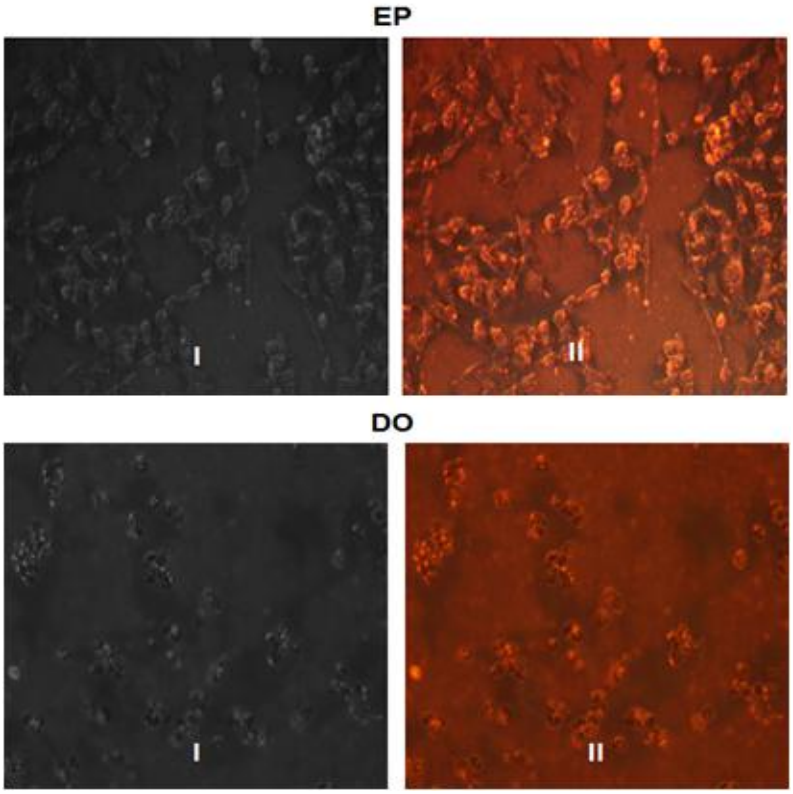

Fig. 4.21 Fluorescence microscopic images of liposomal uptake in B16F10 cells treated with EP & DO lipoplexes

4.6.6 Effect of MRsiRNA down-regulation on EP liposome-mediated transfection

Similar kind of MR down-regulation studies using siRNA were carried out for EP liposomes. Cells were treated with 50pmol of MRsiRNAor same concentration of scrambled siRNA treated with EP lipoplex. After completion of the experiment, β-galactosidase assay was carried out. As shown in Fig. 4.22, there was a small decrease in cytotoxicity of EP liposomes when the receptor was down-regulated with siRNA in A549, down-regulation effect was observed in CHO cells too.

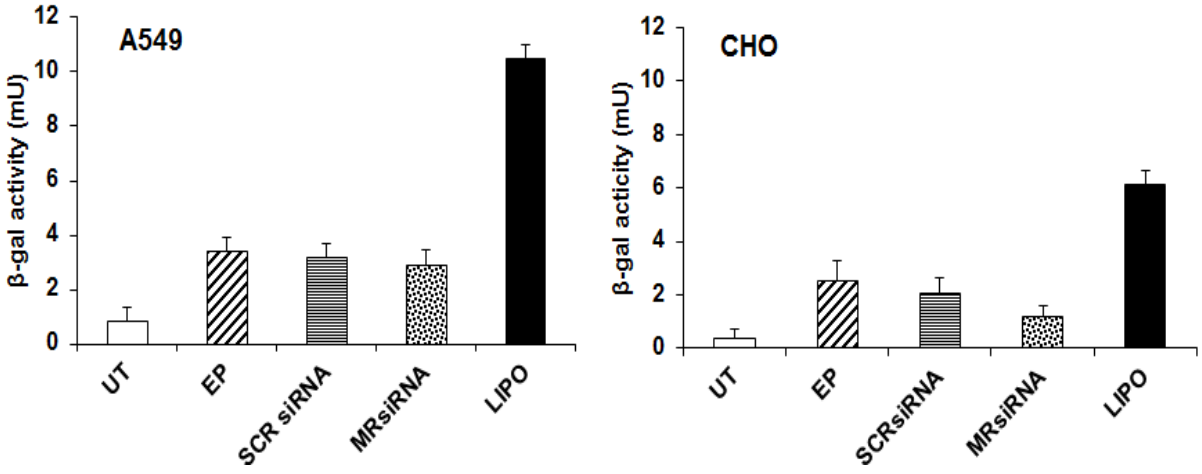

Fig. 4.22 Transfection efficiency of EP after siRNA down-regulation**.** MR down-regulation leads to decrease in β-gal transfection efficiency in A549 but not in normal CHO cell.

Treatment with scrambled siRNA did not induce any effect on toxicity of any of the cells which is obvious. We could not observe any significant effect of MR siRNA down-regulation on transfection efficiency of EP liposomes. All synthetic ligands of MR may not be able to provide a formulation which can compete with natural ligand aldosterone and can target cells in specific manner and this can be the reason why small effect of MR siRNA down-regulation has been found in normal CHO cells.

4.6.7 EP liposome induces cytotoxicity

Similar to the cytotoxic studies carried out for SP liposome, EP liposomes were also tested for their toxicity towards both cancer (MCF-7 and A549) and normal (CHO and HEK293) cells by MTT assay as described earlier. As shown in Fig. 4.23, formulations were found to be toxic towards cancer cells when p53 plasmid DNA was used for transfection while less toxicity was observed for β-gal DNA lipofection.

Toxicity on normal cells was less but if we compare toxicity of SP and EP liposome toward cancer and normal cells, we could observe, SP-p53 liposomal transfection was much toxic towards cancerous cells when compared with EP-p53 liposome, wherein SP liposomes could cause approximately 80% cytotoxicity in MCF-7 cells and 60% in A549 whereas EP liposomal transfection resulted in approximately 70% toxicity in MCF-7 cells and 55% toxicity in A549 cells when β-gal DNA was used for transfection. Similar to the cytotoxicity studies on cancer cells; liposomal transfections were less toxic towards normal cells; moreover, free drug was found to be more toxic to the cells as compared to the liposomal formulations (Fig. 4.24). So if we compare the toxicities of EP and DO, EP lipoplexes were found to kill cells in specific manner.

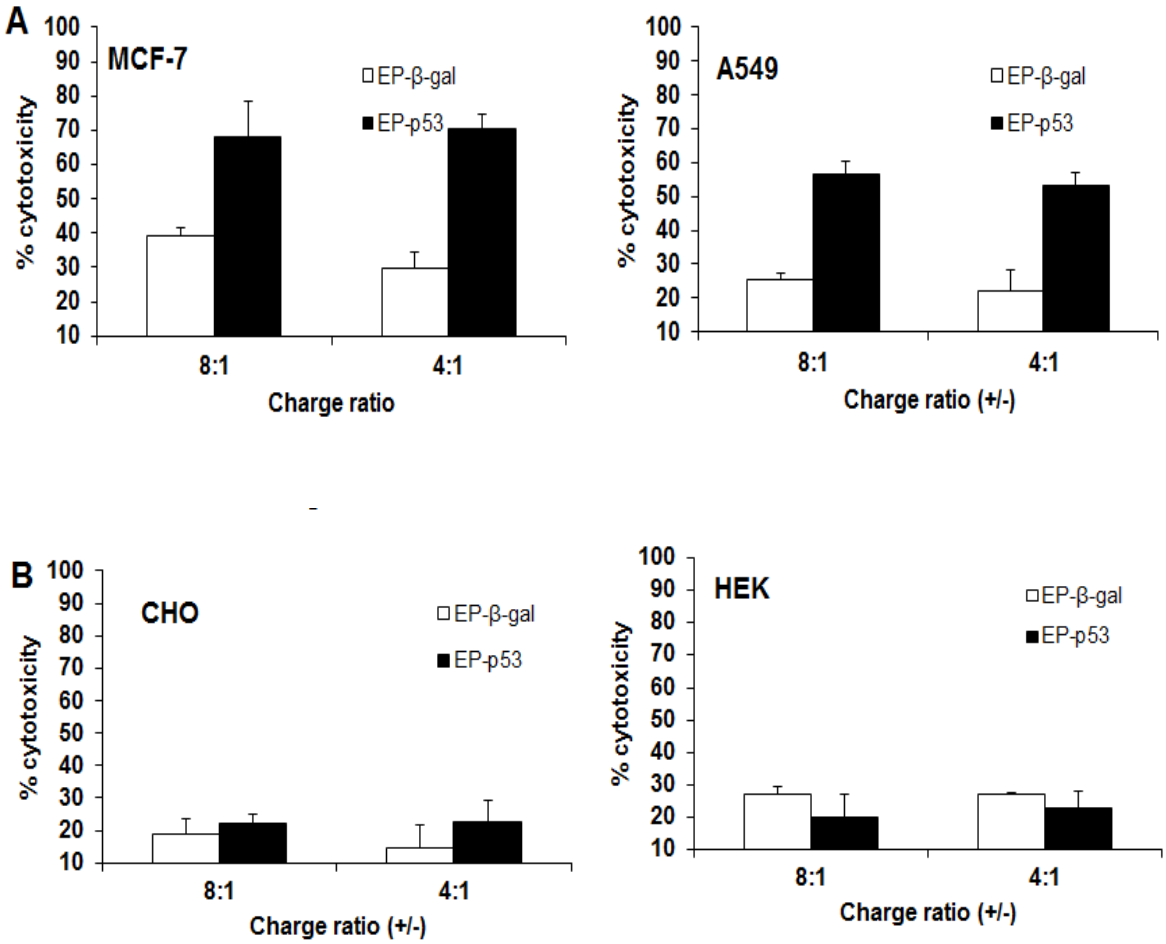

Fig. 4.23 Cytotoxic studies of EP liposomes in cells. MCF-7, A549, CHO and HEK-293 cells were transfected using EP lipoplexed 0.3µg pCMV-β-gal plasmid (white) and p53 (black bar) respectively for 48 hours.

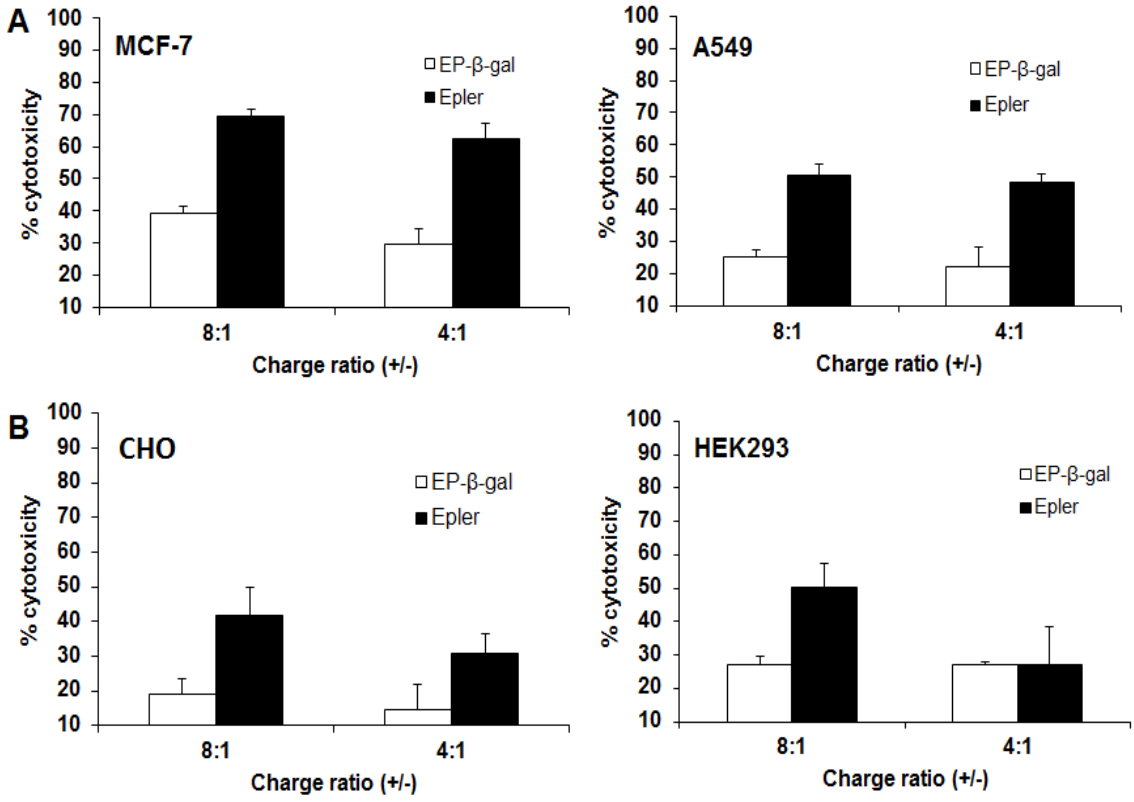

Fig. 4.24 Cytotoxicity studies of EP liposomes and free Eplerenone in cells. MCF-7, A549, CHO and HEK-293 cells were transfected using EP lipoplexed 0.3µg pCMV-β-gal plasmid (white bar) or with equal concentration of free eplerenone (epler) (black bar) respectively for 48 hours.

4.6.8 MR down-regulation leads to small decrease in toxicity on cancer cells

MR was down-regulated in a manner as described in previous section; plated for cytotoxicity test; treated with EP-p53 lipoplexes and kept for 48 h. cytotoxicity was measured using MTT assay. A decrease in toxicity was observed in MR siRNA down-regulated A549 cells as shown in Fig. 4.25. Similar to the transfection data obtained with EP liposomes, significant decrease in cytotoxicity was not observed and in CHO, MRsiRNA down-regulated cells resulted in a small increase in toxicity which indicated that MRsiRNA down-regulation has no effect on normal cells. From this data we observe that the liposomal formulation, SP, can be used for selective delivery of anti-cancer gene, p53 to elicit target-specific toxicity in MR over-expressed cancer cells.

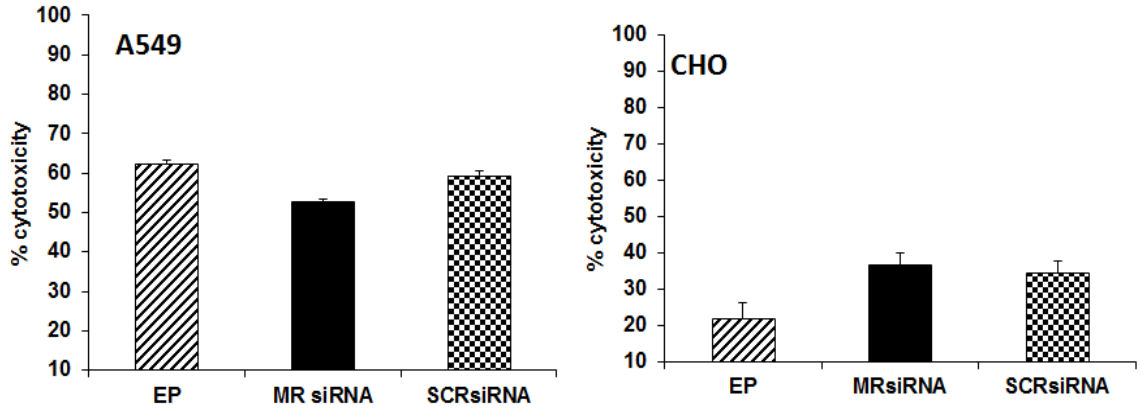

Fig. 4.25 Cytotoxicity study upon MR down-regulation: p53 gene was delivered using EP liposome upon MR down-regulation and after 48h MTT assay was done.

4.7 TARGETED INDUCTION OF APOPTOSIS IN CANCER CELLS

The goal of any therapeutic strategy is to impact on the target tumor cells with limited detrimental effect to normal cell function. Defective apoptosis (programmed cell death) represents a major causative factor in the development and progression of cancer. The challenge lies in the fact that all normal cells also have the capacity to engage the apoptotic program and, furthermore, often do so more readily than their tumorigenic counterparts. The problem of attaining drug selectivity is no more clearly demonstrated than by many of the conventional chemotherapeutics that are designed to exploit the accelerated proliferative response seen in many tumor types (Shapiro & Harper, 1999), with devastating consequences for healthy proliferating cells. So can we really hope to exploit apoptosis as a therapeutic strategy and achieve tumor-selective killing without compromising normal cell function? It is possible if we can exploit the expression and/or function of apoptotic-related molecules that are exclusively inherent to maintaining tumor cell function or molecules that are regulated in a different manner in tumor cells and in normal cells (Kasibhatla & Tseng, 2003). Cancer cells often reside in unique microenvironments armed with a variety of adaptive responses and carry mutations, such as defective apoptotic machinery, that further confer survival advantage. Thus, improving therapeutic efficacy and selectivity and overcoming drug resistance are the major goals in developing anti cancer agents today (Fulda *et al*., 2010). To fulfill these goals, a thorough understanding of apoptotic signaling pathways and how tumor cells resist apoptosis is imperative, because they provide directions to unravel novel therapies and key targets to surpass or supplement current cancer treatments.

Apoptosis is characterized by cell shrinkage, blebbing of plasma membrane, maintenance of organelle integrity, condensation and fragmentation of DNA, followed by ordered removal of phagocytes. It works like a "suicide" program and it causes minimal damage to surrounding tissues. Apoptosis has been sub-classified into two types of death pathways, namely, the extrinsic pathway and the mitochondria-mediated pathway (intrinsic pathway). These two processes however, are not exclusive and evidence suggests that they can be linked and that molecules in one pathway can influence the other (Daniel & Korsmeyer, 2004).

The ability of tumor cells to evade engagement of apoptosis plays a significant role in their resistance to conventional therapeutic regimens. Out of the two major pathways of apoptosis (i.e. intrinsic or extrinsic), intrinsic pathway involves the release of pro-apoptotic proteins that activate caspase enzymes from the mitochondria. This process ultimately triggers apoptosis (Fulda & Debatin, 2006; Letai, 2005). Cell fate is tightly regulated by the interactions between pro and anti-apoptotic proteins which act to tweak the balance between survival and cell death.

In a healthy cell, the outer membranes of its mitochondria display the protein Bcl-2 on their surface. Bcl-2 inhibits apoptosis. Internal damage to the cell causes a related protein, BAX, to migrate to the surface of the mitochondrion where it inhibits the protective effect of Bcl-2 and inserts itself into the outer mitochondrial membrane punching holes in it and causing Cytochrome c to leak out.

The released Cytochrome c binds to the protein Apaf-1 ("apoptotic protease activating factor-1"). Using the energy provided by ATP, these complexes aggregate to form apoptosomes. The apoptosomes bind to and activate caspase-9. Caspase-9 cleaves

and, in so doing, activates other caspases (caspase-3 and -7). The activation of these "executioner" caspases creates an expanding cascade of proteolytic activity which leads to digestion of structural proteins in the cytoplasm, degradation of chromosomal DNA, and phagocytosis of the cell.

The extrinsic pathway begins outside the cell through activation of pro-apoptotic receptors on the cell surface. These are activated by molecules known as pro-apoptotic ligands. Preclinical studies show that ligand binding causes receptors to cluster and ultimately form a death-inducing signaling complex (DISC). Upon DISC activation, the extrinsic pathway has been seen to adopt the same effector caspase machinery as the intrinsic pathway (Ashkenazi, 2002). These pathways are caspase dependent. So, caspases act as the mediator molecules for apoptosis. Xiang *et al*., 1996 discovered that cells can die while displaying morphology similar to apoptosis without caspase activation. Later studies linked this phenomenon to the release of AIF (apoptosis-inducing factor) from the mitochondria and its translocation into the nucleus mediated by its NLS (nuclear localization signal). Inside the mitochondria, AIF is anchored to the inner membrane. In order to be released, the protein is cleaved by a calcium-dependent calpain protease.

4.7.1 Lipoplex induces apoptosis in cancerous cells but not in normal cells

To ascertain the induction of apoptosis in cancer cells following the treatment of SP & EP lipoplexed-p53 (SP-p53), we studied the Annexin V/Propidium iodide (PI) binding profile in one cancer cell (MCF-7) and one normal cell (CHO) using flow cytometric analysis. During the early phase of apoptosis, simultaneous loss of plasma membrane polarity and translocation of phosphatidylserine (PS) from the inner to outer

membrane leaflets is observed. This exposes PS to the external environment. The phospholipid binding protein Annexin V, which has high affinity for PS, binds to cells through externally exposed PS. Positively stained cells with fluorescent-Annexin V correlates with early loss of membrane polarity which is preceded by the complete loss of membrane integrity in the later stages of cell death resulting from either apoptosis or necrosis (Vermes *et al*., 1995).In contrast, PI can only enter cells after loss of membrane integrity. Thus, dual staining with annexin V and PI allows clear discrimination between unaffected cells (annexin V negative, PI negative, lower left quadrant), early apoptotic cells (annexin V positive, PI negative, lower right quadrant), late apoptotic cells (annexin V positive, PI positive, upper right quadrant) and necrotic cells (annexin V negative, PI positive, upper left quadrant).

As can be seen in Fig. 4.26A & B, liposomal treatments (in charge ratio of 8:1 and 4;1) increase the percentage of apoptotic cells in MCF-7 cells while the treatments were found to be less toxic in CHO cells (Fig. 4.27A & B) when comparing treated with the untreated cells.In normal cells (CHO) there was no difference between untreated cells and cells treated with liposomes. The percentage of apoptotic cells has shown a drastic increase from 1.6% to above 20% and reaching 30% in some treatments of MCF-7 cells and an obvious decrease in percentage of healthy cells (Q1) in treated samples. Therefore it is evident that SP & EP liposomal treatment truly induced apoptosis specifically in cancer cells but did not induce apoptosis in normal cells.

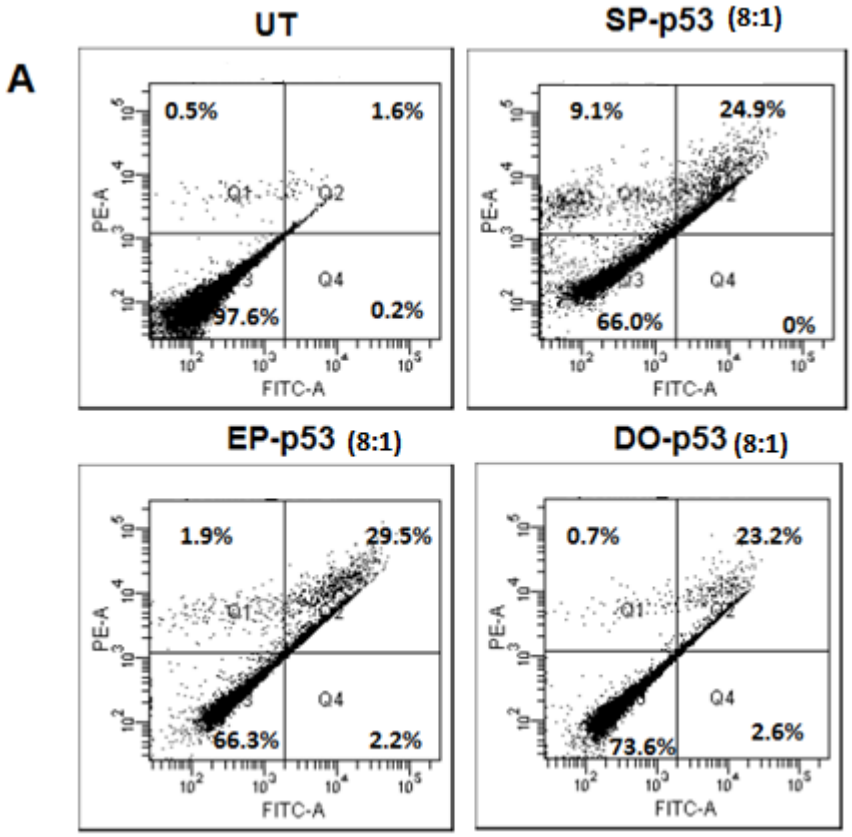

Fig. 4.26 (A) Flow cytometric analysis of apoptosis of MCF-7 cells co-stained with annexin V-FITC and propidium iodide. Vertical and horizontal axes represent PI and FITC labeling respectively. Cells were either left untreated or treated with SP-p53, EP-p53 & DO-p53 lipoplexes for 24h at a charge ratio 8:1

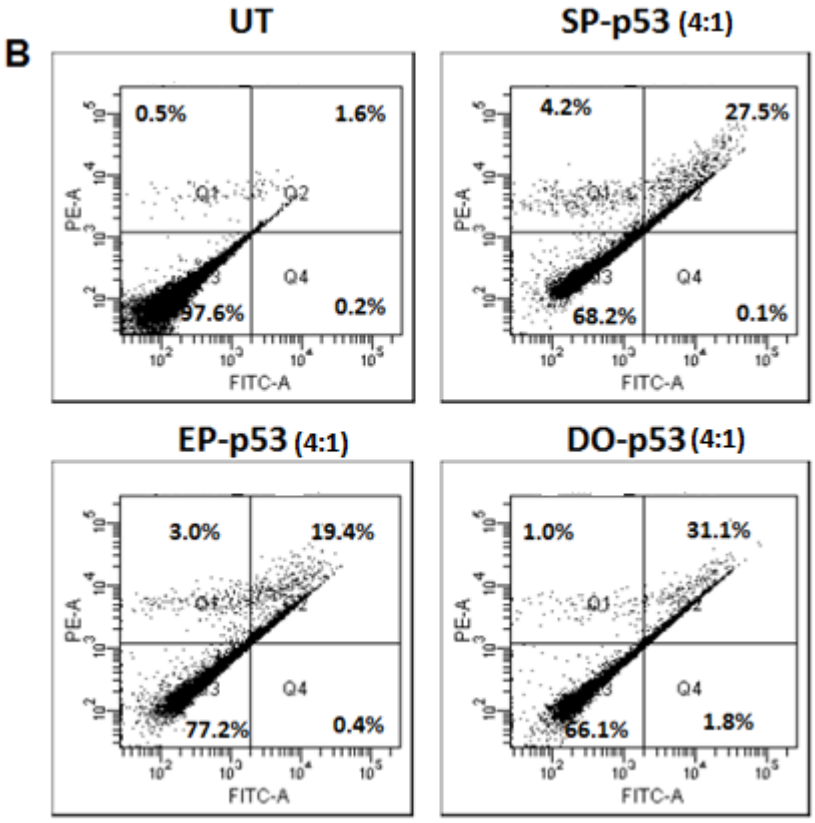

Fig. 4.26 (B) Flow cytometric analysis of apoptosis of MCF-7 cells co-stained with annexin V-FITC and propidium iodide. Vertical and horizontal axes represent PI and FITC labeling respectively. Cells were either left untreated or treated with SP-p53, EP-p53 & DO-p53 lipoplexes for 24h at charge ratio of 4:1.

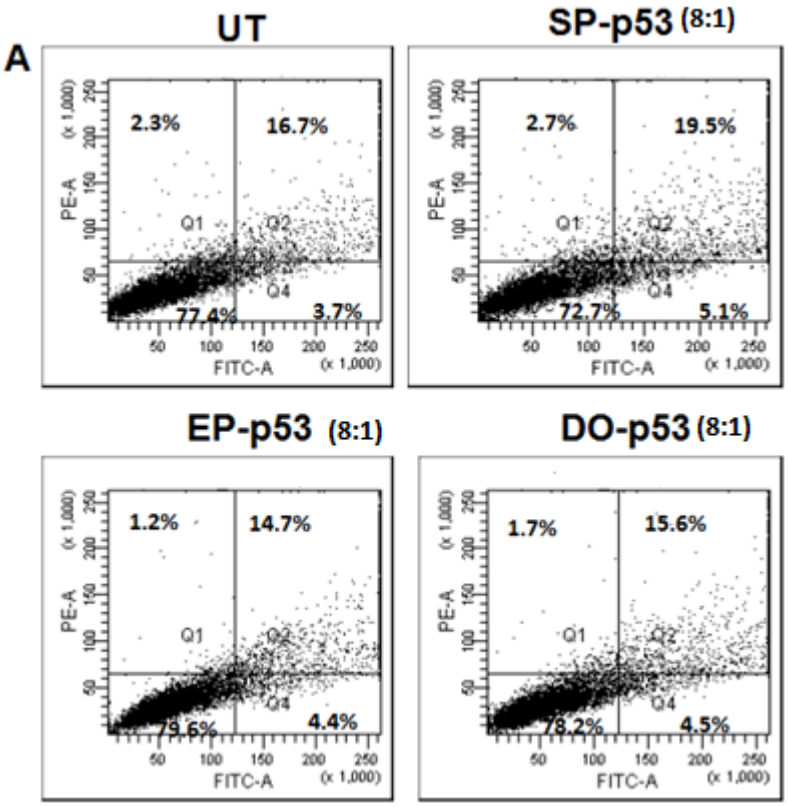

Fig. 4.27 (A) Flow cytometric analysis of apoptosis of CHO cells co-stained with annexinV-FITC and propidium iodide. Vertical and horizontal axes represent PI and FITC labeling respectively. Cells were either left untreated or treated with SP-p53, EP-p53 & DO-p53 lipoplexes for 24h at a charge ratio of 8:1

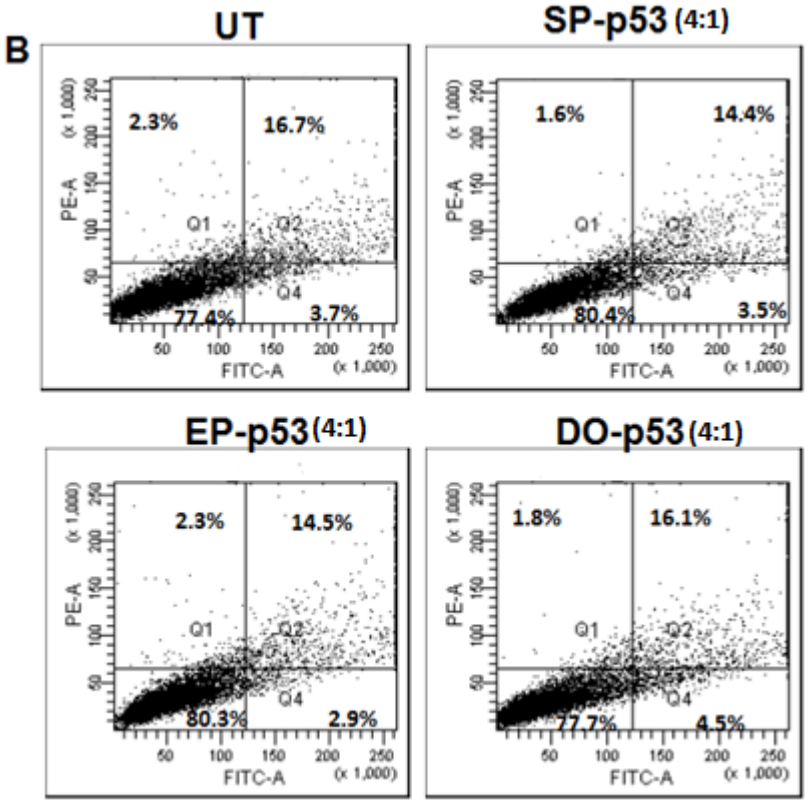

Fig. 4.27 (B) Flow cytometric analysis of apoptosis of CHO cells co-stained with annexin V-FITC and propidium iodide. Vertical and horizontal axes represent PI and FITC labeling respectively. Cells were either left untreated or treated with SP-p53, EP-p53 & DO-p53 lipoplexes for 24h at charge ratio of 4:1

To ascertain that the liposomal formulation is toxic towards cancer cells and has no effect on normal cells, free drug without liposomal encapsulation was also tested in MCF-7 and CHO cells. It is clear from Fig. 4.28, that free drug is equally toxic to the cells irrespective of their characteristics. Hence, we can conclude that our formulations are efficient in delivering genes and targeting cells in a specific manner.

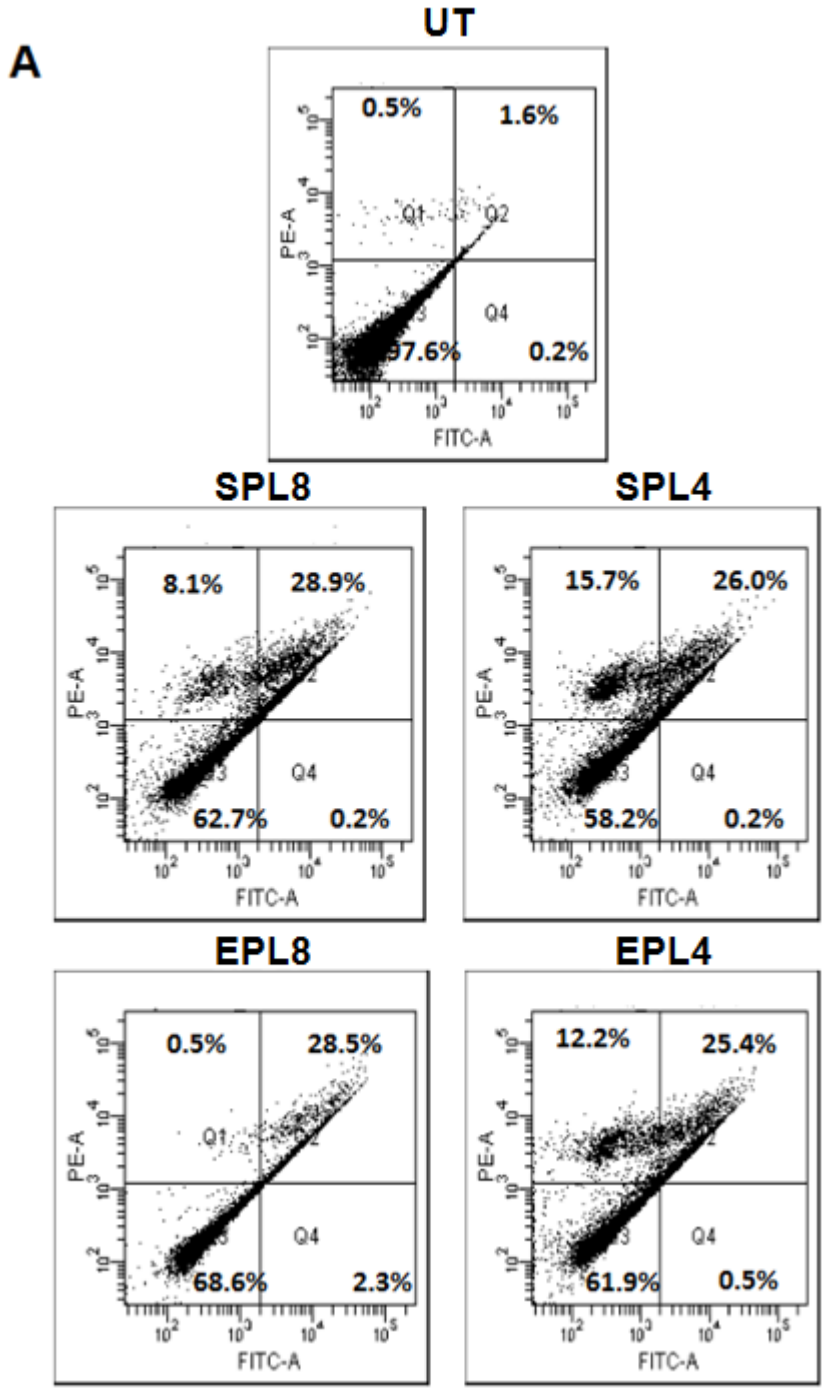

Fig. 4.28 Flow cytometric analysis of apoptosis of MCF-7 cells treated with free drug and co-stained with annexin V-FITC and propidium iodide. Vertical and horizontal axes represent PI and FITC labeling respectively. Cells were either left untreated or treated with different concentrations (equivalent to charge ratio) of free drug i.e. spironolactone & Eplerenone for 24h.

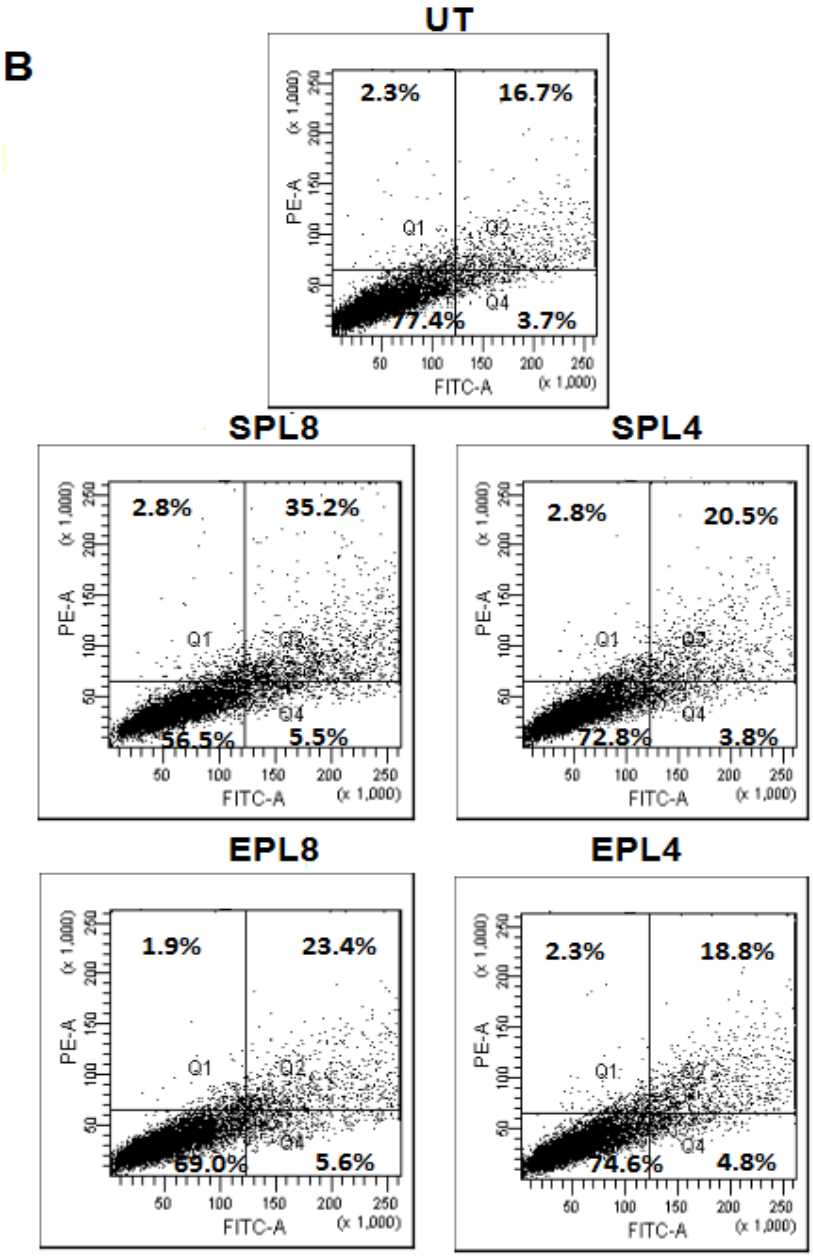

Fig. 4.28 Flow cytometric analysis of apoptosis of CHO cells treated with free drug and co-stained with annexin V-FITC and propidium iodide. Vertical and horizontal axes represent PI and FITC labeling respectively. Cells were either left untreated or treated with different concentrations (equivalent to charge ratio) of free drug i.e. Spironolactone & Eplerenone for 24h.

4.8 WESTERN BLOT STUDIES

4.8.1 Liposome mediated delivery causes apoptosis in cancer cells by initiating intrinsic pathway

Apoptosis is triggered through two signaling pathways: Intrinsic and extrinsic. Intrinsic pathway is often activated in response to signals resulting from DNA damage, loss of cell-survival factors, or other types of severe cell stress. Normally, pro-apoptotic proteins are released from the mitochondria to activate caspase proteases and trigger apoptosis. When these pro-apoptotic signals are not released, the cell cannot die (Cotter, 2009). The intrinsic pathway maintains the balances of activity between pro- and anti-apoptotic members of the Bcl-2 family, which act to regulate the permeability of mitochondrial membrane and subsequently apoptosis. Some members of the Bcl-2 family (such as BAX, BAK) take active part in the permeabilization of the outer mitochondrial membrane causing the release of Cytochrome c, thus acting as pro-apoptotic. On the other hand, others (such as Bcl-2, Bcl-xL) inactivate these pro-apoptotic Bcl-2 proteins by binding to them and ultimately prevent Cytochrome c release, thus acting as anti-apoptotic agent.

The extrinsic pathway begins outside the cell through activation of pro-apoptotic receptors on the cell surface. These are activated by molecules known as pro-apoptotic ligands. Preclinical studies show that ligand binding causes receptors to cluster and ultimately form a death-inducing signaling complex (DISC) (Mayer & Oberbauer, 2003). Upon DISC activation, the extrinsic pathway has been seen to adopt the same effector caspase machinery as the intrinsic pathway (Ashkenazi, 2002).

From Fig. 4.29, the induction of apoptosis is evident by the up-regulation of BAX, Cytochrome c and down-regulation of Bcl-2 proteins in SP-p53 treated MCF-7

cells. Anticancer gene p53 was transfected in cancer cells using SP, EP or one non-targeting liposome DO for 48h and cell lysates were prepared for western blot studies. Results indicated the increase of pro-apoptotic protein BAX and decrease of anti-apoptotic protein Bcl-2 protein upon SP-p53 lipoplex treatment compared to control groups. Mohan *et al*., 2012 studied the involvement of Bcl-2/BAX signaling pathways in the apoptosis of MCF-7 cells induced by a xanthone compound Pyranocycloartobiloxanthone A. Treatment of MCF-7 cells with PA induced apoptosis by down-regulation of Bcl2 and up-regulation of BAC, triggering the Cytochrome c release from mitochondria to cytosol.

A sharp increase of Cytochrome c protein was also observed upon SP-p53 treatment. Increase of Cytochrome c indicates the initiation of apoptosis and subsequent cell death as it is this protein which signals the cells to begin the process of programmed cell death (Goodsell, 2004). It is clear from Fig. 4.30 that when β-gal DNA was used to transfect cells with SP, EP & DO liposomes, no up-regulation or down-regulation of any the mentioned genes was observed. Altogether, this study shows that SP liposome can deliver anticancer gene like p53 in cancer cell and induce apoptosis.

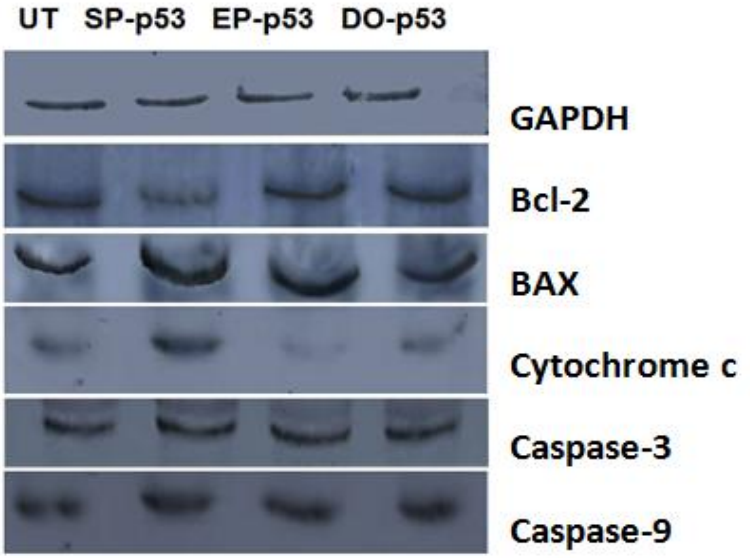

Fig. 4.29 Western blot analysis of MCF-7 cells treated with different groups for 24h Lanewise: (I) Untreated; (II) SP-p53 lipoplex; (III) EP-p53 lipoplex (IV) DO-p53 lipoplex

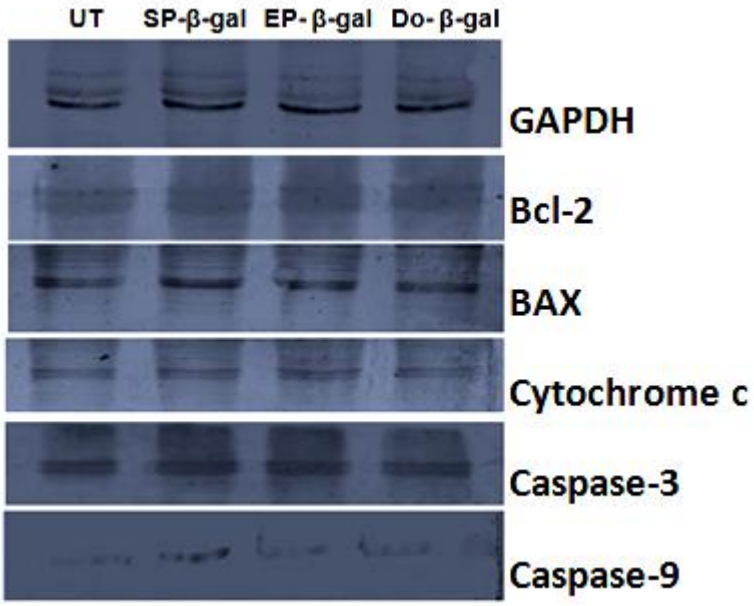

Fig. 4.30 Western blot analysis of MCF-7 cells treated with different groups for 24h Lanewise: (I) Untreated; (II) SP-β-gal lipoplex; (III) EP- β-gal lipoplex (IV) DO- β-gal lipoplex

4.9 MIGRATION AND INVASION ASSAY

4.9.1 SP & EP liposomes inhibit the migration and invasiveness of the cells

Cell migration is a highly integrated, multi-step process that plays an important role in the progression of cancer. The movement of cells from one area to another in response to chemical signal is central to achieving functions such as cell differentiation and tumor metastasis. Cell invasion is similar to cell migration; however, it requires a cell to migrate through extracellular matrix and then become established in a new location. Cancer cells possess a broad spectrum of migration and invasion mechanisms. These include both individual and collective cell-migration strategies. Cancer therapeutics that are designed to target adhesion receptors or proteases have not proven to be effective in slowing tumour progression in clinical trials — this might be due to the fact that cancer cells can modify their migration mechanisms in response to different conditions (Friedl & Wolf, 2003). There are diverse mechanisms that cells can employ to initiate and progress invasion and each offers specific pharmacologic targets for development of anti-metastatic therapies. In order to metastasize through surrounding tissue, tumor cells employ mechanisms that utilize matrix metalloproteases (MMP) and urokinase plasminogen activator (uPA) to digest the ECM. Invasion & migration studies were performed with B16F10 cell line. B16F10 is a well known melanoma cell which shows very high aggressiveness in mouse model. The aggressiveness in cancer cells are attributed partially to both invasive and migration activity.

For migration studies, cells were plated in 24-well transwell plate inserts (corning) with 8μM pore size. After 18-24 h, B16F10 cells were kept untreated or pre-treated with liposomes. 48 h post-treatment, cells in the lower chamber were counted. Less number of cells/count was found to be in the insert treated with SP and EP

liposomes as compared to those with untreated ones. No effect of DO liposomal treatment was found to be in the migration rate of cells. Cells were rather more in number as compared to untreated ones (Fig. 4.31). This can be because of the lipid nature of DO liposome, which is helping the cells to migrate. The data indicates that the liposomal formulation is inhibiting the cells to migrate from inserts to the lower chamber.

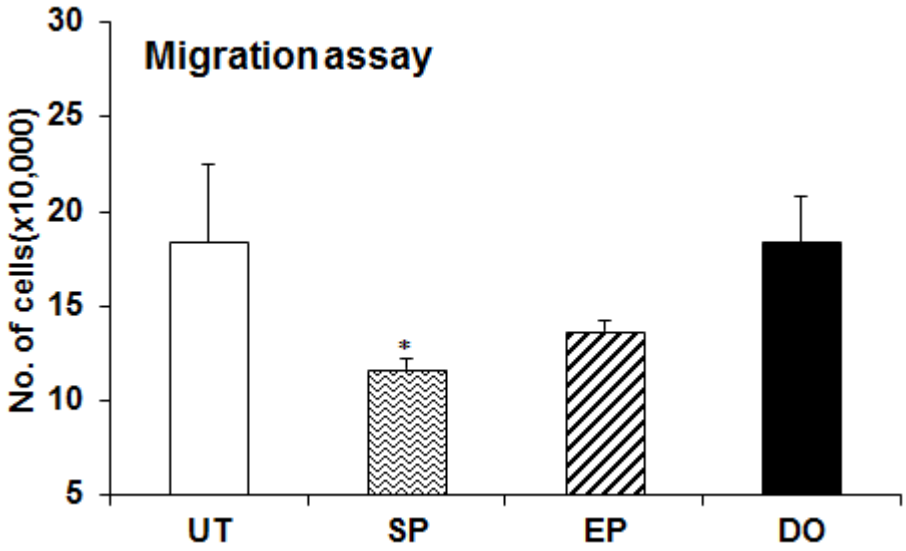

Fig. 4.31 Migration assay: B16F10 cells were kept untreated or pre-treated with liposomes. 48 h post-treatment, cells in the lower chamber were counted

For invasion studies, inserts were coated with 50-100μL of ECM gel before cell plating. After incubating the coated plates, cells were plated and treated with liposomes after 18-24 h. 48 h post-treatment; cell count was done in the lower chamber to check the effect of treatment on cell invasion. Wells treated with SP liposomes has shown a low cell count as compared to untreated ones, wherein cell count was found to be almost half of the untreated. Less number of cells was observed in wells treated with EP & DO liposomes too. This data indicates that the liposomal treatment is inhibiting the cells to cross the coated ECM and getting established in a new location. Number of cells was counted in two different ways: in the medium i.e. those cells that have just crossed the ECM (Fig. 4.32) and on the surface of the well i.e. those cells that have crossed the ECM and got established in new location (Fig. 4.33). Altogether, this experiment shows that the liposomal treatment is reducing the migration and invasiveness (Fig. 4.34) of the cells; and hence metastasis.

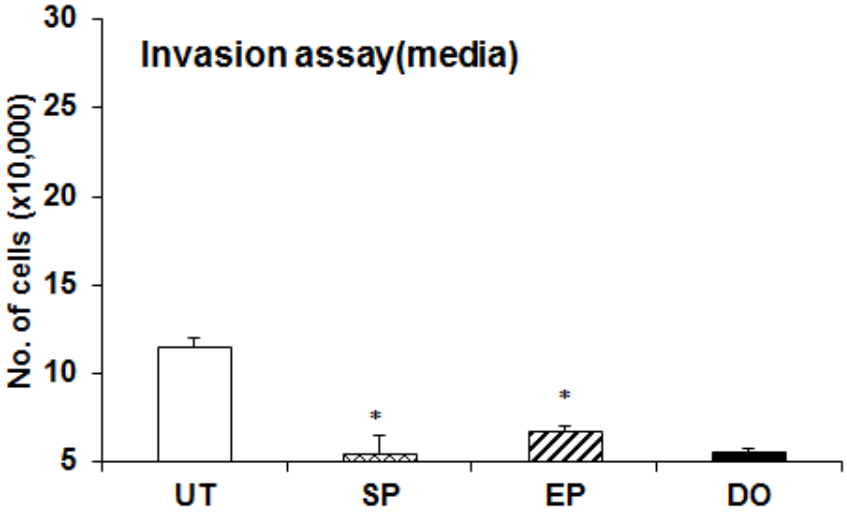

Fig. 4.32 Invasion assay: Inserts were coated with 50-100µL of ECM gel before cell plating. B16F10 cells were plated and treated with liposomes. 48 h post-treatment; cell count was done in the media of the lower compartment to check the effect of treatment on cell invasion

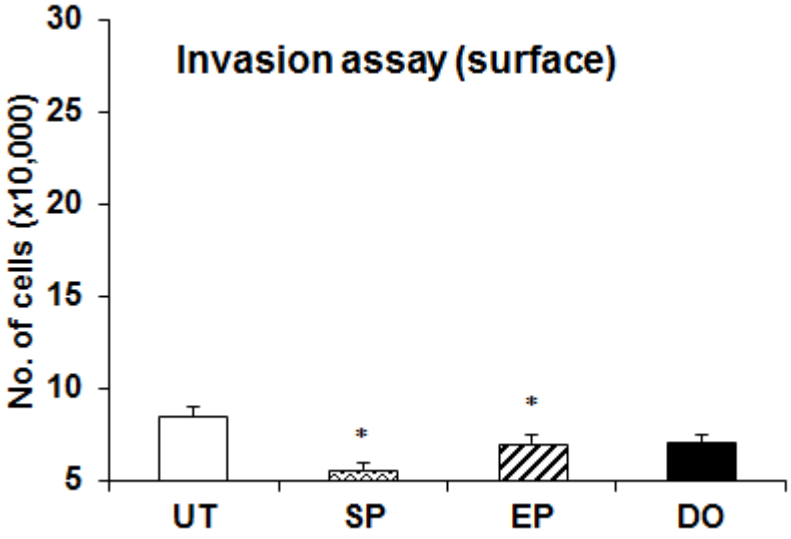

Fig. 4.33 Invasion assay: Inserts were coated with 50-100µL of ECM gel before cell plating. B16F10 cells were plated and treated with liposomes. 48 h post-treatment; cells were counted on the surface of lower compartment to check the effect of treatment on cell invasion

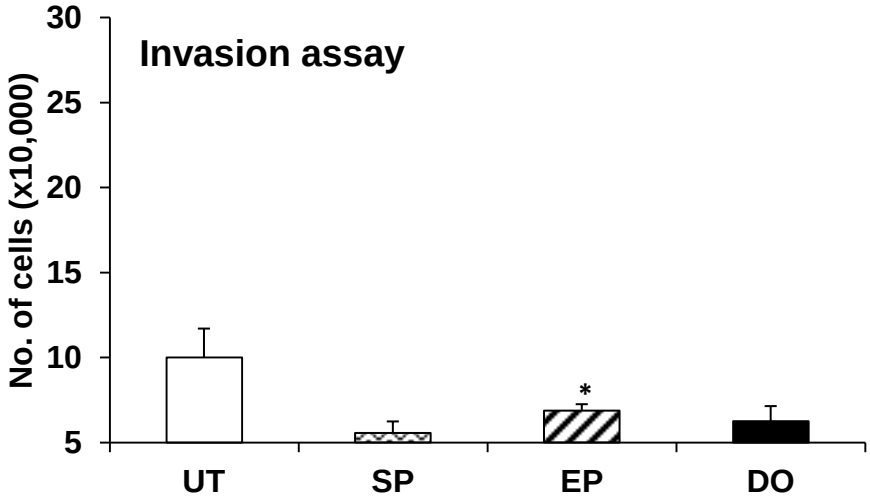

Fig. 4.34 Invasion assay: Inserts were coated with 50-100µL of ECM gel before cell plating. B16F10 cells were plated and treated with liposomes. 48 h post-treatment; cells were counted in the lower compartment to check the effect of treatment on cell invasion

Raw data for size and potential analysis of liposomes & lipoplexes

Table 4.7 A: Size measurement for DO liposome

Run	Angle	KCpS	Z Ave (nm)	Poly Index
1	90	410.2	120.1	0.291
2	90	383.1	125.6	0.299
3	90	392.8	123.2	0.276
Average	-	395.4	123.0	0.289
+/-	-	13.7	2.7	0.011

Table 4.7 B: Measurement of Zeta potential for DO liposome

Run	KCpS	Mobility	Zeta	Width
1	214.4	1.466	5.8	1.6
2	229.6	0.324	4.9	1.6
3	238.1	0.538	3.8	1.6
4	244.0	1.277	6.3	1.6
5	244.8	1.438	5.3	1.6
6	234.4	1.262	6.1	1.6
7	259.8	0.664	4.5	1.6
8	256.7	1.100	4.0	1.6
9	243.0	1.663	3.2	1.6
10	236.7	1.435	4.3	1.6
Average	240.1	1.117	4.8	1.6
+/-	13.0	0.452	1.0	0

Table 4.8 A: Size measurement for SP liposome

Run	Angle	KCpS	Z Ave (nm)	Poly Index
1	90	268.4	188.1	0.535
2	90	269.0	193.9	0.427
3	90	285.7	202.2	0.624
Average	-	274.4	194.7	0.524
+/-	-	9.8	7.1	0.098

Table 4.8 B: Table 4.5 Zeta potential for SP liposome

Run	KCpS	Mobility	Zeta	Width
1	381.5	1.466	18.7	1.6
2	351.6	0.324	4.1	1.6
3	362.1	0.538	6.8	1.6
4	399.0	1.277	16.3	1.6
5	351.8	1.438	18.3	1.6
6	355.3	1.262	16.1	1.6
7	359.8	0.664	8.5	1.6
8	356.7	1.100	14.0	1.6
9	353.0	1.663	21.2	1.6
10	336.7	1.435	18.3	1.6
Average	360.8	1.117	14.2	1.6
+/-	17.5	0.452	5.8	0

Table 4.9 A: Measurement of Zeta size for DO lipoplex

Run	Angle	KCpS	Z Ave (nm)	Poly Index
1	90	245.5	509.8	0.535
2	90	248.9	499.6	0.518
3	90	253.6	502.1	0.424
Average	-	249.3	503.8	0.492
+/-	-	4.06	5.3	0.059

Table 4.9 B: Measurement of Zeta potential for DO lipoplex

Run	KCpS	Mobility	Zeta	Width
1	199.1	0.116	1.5	1.6
2	219.9	0.192	2.4	1.6
3	115.9	0.186	2.4	1.6
4	216.4	0.241	3.1	1.6
5	134.6	0.188	2.4	1.6
6	206.8	0.117	1.5	1.6
7	229.4	0.117	1.5	1.6
8	207.9	0.190	2.4	1.6
9	198.2	0.185	2.4	1.6
10	178.2	0.198	2.5	1.6
Average	190.64	0.173	2.21	1.6
+/-		0.039		0

Table 4.10 A: Measurement of Zeta size for SP lipoplex

Run	Angle	KCpS	Z Ave (nm)	Poly Index
1	90	133.5	448.8	0.387
2	90	134.9	442.6	0.519
3	90	129.6	418.6	0.484
Average	-	132.6	436.6	0.463
+/-	-	2.7	15.9	0.06

Table 4.10 B: Measurement of Zeta potential for SP lipoplex

Run	KCpS	Mobility	Zeta	Width
1	135.0	0.148	5.8	1.6
2	128.9	0.211	4.9	1.6
3	138.4	0.244	3.8	1.6
4	144.0	0.245	6.3	1.6
5	142.8	0.246	5.3	1.6
6	126.4	0.242	6.1	1.6
7	138.8	0.229	4.5	1.6
8	144.7	0.233	4.0	1.6
9	143.0	0.253	3.2	1.6
10	144.7	0.235	4.3	1.6
Average	138.67	0.228	4.8	1.6
+/-	6.63	0.030	1.0	0

Table 4.11 A: Size measurement for EP liposome

Run	Angle	KCpS	Z Ave (nm)	Poly Index
1	90	256.0	152.3	0.527
2	90	254.0	156.5	0.550
3	90	243.8	153.0	0.580
Average	-	251.3	153.9	0.552
+/-	-	6.5	2.3	0.026

Table 4.11 B: Zeta potential for EP liposome

Run	KCpS	Mobility	Zeta	Width
1	246.7	1.685	21.5	1.6
2	257.8	1.099	14.0	1.6
3	251.1	0.872	11.1	1.6
4	258.5	0.781	9.9	1.6
5	254.6	0.597	7.6	1.6
6	240.6	1.440	18.3	1.6
7	262.5	0.720	9.2	1.6
8	230.0	1.128	14.4	1.6
9	242.5	1.266	16.1	1.6
10	235.5	1.425	18.1	1.6
Average	248.0	1.101	14.0	1.6
+/-	10.7	0.356	4.5	0

Table 4.12 A: Measurement of zeta size for EP lipoplex

Run	Angle	KCpS	Z Ave (nm)	Poly Index
1	90	56.8	618.1	0.515
2	90	59.3	623.8	0.477
3	90	62.8	598.1	0.484
Average	-	59.63	613.3	0.492
+/-	-	3.01	13.4	0.02

Table 4.12 B: Measurement of zeta potential for EP lipoplex

Run	KCpS	Mobility	Zeta	Width
1	211.5	1.013	3.7	1.6
2	251.6	1.018	4.1	1.6
3	262.3	0.938	4.8	1.6
4	219.0	1.007	4.3	1.6
5	251.3	0.438	4.2	1.6
6	255.1	0.762	3.1	1.6
7	239.8	0.774	3.5	1.6
8	256.2	1.005	4.3	1.6
9	243.0	0.763	4.2	1.6
10	236.7	0.435	4.8	1.6
Average	242.6	0.815	4.1	1.6
+/-	16.5	0.226	0.5	0

Cancer is one of the leading causes of deaths worldwide and has a major impact on society across the world. Chemotherapeutic drugs or agents have major disadvantages because of non-specific killing of normal cells and often create adverse side effects. There is intensive on-going research to improve the treatment of cancer by targeted **gene/drug** delivery systems that includes new chemical entity development and the development of novel ways to deliver existing drugs leading to improved therapeutic index and reduced side effect. *In vivo* gene transfer using viral vectors is today the most commonly used approach (Lim *et al*., 2010). This approach takes advantage of the viruses' ability to deliver their genetic material to target cells, including non-dividing cells, and to induce long-term transgene expression. The disadvantages of viral delivery include generation of immune responses to expressed viral proteins that subsequently kill the target cells required to produce the therapeutic gene product, random integration of some viral vectors into the host chromosome, clearance of viral vectors delivered systemically, difficulties in engineering viral envelopes or capsids to achieve specific delivery to cells (Templeton, 2001). Improved delivery is made possible by encapsulating the drug in novel drug delivery systems such as, liposomes, nanoparticles, micelles, microspheres and emulsions.

Liposomes are the extensively studied and most successful for systemic and targeted delivery. There are several studies going on for the development of cancer cell targeted delivery systems using liposomes. Currently, liposomal nanoparticles

(LNs) encapsulating therapeutic agents, or liposomal nanomedicines, represent an advanced class of drug development, with several formulations in clinical trials. Over the past 20 years, a variety of techniques have been developed for encapsulating both conventional drugs (such as anticancer drugs and antibiotics) and the new genetic drugs like plasmid DNA containing therapeutic genes, antisense oligonucleotides and small interfering RNA (Bolhassani *et al*., 2011). Intercellular as well as cell surface receptors, those behave differently in normal and cancer cells; have emerged as lucrative candidate for targeted therapy (Sharma *et al*., 2013; Puri *et al*., 2009). Since steroid hormone receptors (SHRs) are ubiquitously expressed in cells, targeting these receptors–could be an avenue to target various cancer cells using a single delivery system.

SHRs maintain a cytosolic inactive state by association with heat shock proteins and/or other proteins such as co-repressors. Upon ligand binding, the SHRs undergo conformational changes that involve release from the repressor proteins, and translocation to the nucleus where it can bind to specific hormone responsive sequences in the DNA of genes regulated by steroid hormones (Beato & Klug, 2000; Aranda & Pascaul, 2001). In one of the previous studies, glucocorticoid receptor (GR) mediated delivery system was developed for the systemic delivery of genes in cancer cells. Dexamethasone, a synthetic ligand of GR was used in the liposomal formulation

to exploit the LBD of GR in cancer cell (Mukherjee *et al*., 2009). In the present study, we have chosen mineralocorticoid receptor (MR), another member of SHRs' family towards the development of targeted delivery system.

5.1 Mineralocorticoid receptor mediated transfection of exogenous gene in cancer cells

Spironolactone, a synthetic MR ligand; when formulated with a cationic lipid (DODEAC) and co-lipid cholesterol, has efficiently formed a stable liposome (SP). The SP liposomal formulation forms stable lipoplex with plasmid DNA at different liposome to plasmid DNA charge ratios and is able to transfect cancer cells with efficacy (Fig. 4.2). Ratio of number of positive charges originating from cationic lipid and number of negative charges from DNA's phosphates of lipoplexes are important physicochemical characteristics for the transfection efficiency of cationic lipid formulation into liposome. Lipoplexes with a charge ratio of 8:1 to 2:1 have given a stable formulation while 1:1 lipoplex was found to be weak.

Liposomal formulation SP shows highest transfection efficiency when liposomal formulation contains DODEAC, Cholesterol and Spironolactone at a molar ratio of 1:1:0.75 compared to other compositions (Fig 4.3). SP liposome shows higher transfection efficiency at 8:1 & 4:1 liposome to plasmid DNA charge ratios in cancer cell. Since, spironolactone share some structural similarity with cholesterol, we

incorporated cholesterol along with spironolactone. The novel liposomal formulation (SP) was able to transfect cancer cells preferentially with higher efficacy compared to normal cells. It is reported that spironolactone is specific to MR (Rogersen *et al*., 2004) but mineralocorticoid ligands have the ability to bind to both MR as well as GR as they share structural similarities in their LBD. The human MR of 984 amino acids has 57% amino acid identity with GRα in the ligand binding domain (LBD), and 94% in the DNA binding domain (DBD) (Funder, 1997). The receptor was inhibited with RU486 (which is common to both GR and MR) and Eplerenone (which is MR specific). Receptor inhibition with either of the inhibitors resulted in decrease in transfection efficiency in cancer cells (A549 and MCF-7) while there was no effect of inhibition in normal cells (CHO and HEK293). Previously, Glucocorticoid receptor was targeted by directly incorporating Dexamethasone, synthetic GR ligand, alongside the regular co-lipid cholesterol in the cationic lipid–associated gene delivery formulation. Similarly, we incorporated Spironolactone (Spiro, the synthetic MR-ligand) in cationic lipid formulation so that the cationic lipid/DNA complex (lipoplex) would be able to target cellular MR by its binding to MR-LBD and safely localize the Spiro-associated–lipoplex and the genetic cargo inside the nucleus.

Eplerenone, which is another mineralocorticoid receptor antagonist that acts by binding to MR and blocking the natural ligand aldosterone, was used for another

liposome formulation (EP) to test whether all steroid hormone ligands can be exploited for stable liposome formulation and cancer cell targeting or not. EP lipoplexes were formed with different charge ratio (i.e 8:1, 4:1, 2:1, 1:1) and the stability of the lipoplexes were checked by DNA-binding studies. Lipoplex with charge ratio 1:1 did not form a stable complex while others did and it can be because of the non-availability of cations for complexion with anionic DNA.

EP liposomes lipoplexed with β-gal DNA were used for transfecting cells of cancer and non-cancer nature and after 48 h of treatment, cells were assayed for β-galactosidase activity. Results similar to SP liposome mediated transfections were obtained with EP liposomes and were found to be efficient in cancer cells (A549 and CHO), while no effect of EP liposome treatment was observed in normal cells. Liposomes with a molar ratio of 1:1:1 i.e. EP(M1) & 1:1:0.75 i.e. EP(M2) were found to be transfection efficient at a charge ratio of 8:1 and 4:1(Fig. 4.17). But while comparing the transfection efficiencies of SP & EP formulations, we find that SP formulations are far more efficient in transfecting cells as compared to EP. Though transfection with EP lipoplexes did not affect normal cells (CHO and HEK293), but were found to be less transfection efficient than SP formulation. From this data, we conclude that all steroid hormone receptor ligands (or specifically MR ligands) cannot be exploited for the formulation of efficient delivery vehicle.

Mukherjee *et al*., 2009 had shown that GR behaves aberrantly in cancer cells due to the compromised role of heat shock protein 90 (Hsp90), a chaperonic protein which maintains the structural integrity of ligand binding domain (LBD) of GR. GR mediated transfection was possible in cancer cells over normal cells. When Hsp90 was inhibited in normal cells, there was GR mediated transfection in normal cells too like cancer cells. It shows the compromised role of Hsp90 in cancer cells allows Dexamethasone associated liposomal formulation to act via GR (Mukherjee *et al*., 2009). Hsp90 is also involved in maintaining structural integrity of other nuclear hormone receptors including mineralocorticoid receptors (Smith *et al*., 1992). It can be said that the compromised role of Hsp90 in cancer cells could allow mineralocorticoid receptor to behave adversely and also help MR-mediated liposomal delivery of genetic cargo into nucleus like GR.

To understand the MR specific delivery of SP, mineralocorticoid receptor expression was down-regulated using MR-siRNA and transfection was studied. MR is overexpressed in many cancers (Santanders *et al*., 1965) and this could be the reason of receptor down-regulation in cancer cells at a low siRNA concentration as compared to normal cells. The extent of down-regulation of mineralocorticoid receptor was measured by reverse transcriptase PCR studies using MR primers (Fiore *et al*., 2006) after 24 h, 48 h and 72 h. After PCR, the samples were run on 1% agarose gel. The

results obtained indicated that after 48h of MR-siRNA treatment there was MR down-regulation in cancer cells and transfection efficiency of SP liposome was significantly down in MR-down-regulated cell line. But, there was no change in transfection values in normal cells before and after the MR-siRNA treatment. No change in cytotoxicity in normal cell was observed even after the down-regulation of MR. siRNA mediated down-regulation of MR expression caused decrease in the MR expression level leading to the increase in cell viability and thus a decrease in cellular toxicity. Altogether, this shows that SP liposome works via MR which behaves differently in cancer cells that helps SP to deliver genes preferentially in cancer cells over normal cells. No significant effect of siRNA down-regulation on transfection and cellular toxicity with EP liposomal formulation was observed. Cells were treated with 50pmol of siRNA before liposomal treatment. Cells were checked for any effect of receptor down-regulation on transfection efficiency and cytotoxicity. It was observed that there was a small decrease in transfection efficiency of EP liposomes when the receptor was down-regulated with siRNA in A549, down-regulation effect was observed in CHO cells too. Since, EP having MR ligand Eplerenone associated with it was found to be able to transfect cells and cause toxicity in cell-specific manner like SP liposome but could not affect transfection and toxicity even after MR siRNA down-regulation, further investigation is required.

Till now, we observed a difference in transfection efficiency and toxicity of SP and EP liposomes, but this difference could be because of difference in liposomal uptake efficiency in these cells. SP and EP lipoplex containing rhodamine-linked "red-fluorescent" lipid was treated to both cancer (MCF-7 and B16F10) and normal CHO cells. After 4 hr of treatment, respective cellular lysates were quantitatively estimated for its red-fluorescence content i.e. uptake efficiency by a microplate fluorescence reader. We found that there was no significant difference in lipoplex uptake of respective cells and lipoplex uptake was unrelated to transfection efficiency.

5.2 Targeted induction of apoptosis in cancer cells

As our liposomal formulations (SP& EP) were able to deliver reporter gene into cancer cells preferentially over normal cells, we wanted to observe how SP and EP mediated delivery of an anti-cancer gene p53 affects cancer & normal cells. So, towards probing the effect of SP-& EP-mediated p53 gene delivery in inducing apoptosis in cancer cells, Annexin V/Propidium iodide (PI) binding based flow cytometric apoptosis assay was performed. Before apoptosis, the cells were tested for SP-& EP-mediated cytotoxicity. In MCF-7 and A549 cells, toxicity was observed when p53 plasmid DNA was used for transfection. Cells transfected with control β-gal DNA showed no toxicity (Fig. 4.12; Fig.4.23). This shows that liposome itself is non-toxic towards cancer and normal cells, while the formulation having p53 is toxic towards cancer cells. On the other hand, we wanted to see whether SP & EP liposome itself possesses any cytotoxic effect to cells. We found that SP-β-gal & EP- β-gal

lipoplex causes no significant cytotoxicity in normal and cancer cells but same amount of free drug spironolactone & Eplerenone causes cell death (Fig. 4.13; Fig. 4.24).

Data indicates that the lipoplex SP-p53 and EP-p53 could induce apoptosis in cancer cells as revealed by flow cytometric studies (Fig.4.26). Although, EP liposome could not affect transfection upon MRsiRNA down-regulation, but induce apoptosis in cancer cells. The treatment of SP-p53 & EP-p53 has no cytotoxic effect on normal cells. We found that SP-β-gal lipoplex and EP- β-gal lipoplex causes no significant cytotoxicity in normal and cancer cells but same amount of free drug spironolactone causes cell death irrespective of the cell nature. To our knowledge, this is the first study showing that MR, specifically SP formulation, could be utilized for inducing apoptosis in cell specific manner.

Most of the anticancer, agent-mediated cancer cell killing occurs through induction of apoptosis, the important process involved in programmed cell death (PCD). The ability of tumor cells to evade engagement of apoptosis plays a significant role in their resistance to conventional therapeutic regimens. Out of the two major pathways of apoptosis (i.e., intrinsic or extrinsic), intrinsic pathway involves the release of pro-apoptotic proteins that activate caspase enzymes from the mitochondria. This process ultimately triggers apoptosis (Fulda & Debatin, 2006). The components

of extrinsic pathway depend on intrinsic components. In a recent study, nuclear hormone receptors (NHR) in only cancer cells were easily targeted and manipulated for nuclear delivery of cationic lipid components when the lipid was pre-associated with the NHR-ligand (Mukherjee *et al*., 2009). SP-p53 lipoplex was found to trigger intrinsic pathway of apoptosis in cancer cells (Fig. 4.29). MCF-7 cells were transfected with p53 gene using targeted liposome SP and western blot data shows the increase of pro-apoptotic protein BAX and the decrease of anti-apoptotic protein Bcl-2. There was sharp increase of Cytochrome c protein indicated apoptotic cell death which was also confirmed by FACS study. Mohan *et al*., 2012 studied the involvement of Bcl-2/BAX signaling pathways in the apoptosis of MCF-7 cells induced by a xanthone compound Pyranocycloartobiloxanthone A. Treatment of MCF-7 **c**ells with PA induced apoptosis by down-regulation of Bcl-2 and up-regulation of BAX, triggering the Cytochrome c release from mitochondria to cytosol. Increase of Cytochrome c indicates the initiation of apoptosis and subsequent cell death as it is this protein which signals the cells to begin the process of programmed cell death (Goodsell, 2004). In a healthy cell, the outer membranes of its mitochondria display the protein Bcl-2 on their surface. Bcl-2 inhibits apoptosis. Internal damage to the cell causes a related protein, BAX, to migrate to the surface of the mitochondrion where it inhibits the protective effect of Bcl-2 and inserts itself into the outer mitochondrial membrane punching holes in it and causing Cytochrome c to leak out.

No change in apoptotic regulatory proteins was observed when cells were treated with EP-p53 lipoplex (Fig. 4.29). All synthetic ligands of MR may not be able to provide a formulation which can compete with natural ligand aldosterone and can target cells in specific manner and this can be the reason why small effect of MR siRNA down-regulation has been found in cancer cells.

A number of recent studies have defined a form of cell death that proceeds independently of Apaf-1 (extrinsic pathway) and caspase activation but is subject to regulation by factors typically associated with the apoptotic cascade. Following a variety of apoptotic stimuli, apoptosis inducing factor (AIF) is released from mitochondria via a mechanism regulated by Bcl-2 (Susin *et al*., 1999) and engages a form of cell death characterized by a number of biochemical and morphological alterations, none of which are altered by the caspase inhibitor zVAD-fmk (Cregan *et al*., 2002). These observations appear to define a regulated form of cell death that retains certain features of apoptosis but cannot be classified as necrosis. Recent descriptions of an alternative Apaf-1-independent but caspase-dependent form of cell death also imply the existence of a novel mechanism for the activation of caspases (Haraguchi *et al*., 2000; Rao *et al*., 2002). These studies describe one or more forms of Apaf-1-independent death engaged by specific stimuli, including endoplasmic reticulum stress (Rao *et al*., 2002), serum withdrawal (Haraguchi *et al*., 2000) that can

be suppressed by Bcl-2, caspase inhibitors, and significantly attenuated by the overexpression of catalytically inactive forms of caspases-9 and-12 (Rao *et al*., 2002). This series of observations indicates a complexity of caspase-dependent cell death that is not completely understood.

This study shows a synthetic ligand of MR (SP) when incorporated into liposomal formulation can target MR and deliver gene in cancer cells. In other words, SP formulation when complexed with anticancer gene, p53 is able to trigger intrinsic pathway for induction of apoptosis. EP-p53 also resulted in induction of apoptosis in cancer cells specifically but the mechanism is still not clear and requires further investigation. This opens a new way of developing targeted delivery system for improved anti-cancer therapeutics.

5.3 Inhibition of cell metastasis/ antimetastatic effect

Metastasis is a complex process that involves the spread of a tumor or cancer to distant parts of the body from its original site. However, this is a difficult process. To successfully colonize a distant area in the body a cancer cell must migrate and invade through tissues and penetrate the basement membrane. Cell migration and invasion are crucial steps in the pathophysiology of cancer. Indeed, the capacity to produce metastases, very different among cancers, is the main features of malignant tumors and it is one of the main causes of death for cancer. This is due to the fact that

metastases are constituted by cells much more resistant, aggressive and efficient than those forming the primary tumor (Bozzuto *et al*., 2010).

Cell metastasis experiment was performed in B16F10 cells which are very aggressive in nature in terms of migration and invasion. For migration assay, cells were directly plated in trans-well membrane inserts of pore size 8µm. Cells were treated with different formulations (SP, EP & DO) and post-treatment were checked for migration activity. Treatment of cells with targeted SP & EP liposomes led to an inhibition in the migration activity of cells; while the non-targeted liposome, DO did not inhibit the migration activity. Ogasawra *et al*., 2001 utilized Transwell cell culture chambers in a 24-well format to screen 75 types of natural compounds from a variety of chemical classes for their ability to inhibit migration of the murine colon adenocarcinoma cell line 26-L5.For invasion assay, inserts were coated with ECM gel prior plating. Cells were treated with different formulations and checked for invasiveness. Cells in the medium of lower compartment and those that got attached (located) on the surface were counted. SP & EP liposomal treatment led to the decrease in invasiveness of the cells (Fig. 4.34). Similar kind of results was obtained in migration studies. Cells treated with DO liposomes also led to decrease in invasiveness of the cells while no effect of the same was found on cell migration. Though, the EP liposomal formulations were not able to transfect cells efficiently, but

could induce apoptosis in cancer cells and inhibit the metastatic activity of cells to a significant extent. So far, there are no reports mentioning the role of mineralocorticoid receptor ligand in inhibition of metastasis. The potential role of MR in cancer therapeutics is being demonstrated for the first time through this study. This data adds to our knowledge that SP & EP can inhibit tumor metastasis and MR is a valid therapeutic target for cancer.

So, the present study has opened a novel way of targeting drugs to cancer cells through mineralocorticoid receptor mediated pathway.

Liposomes are extremely useful and powerful carrier system for effective and controlled delivery of drugs. Liposomes have moved a long way to become a pharmaceutical carrier of choice for numerous practical applications. The range of medical applications of liposomes extends from chemotherapy of cancer to gene therapy. Chemotherapeutic drugs or agents have major disadvantages because of non-targetibility and often create adverse side effects towards normal tissues. There are several studies going on for the development of cancer cell targeted delivery systems. Intercellular as well as cell surface receptors, those behave differently in normal and cancer cells; have emerged as lucrative targets for targeted therapy. Steroid hormone receptors (SHRs) ubiquitously express in many cells and hence, targeting of this class of proteins could help to target various cancer cells using a single delivery system. SHRs maintain a cytosolic inactive state by association with heat shock proteins and/or other proteins such as co-repressors. Upon ligand binding, the SHRs undergo conformational changes that involve release from the repressor proteins, and translocation to the nucleus where it can bind to specific hormone responsive sequences in the DNA of genes regulated by steroid hormones.

Liposome technology creates an ample opportunity for formulation and delivery of a wide variety of difficult-to-deliver therapeutic agents, including genes, peptides, siRNA or RNAi, protein, and growth hormones. Cationic lipid/co-lipid formulations are extremely powerful tools for delivery of anticancer agents/genes. In the present

thesis, we show that spironolactone (SP) and Eplerenone (EP), when formulated with a cationic lipid (DODEAC) and co-lipid cholesterol, has efficiently formed stable liposome. Lipoplexes are able to transfect cells with efficacy, induce apoptosis in cancer cells and inhibit migration & invasion.

Specific conclusions of the thesis are:

- Spironolactone and Eplerenone (mineralocorticoid receptor ligands) when associated with aqueous formulation of cationic lipid or liposome gave a stable formulation at a charge ratio of 8:1 , 4:1 and 2:1 as concluded by DNA-binding studies. Lipoplexes with equal concentration of liposomes and DNA (1:1) was found to be unstable.
- Lipoplexes with β-gal DNA are able to transfect cancer cells with efficacy in cell specific manner with the maximum transfection efficiency at a charge ratio of 8:1 as assayed by β-galactosidase activity.
- There was no significant difference in lipoplex uptake of respective cells and lipoplex uptake was unrelated to transfection efficiency.
- MR down-regulation studies using MRsiRNA proved that the transfection of cancer cells was MR-mediated, wherein MR down-regulation was observed in MR over-expressed cells while no effect on normal cells.

- Formulation (SP) can be used for selective delivery of anticancer gene p53, to elicit target-specific toxicity in MR over-expressed cancer cells.
- Lipoplex SP-p53and EP-p53 could induce apoptosis in cancer cells as revealed by cytotoxicity and flow cytometric studies. Although, EP liposome could not affect transfection upon MRsiRNA down-regulation, but induce apoptosis in cancer cells.
- MR-mediated SP liposomal delivery system induced apoptosis in cancer cells in a targeted manner by initiating intrinsic pathway of apoptosis as analysed by Western blots.
- No change in apoptotic regulatory proteins was observed when cells were treated with EP-p53 lipoplex.
- Lipoplex treatment interfere with the migration and invasion of the cells. Treatment of cells with SP and EP lipoplexes inhibited cell migration and invasion to a significant extent.

In brief, we conclude that this is the first study demonstrating mineralocorticoid receptor to be an effective target for the delivery of anticancer agents in cancer cells and this opens a novel way of developing targeted delivery system for improved anti-cancer therapeutics.

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Future scope of work

- The present work elucidates the potential of Mineralocorticoid receptor ligand in cancer cell targeting. Although Spironolactone associated liposome has shown a broad spectrum of transfection activity, cytotoxicity, apoptosis and anti-metastatic activity, extensive studies are still required for other ligand, Eplerenone ,which has proven to have anti-apoptotic and anti-metastatic potential but could not work similarly like SP formulation in terms of transfection efficiency and receptor targeting.
- This study indicates that mineralocorticoid receptor can be a good target for the delivery of anticancer agents in cancer cells and opens a new possibility for targeted anticancer therapy but further mechanistic studies are yet to be carried out as all the ligands do not act similarly for cancer targeting.
- Tumor targeting with receptor ligand provides a basis for the development of new diagnostic and therapeutic approaches for cancer.
- Preclinical and clinical studies are yet to be carried out for cancer therapeutics
- Liposomal formulations can be used to target other MR-related diseases

Limitations of the research

Although a variety of receptor-targeted therapeutic approaches have shown promising results *in vitro* and in pre-clinical models *in vivo*, several important parameters need to be determined and optimized to ensure successful translation of these technologies to clinical application. Ongoing and future work in the field need to correlate the extent of receptor expression to the amount of ligands needed for effective targeting. It is envisioned that continued research in this area will also enable identification of tumors where receptor-targeted approaches can provide additional advantages over non-targeted ones. This information is highly critical, because although several cancers are known to have upregulation of certain receptors, the level of upregulation may or may not be sufficient to warrant additional benefit from active targeting over and above passive accumulation. Furthermore, cancers with high receptors expressions are often found to have widespread hypoxic regions that become barriers for liposomes to permeate and penetrate throughout the tumor volume effectively. Therefore, in such cases even if the particles themselves may have excellent receptor-targeting capability, getting enough particles to penetrate throughout the tumor volume can be a challenge, and this will be a critical component of ongoing and future research.

List of Publications

Papers published in Peer Reviewed Journals

- **Sharma P**, Banerjee R and Narayan KP (2015) Mineralocorticoid receptor mediated liposomal delivery system for targeted induction of apoptosis in cancer cells (accepted in Biochimica et Biophysica Acta-Biomembranes)
- **Sharma P**, Banerjee R and Narayan KP (2015) **:** Development of steroid hormone receptor-targeted stable liposomes for delivery in cancer cells (under review in Data in brief)
- **Sharma P**, Banerjee R and Narayan KP (2013) Gene Therapy: Potential Use of Liposomes. Int. J. Pharm. Sci. Rev. Res., 23(1): 126-132
- **Sharma P**, Banerjee R and Narayan KP (2014) Liposomes for controlled drug delivery: drugs of the future. Journal of Pharmacy Research. 8(5),637-641.

Abstracts published in Conferences/Seminar/Symposia

- **Priyanka Sharma**, Rajkumar Banerjee, Kumar Pranav Narayan (2014) Steroid hormone receptor mediated liposomal delivery system induces apoptosis in cancer cells in a targeted manner. 5th International Conference on Stem Cells and Cancer (ICSCC-2014): Proliferation, Differentiation and Apoptosis held at JNU, Delhi.
- **Priyanka Sharma**, Rajkumar Banerjee, Kumar Pranav Narayan (2014) Phytoextracts from highly saponin producing plants induces cytotoxicity and apoptosis in cancer cells. International Conference on Chemical Biology (ICCB-2014) held at CSIR-IICT, Hyderabad.

List of conferences

- Presented poster at 5th International Conference on Stem Cells and Cancer (ICSCC-2014): Proliferation, Differentiation and Apoptosis on the topic entitling "Steroid hormone receptor mediated liposomal delivery system induces apoptosis in cancer cells in a targeted manner" held at Jawaharlal Nehru University, New Delhi.
- Presented poster at International Conference on Chemical Biology on the topic entitling "Phytoextracts from highly saponin producing plants induces cytotoxicity and apoptosis in cancer cell" held at CSIR-IICT (Indian Institute of Chemical Technology), Hyderabad.
- Attended National Symposium on Human Diseases held at BITS Pilani, Hyderabad Campus.

Award

- Won best poster presentation award for poster titled " Steroid hormone receptor mediated liposomal delivery system induces apoptosis in cancer cells in a targeted manner" held at Jawaharlal Nehru University, New Delhi

Biography of Priyanka Sharma

Priyanka Sharma has done her M.Sc. Biotechnology and M.Phil. Biotechnology from Himachal Pradesh University, Shimla. She had been the recipient of **Himachal Pradesh University-Junior Research Fellowship** (HPU-JRF) of the year 2008-2009 for carrying out M.Phil & Ph.D. She had been a teaching assistant at Jaypee Institute of Information Technology, Noida for one year after that she joined Ph.D Program at BITS Pilani, Hyderabad campus. She had been working as a research scholar at BITS Pilani, Hyderabad Campus from 2011-2015 under the supervision of Dr Kumar Pranav Narayan.

Priyanka Sharma has attended International and National Conferences and had got Best Poster Presentation Award for the topic entitled " **Steroid hormone receptor mediated liposomal delivery system induces apoptosis in cancer cells in a targeted manner**" in 5th International Conference on Stem Cells and Cancer (ICSCC-2014): Proliferation, Differentiation and Apoptosis held at JNU, New Delhi.

Biography of Dr Kumar Pranav Narayan

Dr Kumar Pranav Narayan is Assistant Professor in Biological Sciences since 2008. He has interest in cancer therapeutics using targeted gene delivery system and phyto-compound for targeted therapeutics. He has also worked in the field of agriculture microbiology such as molecular biodiversity of cyanobacteria and bacteriocinogeny for the development of competitive strain of *Mesorhizobium ciceri* as potential biofertilizer.

Dr Narayan received Doctoral degree from Jamia Millia Islamia, New Delhi under joint supervision of Dr. Y. D. Gaur, Division of Microbiology, Indian Agricultural Research Institute, New Delhi and Dr. Arif Ali, Professor, Department of Biosciences, Jamia Millia Islamia, New Delhi. He had been Postdoctoral Senior Research Fellow in Division of Microbiology, Indian Agricultural Research Institute, New Delhi in the field of molecular biodiversity analysis of cyanobacterial germplasm and Postdoctoral Research Associate in Lipid Science and Technology, Indian Institute of Chemical Technology (IICT), Hyderabad in the field of Gene Therapy for Cancer.