Possible Mechanism of Action of Albumin Conjugated Curcumin on Lung Cancer Cells.

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DECLARATION

I, **SREEKUTTY.J**, hereby declare that the thesis work entitiled '**Possible Mechanism of Action of Albumin Conjugated Curcumin on Lung Cancer Cells**' was done by me under the direct guidance of **Dr. Lissy K Krishnan, Scientist G, Division of Thrombosis Research**, Biomedical technology wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram, Kerala, India. External help sought are acknowledged.

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CERTIFICATE

This is to certify that the thesis work entitiled '**Possible Mechanism of Action of Albumin Conjugated Curcumin on Lung Cancer Cells**' submitted by **Sreekutty J** (2016/MPhil/07) in partial fulfillment for the Degree of Mater of Philosophy in Biomedical Technology was done under my supervision and guidance at Division of Thrombosis Research, Biomedical technology wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram, Kerala, India.

Place: Thiruvananthapuram
Date: 19/7/2017

Dr. LISSY K KRISHNAN

The Thesis Entitiled

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Submitted by

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Abbreviations

%	Percentage
µl	Microlitre
µm	Micrometer
BSA	Bovine serum albumin
FACS	Flourescence activated cell sorting
FITC	Fluorescein isothiocyanate
g	Gravity
kDa	Kilodalton
L	Litre
mg	Milli gram
ml	Milli litre
NaCl	Sodium chloride
NaOH	Sodium hydroxide
PBS	Phosphate buffered saline

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Synopsis

Anticancer drug**,** also called antineoplastic drug, is a molecule effective in the treatment of malignant, or cancerous disease. The initial requirement for drug action is adequate delivery to the target site. The treatment of cancer is complicated in that the drugs used target human cells, albeit cells that have undergone genetic changes and are dividing at a fast and uncontrolled rate. Anticancer agents are generally toxic to normal cells and can cause numerous side effects, some of which are life-threatening. Such side effects include hair loss, sores in the mouth and on other mucous membranes, cardiac anomalies, bone marrow toxicity, and severe nausea and vomiting.In order to reduce such complications there has been a surge of interest in herbal remedies for treatment of cancer.One such compound is curcumin.

Curcumin, is the active component of turmeric, a common Indian spice, which is derived from the dried rhizome of the *Curcuma longa* plant. It is extensively used in Ayurveda, Unani, Siddha, and Chinese medicine for the management of various diseases such as wound, inflammation, and cancer and used in curries and dishes especially in spicy dishes in India. Curcumin is lipophilic in nature which shows low solubility and stability in aqueous solution. Various studies have shown that curcumin modulates numerous targets. These include the growth factors, growth factor receptors, transcription factors, cytokines, enzymes, and genes regulating apoptosis. The reasons for reduced bioavailability of albumin within the body are low intrinsic activity, poor absorption, and high rate of metabolism, inactivity of metabolic products and/or rapid elimination and clearance from the body.In order to increase the bioavailability, we conjugate curcumin(Curc) with serum albumin (Alb) to obtain Curc-Alb.

Albumin is a multifaceted, highly soluble, stable, non-toxic, non-poisonous, biocompatible and biodegradable plasma protein. Because of its versatile nature, it can be used for the delivery of the drugs, hormones, metals and fatty acids by binding to its specific binding sites. Albumin is best known for its remarkable ligand binding capacity. It is a versatile protein with antioxidant, immunomodulatory, detoxifying properties and can act as a potent drug carrier. It is important to understand if Alb-Curc enters the cancer cells and normal cells in dose dependent and time dependent manner so that the therapeutic regime can be modulated. So the research question of this study is designed to find out if after entering cell cytoplasmCurc-Alb activates mitochondrial proteins to induce cell death.

This dissertation is divided into 4 chapters:

Chapter I gives a brief introduction to the research problem and recent review of literature which includes, currently used anticancer drugs, their side effect,mechanism of drug action,brief introduction about curcumin, their molecular targets, which includes transcription factors, cytokines and growth factors, receptors, protein kinase, effect of curcumin on apoptotic genes and tumour suppressing genes. The properties of albumin as a drug carrier is also briefly reviewed.Finally, chapterI describes the gaps identified, the study hypothesis, and specific objectives of the study.

This study hypothesize: (1) Curcumin conjugated to albumin (Curc-Alb) may be tagged with FITC and endocytosis into various cells may be analysed qualitatively and quantitatively; (2) Dose response of Curc-Alb for inducing apoptosis may be demonstrated using simple apoptosis assays; (3) Caspase activation by Curc-Alb may be understood by live cell imaging using FRET based probes.

The objectives of the study are: (1) To prepare fluorescent tagged Curcumin-Albumin conjugate; (2)To establish the Curc-Alb concentrations that cause cytotoxic response in lung cancer cell line A549; (3)To establish endocytosis of the conjugate into cancer cells and normal cells using fluorescent Curc-Alb; (4) To establish induction of apoptosis and death of cancer cells and normal cells in a dose -dependent manner using Annexin V binding assay; and (5) To confirm caspase activation by Alb-Curc using FRET based probe for live cell imaging.

Chapter II gives the details of materials and methods used along with sufficient description of procedures used inthe study. The procedures described are: preparation of Curc-Alb conjugate,preparation of FITC-tagged Curc-Alb, detection of binding ratio by estimation of protein and curcumin on the conjugate,MTT assay to determine toxicity profile for A549 cancer cells and primary human adipose derived mesenchymal stem cells (h-ADMSC), qualitative and quantitative analysis of Curc-Alb endocytosis, quantitative apoptosis assay using AnnexinV/PI kit, and live cell imaging using FRET based probe to demonstrate caspase activation.

Results and discussion of the study are presented in Chapter III. The preparation of soluble Curc was achieved by conjugating the drug with albumin. The anti-proliferative activity of

Curc-Alb conjugate is clearly demonstrated. The cytotoxic response is found to be dependent on both dose and time. Also it was found that Curc-Alb is less toxic to primary cells, such as ADMSC. The uniform distribution of Curc-Alb within the cell was observed by Fluorescence microscopy. The fluorescence intensity is considered as a measure of number of Curc-Alb molecules that enter the cells. About 15 fold higher numbers of Curc-Alb molecules were found to have internalized in A549 whencompared to ADMSCs at similar concentrations and time period. Selectivity for the conjugate may be dependent on the cell's ability to internalize the conjugate. The increased numbers of molecules of Curc-Alb in the cytoplasm of A549 reflected on their increased apoptotic activity. Dose response of apoptosis is demonstrated by Annexin binding assay. Both entry of Curc-Alb into ADMSC and cell death were significantly less in ADMSC as compared to that in A549. Caspase activation by Curc-Alb is evident from FRET-based live cell imaging. Treatment of A549 cells with similar concentrations of free curcumin dissolved in DMSO and Curc-Alb conjugate showed markable difference with respect to apoptosis. This result suggest that Curc-Alb enters the cell cytoplasm more easily and activate caspase pathway to induce apoptosis.

Chapter IV summarizes the study and conclusion. Based on the results it may be concluded that conjugate have high potential for treatment of cancer. It may be expected that the conjugate has better bioavailability and more chance of internalization. Thus side effects of drug action on normal cells may be less as compared to many other drugs that are currently in clinical use. More *in vivo* studies are indicated for proving this concept.
Bibliography section has listed all relevant references cited in this dissertation.

Chapter I

INTRODUCTION

Anticancer drug**,** classified also as antineoplastic drug, is considered to be effective in the treatment of malignant, or cancerous disease. These drugs are classified into various categories based on their action, such as alkylating agents, antimetabolites and hormones. Anticancer drug action depends on many factors, such as type and location of the cancer, its severity, whether drug treatment should be associated surgery or radiation therapy, the side effects associated with the drug which limits the dose and treatment cycles. Therefore, for development and use of anticancer drugs, their mechanismof action and dose dependency and kinetics of action should be established. Also how each drug can affect the normal cell is an important criterion to decide effectiveness of the drug and its application in the clinics.

1.1 Mechanism of Drug action

There are several major targets of drug action: nucleic acids, specific metabolic and signaling enzymes, and microtubules. When nucleic acids are the target, it is generally an action on the integrity or synthesis of DNA rather than of RNA that is presumed to cause cell death. There are several mechanisms by which drugs can bind DNA, the best understood being alkylation of nucleophilic sites within the double helix. A second mechanism of drug binding to nucleic acids is intercalation, the insertion of a planar ring structure between two adjacent nucleotide bases of DNA. This mechanism is characteristic of many antitumor antibiotics. The antibiotic molecule is non-covalently bound to DNA and distorts the shape of the double helix, resulting in inhibition of RNA or DNA synthesis. Many agents capable of classical intercalation, such as doxorubicin and mitoxantrone, are also inhibitors of topoisomerase II, and may produce DNA strand breaks by inhibition of the religation function of this enzyme. A third mechanism of nucleic acid damage is illustrated by the anticancer drug bleomycin. The amino terminal tripeptide of the bleomycin molecule appears to intercalate between guanine cytosine base pairs of DNA. The opposite end of the bleomycin peptide binds Fe (II) and serves as a ferrous oxidase, able to catalyze the reduction of molecular oxygen to superoxide or hydroxyl radicals that produce DNA strand scission. The microtubule spindle structure provides a third target for chemo- therapeutic agents, classically the *Vinca* alkaloids vincristine and vinblastine, but more recently vinorelbine. The *Vinca* alkaloids exert their cytotoxic effects by binding to specific sites on tubulin, inhibiting the assembly of tubulin into microtubules and ultimately the dissolution of the mitotic spindle structure. The microtubule system in cells performs a variety of important functions, including transport of

solutes, cell movement, and chromosomal separation, and provides structural integrity, any one of which could potentially be disrupted by tubulin binding agents. The taxanes are a newer class of agents, consisting of the natural plant alkaloid paclitaxel and a semisynthetic derivative docetaxel. These novel plant alkaloids inhibit cell division by stimulating tubulin polymerization, thus enhancing the formation and stability of microtubules. Paclitaxel treated cells accumulate large numbers of microtubules, free and in bundles,that disrupt microtubule function and ultimately cause cell death. Many anticancer drugs require activation before they are able to exert a cytotoxic effect. The activation process may involve chemical or enzymatic reactions in either normal or tumor tissues.Cisplatin, for example, undergoes a chemical reaction with water molecules intracellularly, resulting in the generation of a positively charged aquated species that attacks nucleophilic sites on DNA. In contrast, the activation of cyclophosphamide is mediated primarily by CYP 2B6 (one of the P450 enzymes), resulting in the release of active alkylating species into the systemic circulation. Intracellular activation by tumor cells is a critical determinant of effect for most antimetabolites. Nucleoside analogs such as araC, fludarabine, and gemcitabine require phosphorylation to active nucleotide triphosphate forms and incorporation into DNA before they are able to exert a cytotoxic effect. Nucleobase analogs such as 6-mercaptopurine, and 6-thioguanine undergo phospho-ribosylation to the nucleoside monophosphate forms, which are active inhibitors of de novo purine nucleotide synthesis. Amination of 6-mercaptopurine to thioguanine monophosphate followed by phosphorylation, reduction to the deoxynucleotide, and a subsequent phosphorylation results in 2′deoxy thioguanine triphosphate, which is a substrate for incorporation into DNA. Phosphoribosylation also converts 5fluorouracil to the monophosphate, which is then phosphorylated to the diphosphate, reduced to the deoxynucleotide, and dephosphorylated to the active 5fluorodeoxyuridine monophosphate (FdUMP), which inhibits thymidylate synthase.

1.2 Drug delivery for anticancer action

The initial requirement for drug action is adequate drug delivery to the target site. This depends largely on blood flow in the tumor bed and the diffusion characteristics of the drug in tissue. In order to produce cytotoxicity, most anticancer drugs require uptake into the cell. A number of mechanisms exist for the passage of drugs across the plasma membrane, including passive diffusion, facilitated diffusion, and active transport systems.

Passive diffusion of drugs through the bilayer lipid structure of the plasma membrane is a function of the size, lipid solubility, and charge of the drug molecule. The passage of

physiologically important hydrophilic compounds across the plasma membrane is usually mediated by a specific receptor, or carrier, in the plasma membrane that facilitates the translocation of the substance into or out of the cell. Many antineoplastic drugs, particularly those that are structural analogs of natural compounds, gain entry into the cell by carrier mediated mechanisms. Naturally occurring nucleosides are transported by both facilitated diffusion (equilibration) and by concentrative mechanisms. Nucleoside analogs that are important in cancer therapy also utilize these transporters, but some specificity is emerging. For instance, cytarabine, floxidine, and pentostatin appear to utilize equilibration transporters, whereas fludarabine, gemcitabine, and cladribine are substrates for concentrative transport systems in addition to equilibration pathways. Transport of reduced folates and methotrexate is an active energy dependent process which can be mediated by two distinct mechanisms: a membrane carrier system capable of the rapid transport of reduced folates and of 4 amino analogs of folic acid and a group of membrane bound folate receptors termed the folate binding proteins which are brought into the cell by endocytosis to release ligand before recycling back to the membrane.

The importance of transmembrane movement of a drug to facilitate its pharmacologic effect depends on several factors including the rate of drug delivery to the tissue, the affinity of the transport process, and the nature of the intracellular biochemical events required for drug action. If a drug requires intracellular activation, such as phosphorylation of nucleoside analogs or polyglutamylation of methotrexate, before it can exert a cytotoxic effect, then the ratelimiting step in drugaction could be activation rather than transport, if the rate of activation is slow relative to the rate of influx into the cell(Burnette.,2012).

1.3 Problems associated with anti-cancer drugs

The treatment of cancer is complicated because of the inability of drugs to target exclusively the cancer cells that have undergone genetic changes and are dividing at a fast and uncontrolled rate. Certain anticancer drugs can differentiate to some degree between normal tissue cells and cancer cells. In fact, the rate at which cancer cells proliferate may play a role in the apparent selectivity of the drugs. The specificity of anticancer drugs plays an important role in reducing the severity of side effects associated with the drug's use. This is because in many ways cancer cells are similar to normal human cells and generally anticancer agents are also toxic to normal cells. This toxic effect can cause numerous side effects, some of which are life-threatening. Such side effects include hair loss, sores in the mouth and on other

mucous membranes, cardiac anomalies, bone marrow toxicity, and severe nausea and vomiting. The bone marrow toxicities result in anaemia as well as in decreased resistance to infectious agents. Some drugs can cause permanent infertility. Such adverse effects may require that the drug dosage be reduced or the drug regimen be changed to make the drug tolerable to the patient. In rare instances prolonged use of anticancer drugs can lead to the development of secondary cancers.

1.4 Development of new drugs

Due to these types of problems associated with currently available cancer drugs, efforts are ongoing in many laboratories to develop effective drugs which has lower side reaction on normal cells. Synthetic chemists make efforts to synthesize molecules that act on cells at molecular level to prevent cancer growth. Many types of natural medicines in crude form and molecules in pure form are being tested for their drug action and effectiveness. Crude forms are likely to have more side effects because there are many components in the preparation that may have toxic effects on continuous use and when used in high quantities. Therefore, purified molecules have better prospects and its mechanism of action and possible side effects can be better understood by modern techniques in cell biology.

Curcumin, is one such component purified from turmeric, a common material used in curries and dishes especially in spicy dishes in India, Pakistan, Bangladesh, and other countries of Asia mainly as coloring agent. Turmeric is obtained from the dried rhizome of the *Curcumalonga* plant. Turmeric contains three principal components, curcumin, demethoxycurcuminand bis-demethoxycurcumin, of which curcumin is the most abundant and potent molecule. Chemical structure of curcumin was characterized in 1910 by Milobedeska and colleagues, and synthesis was confirmed by Lampe and colleagues in 1913 (Milobedeska et al., 1910, Lampe et al 1913). Curcumin is lipophilic in nature which shows low solubility and stability in aqueous solution. It is extensively used in Ayurveda, Unani, Siddha, and Chinese medicine for the management of various diseases such as wound, inflammation, and cancer. The mechanism of action of curcumin on cells have been widely studied. But due to poor aqueous solubility and quick degradation in aqueous solution, the potential of the drug has not been utilized effectively. However, literature continues to expand with knowledge on its action on cells at molecular level as seen below.

II.Review of literature

II.1 Characteristics of Curcumin

Curcumin is a symmetric molecule, also known as diferuloyl methane. The IUPAC name of curcumin is 1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione, with chemical formula C21H20O6, and molecular weight of 368 (Jovanovic et al., 2001). It has three chemical entities in its structure: two aromatic ring systems containing o-methoxy phenolic groups, connected by a seven carbon linker consisting of an α,β-unsaturated β-diketone moiety(Priyadarsini et al., 2013) . The diketo group exhibits keto-enoltautomerism, which can exist in different types of conformers depending on the environment. In the crystal state it exists in a cis-enol configuration, where it is stabilized by resonance assisted hydrogen bonding and the structure consists of three substituted planar groups interconnected through two double bonds. In most of the non-polar and moderately polar solvents the enol form is generally more stabilized than the keto form by 5 to 8 kcals/mol depending on the nature of the solvent. Due to extended conjugation, the π electron cloud is all along the molecule. In solution it exists as cis-trans isomers where the trans-form in which the two phenolic-methoxy groups are on the opposite sides of the curcumin backbone is slightly more stabilized than the cis-form, where the phenolic methoxy groups are in the same side up the backbone. The computed dipole moment of curcumin in the ground state is 10(Gianluca et al., 2011). It is a hydrophobic molecule with a logP value of ~3.0. It is almost insoluble in water and readily soluble in polar solvents like dimethyl sulphoxide (DMSO), methanol, ethanol, acetonitrile, chloroform, ethyl acetate, etc. It is sparingly soluble in hydrocarbon solvents like cyclohexane and hexane. The absorption spectrum of curcumin has two strong absorption bands, one in the visible region with maximum ranging from 410 to 430 nm and another band in the UV region with maximum at 265 nm region. The molar extinction coefficient of curcumin in methanol is 55,000 dm3 ·mol−1 ·cm−1 at 425 nm. Curcumin is a weak Brönsted acid, with three labile protons, and accordingly three pKas have been estimated corresponding to three prototropic equilibria. Both NMR and absorption spectrometry have been used to estimate the pKa. The first pKa in the pH range of 7.5 to 8.5 changes curcumin from yellow to red. The chemical reactivity and solubility of the anionic curcumin, i.e., in the basic pH range increases and this form of curcumin is more water soluble than the neutral form. The absorption maximum of fully deprotonated (red in colour) curcumin in alkaline pH (>pH 10) is at 467 nm and the molar extinction coefficient is 53,000 dm3 mol^{-1} cm^{-1}. There is still a debate which one, i.e., the enolic OH or the phenolic OH is

the most acidic. Although calculations indicate that the enolic OH is the most acidic group, the pH dependent spectral changes are difficult to distinguish between the two protons. From H-NMR studies the pKa of 12.5 for the deprotonation of the enolic proton and another pKa at 13.6 for the phenolic protons. These values for enolic protons however differ significantly from those reported by other methods. With the availability of other spectroscopic techniques, it should be possible to resolve these differences in the estimation of pKa in the future. Aqueous curcumin solutions can be prepared by adding surfactants, lipids, albumins, cyclodextrins, biopolymers etc. Micelle solutions using surfactants are the best suited for preparing high concentration curcumin in water. However while using aqueous surfactant solutions in biological systems, care must be taken by performing proper control experiments, as surfactants can interfere in biological studies. Curcumin has three reactive functional groups: one diketone moiety, and two phenolic groups(Priyadarsini et al., 2013). Important chemical reactions associated with the biological activity of curcumin are the hydrogen donation reactions leading to oxidation of curcumin, reversible and irreversible nucleophilic addition (Michael reaction) reactions, hydrolysis, degradation and enzymatic reactions. All these have significant role in different biological activities of curcumin. Extensive studies have been made to understand various functions of curcumin which enables it to be considered as a drug to treat various disease.

II.2 Curcumin: Modulator of Molecular Pathways

Tumorigenesis and tumor progression are thought to occur as a result of some changes in the different types of genetic pathways (Rahmani et al 2013,Babiker et al., 2014). Curcumin, the chief constituent of turmeric, shows a vital role in cancer prevention and treatment through modulation of various biological activities including molecular cascades.

II.3 Effect of Curcumin on Tumor Suppressor Genes.

Tumor suppressor genes play a vital and significant role in the inhibition of cancer formation and its progression. When an alteration or mutation occurs in a gene, then tumour suppressor gene lose its ability to perform normal function.Tumor suppressor gene p53 is the guardian of all genes that regulates various cellular and molecular pathways and prevents cancer formation. Numerous *in vivo* and *in vitro* reports showed that turmeric and its constituents have a significant role in cancer prevention or inhibition. An important study showed that curcumin down-regulates the expression of p53, as well as the survival genes egr-1, cmyc and bcl-XL in B cells (Han et al., 1999).

Another report has also indicated that curcumin inhibits cell cycle progression of immortalized human umbilical vein endothelial cells (HUVEC) by upregulating the CDK inhibitors p21WAF1/CIP1, p27KIP1, and p53 (Park et al., 2002). Further studies reported that curcumin mainly acts in p53 dependent manner and also showed that wild p53was highly susceptible to curcumin toxicity (Sa et al., 2008). Another tumor suppressor gene, phosphatase and tensin homolog deleted on chromosome ten (PTEN) has a role in the progression of the cell cycle and apoptosis. Alteration or mutation of PTEN gene has been noticed in several types of cancers. A study of the curcumin has shown that PTEN increases the curcumin-induced apoptosis, whereas inactive PTEN decreases/inhibits the curcumin-induced apoptosis (Shankaret al., 2007). A study showed that difluorinated curcumin (CDF), a non-toxic analog, modulates the expression of miR-21 and PTEN in pancreatic cancer.The retinoblastoma is a type of tumour suppressor gene and shows an important role in the control of cell cycle.pRb, the protein coded for by the *RB1* gene, shows an important role in cell cycle regulation, promoting G1/S arrest and growth restriction via inhibition of the E2F transcription factors (Weinberg et al., 1995). It is inactivated through hyper phosphorylation catalyzed by the cyclin D-cyclin-dependent kinase 4 (cyclinD-cdk4) and cyclin E-cdk2 complexes. Various medicinal plants and their constituents show a vital role in the regulation of Rb genes via regulation of phophorylation. In this view, curcumin, chief constituents of turmeric also shows an important role in modulation of Rb gene via reductionin hyper-phosphorylation. An important study based on prostate cancer cells has revealed that curcumin induced the expression of cyclin-dependent kinase (CDK) inhibitors p16,p21, and p27 and inhibited the expression of cyclin E andcyclin D1 and hyper-phosphorylation of retinoblastoma (Rb)protein (Srivastava et al., 2007) and another study has shown that suppression of cyclin D1 by curcumin led to inhibition of CDK4-mediated phosphorylation of retinoblastoma protein (Mukhopadhyay et al., 2001).

II.4 Effect of Curcumin on Apoptotic Genes.

Apoptosis is one of the prerequisites to maintain the normal and healthy internal milieu.Any alteration or change in the normal process ofapoptosis may increase cell survival and support the tumor development and progression (Hollowood et al., 1991, Sachs et al., 1993). Curcumin plays a vital role in the upregulation of different pro-apoptotic genes and at the same time downregulates some of the anti-apoptotic genes and by this way balances the apoptosis process. An interesting study showed that curcumin induces apoptosis in scleraderma lung fibroblasts (SLF) without affecting normal lung fibroblasts (Tourkinaet

al.,2004). Furthermore, curcumin has shown antitumor activity and was involved in the apoptosis induction and the modulation of key apoptotic proteins such as Bax and bcl-2 (Yu et al., 2011). A study has reported that growth arrest and apoptosis of B cell lymphoma occur through the down regulation of c-myc, bcl-XL, and p53 with the treatment of curcumin (Ramachandran et al., 2002). Another report in human breast cancer cell line showed that CD437 induces G0-G1 arrest and apoptosis via regulation of p21WAFI/CIPI, Bcl-2, and Bax in a p53 independent manner (Shao et al., 1995). Another study on p53-null cells, as well as TR9-7 cells, reported that curcumin induces apoptosis in tumor cells via a p53-dependent pathway and Bax act as downstream effectors of p53(Choudhuri et al., 2002).Curcumin induces apoptosis in a range of tumor cell lines through activation of caspase-3, cytochrome-c release, and down regulation of bcl-2(Bae et al., 2003, Mukherjee et al., 2007). Curcumin has shown anapoptotic effect by inhibiting various genes such as protein tyrosine kinase, protein kinase C, c-myc mRNA expression,and bcl-2 mRNA expression (Chen et al., 1998) and also mitochondrialpathway. Earlier studies have shown that curcumin possesses an apoptotic activity in different types of cancer cell such as human colon cancer cells, stomach, and skin tumors,breast cancer cells, and prostate cancer cells (Agarwal et al., 2003). Study of colon cancer cell line showed that apoptosis was increased in response to curcumin(Moragoda et al., 2001). Curcumin also showed a vital role in decreasing cell proliferation in a dose dependent manner. Curcumin may lower the incidence of various cancers, including urothelial malignancies (Kamat et al., 2007) and also may induce apoptosis in MBT-2 cells and G2/Marrest of T24 cells (Parket al.,2001).Experimental studies showed that the down-regulation of the expression of anti-apoptotic protein occurs with curcumin treatment (Herrmann et al., 1998).

II.5 Effect of Curcumin on Angiogenesis

Angiogenesis is a complex process involving widespread interaction between the cells, soluble factors, and ECM components (Liekens et al., 2001). It also shows a vital effect in tumor growth and is triggered by chemical signals from tumour cells in a phase of rapid growth (Folkman et al., 1971). There are several angiogenic factors such as vascular endothelial growth factor via (VEGF), basic fibroblast growth factor (bFGF), angiogenin, transforming growth factor. These factors show critical role in cancer angiogenesis by releasing molecules and sending signals to surrounding normal host tissue (Shishodia et al., 2007). VEGF is a crucial survival factor for endothelial cells in the process of physiological,tumour angiogenesis and induces the expression of anti-apoptotic proteins in

the endothelial cells (Gerber et al 1998). There are certain drugs,like Bevacizumab (Avastin), available as an inhibitor of VEGF action in the treatment of cancer.These drugs are expensive and a large group of the population cannot afford their cost.However, a safe and affordable natural product is needed to control the cancer development. Earlier studies have shown that curcumin is an inhibitor of VEGF in different types of cancer, including orthotopically implanted pancreatic tumors (Kunnumakkara et al., 2007). Important record via *invitro* and *invivo* studies showed that curcumin suppresses the proliferation of human vascular endothelial cells and also abrogates the FGF-2-induced angiogenic response (Singh et al., 1996,).Moreover, curcumin has the ability to inhibit both VEGF and its receptor in various cancer types; it might be usefulas an anti-angiogenic agent (Chadalapaka et al., 2008). Besides, curcumin plays a major role in the suppression of transcriptional activity ofAP62 and HIF-1 and causes a reduction in the expression of VEGF (Singh et al., 1996). Results of an important study suggested that curcumin potentiates the antitumor effects of gemcitabine in pancreatic cancer via suppressing proliferation, angiogenesis, NF-kB or NF-kB regulated genes.

II.6 Effect of Curcumin on Phase I and Phase II Genes/ Enzymes.

Xenobiotics are molecules introduced into the body from the environment and not produced inside the body. The body then metabolizes them through two phases of transformation: Phase I and Phase II. The turmeric has shown a significant effect on the regulation of xenobiotic metabolism via inhibition of the phase I and activates the phase II gene/enzymes. In the phase I reactions,addition of a functional polar group normally results in a relatively small increase in hydrophilicity and may cause metabolic activation. Cytochrome P450 (CYP) is the main enzymes in phase I and shows the vital effect on the activation of carcinogens.

So, control of CYP450 activity is the main issue in cancer prevention through increasing the degree of cellular safety. A study in rat model showed that curcumin inhibits the alkylation reaction catalysed by CYP1A1, 1A2 (Thapliyal et al., 2001). In another interesting study, it was reported that CYP plays a vital role in the formation of aflatoxine-DNA adduct and this intermediate product is suppressed or inhibited by curcumin treatment. Curcumin has shown its effect in hampering CYP1A1 activity in DMBA-treated cells and also inhibited the metabolic activation of DMBA and decreased the DMBA-induced cytotoxicity.In the Phase II reactions, conjugation with a small hydrophilic endogenous substance increases the hydrophilicity and facilitates the emission. However, the activation of Phase II enzymes such

as Glutathione S transferase in the treatment and suppression of cancer is critical and is a significant strategy. Several earlier studies reported that turmeric and its constituents play a significant role in the prevention of cancer via the activation of GST genes.An important finding showed that turmeric/curcumin enhances the activity of Phase II enzyme GST (Singh et al., 1996).Curcumin also elevates the protein as well as mRNA expressions of GSTs and NQO1 in mouse tissues, suggesting a role of curcumin in transcriptional regulation of phase IIenzymes (Garg et al., 2008).

II.7 Androgen Degradation/Down Regulation by Curcumin.

The androgen receptor (AR) is a ligand-activated steroidhormone receptor that plays a vital and significant role in developing the function of normal prostrate as well as in prostate cancer development and progression (Gao et al., 2005).Change in the function or over expression of AR has been observed in cancer (Rahmani et al., 2013). The treatment basis on allopath like hormone therapy was considered as a potential treatment, but its limitation/disadvantage is prostate cancer cells become progressive and may lead to metastasis (Feldman et al.,2001). The regulation of AR activities is a critical step in the control or suppression of tumor development and progression. An important study of curcumin on androgen dependent LNCaP prostate cancer cellline and an androgen independent PC-3 prostate cancercell showed that AR protein level is down-regulated. Earlier studies had shown that curcumin down-regulates the transactivation and expression of AR and AR-related cofactors(Nakamura et al., 2002).The constituent has a potential therapeutic effect on prostate cancer cells through the down-regulation of AR andAR-related cofactors AP-1, NF, and CBP. Another study reported that curcumin acts as an inducer of apoptosis in both androgen-dependent and hormone refractory prostate cancer cells. Curcumin blocks the activation of androgen and IL-6 on prostate-specific antigen expression in human prostatic carcinoma cells (Tsui et al., 2008).

II.8 Effect of Curcumin on PI3 K/Akt Pathways.

PI3 K/Akt signalling pathway is important and critical in mediating cell survival, proliferation, migration, and angiogenesis. PI3K catalyses the production of the lipid secondary messenger Phosphatidylinositol-3,4,5-triphosphate including the serine/threonine kinase Akt. Mutation and/or loss of PTEN function plays an important role in the activation of PI3 K and is associated with the growth and progression of various types of cancers (Roy et al., 2002). The inhibition of the PI3K/Akt and activation of PTEN pathway is a good

strategy in the prevention of cancer. An important study showed that curcumin inhibits the phosphorylation of Akt, mTOR, and their downstream substrates, and this inhibitory effect acts down stream of phosphatidylinositol 3-kinase and phosphatidyl inositol dependent kinase1 (Yu et al., 2008).

II.9 Effect of Curcumin on Cycloxygenase Enzyme.

COX is an inducible enzyme in the conversion of arachidonic acid to prostaglandins (PGs). There are two types of cycloxygenase: COX1 plays a vital role in physiological functions and COX2 is upregulated or overexpressed in various types of cancers (Gupta et al., 2010). It was previously stated that curcumin inhibits the critical stage of tumor initiation and promotion (Huang et al., 1995, Rao et al., 1995) and COX inhibition (Huang et al., 1989). Curcumin also inhibits the COX2 expression on colon cancer cell lines (Goel et al., 2008). Earlier studies reported that curcumin plays an important role in the down regulation of the expression of COX-2 and finally prevents or suppresses the cancer progression (Aggarwal etal., 2003).Moreover, curcumin plays a significant role in the cancer prevention via controlling the activities of various genes in the initiation, promotion, and progression stage of tumor development.

II.10 Effect of Curcumin on Oncogene.

Alteration or mutation of proto-oncogene is the key factor in the development and progression of various types of tumours. An activated oncogene has been noticed in various types of cancer (Slamon et al., 1989). Safe route of inactivation of an oncogene is a prime interest in the prevention of tumor. Several earlier investigations reported that curcumin shows a significant effect in cancer prevention via the inactivation of oncogene.Curcumin down-regulated N-Myc (Elamin et al., 2010) in various cancer types and decreased the expression of proto-oncogenes such as *ras* and *fos* in tumorous skin (Limtrakul et al., 2001). A report on the effect of curcumin in hepatocellular carcinoma revealed that curcumin blocked transactivation of the c-Met promoter through AP-1.Another finding on curcumin effect in the down regulation of oncogene showed that curcumin induced the anti-proliferative, anti-migratory and apoptotic effects via the down-regulation of various genes, includingc-Myc, N-Myc, cyclin D1, and anti-apoptotic factors Bcl-2 and Bcl-xL.Several other studies showed the effect of curcumin in the inhibition or down-regulation of various oncogenes such as EGFR, HER-2, PI3K/Akt, and MAPK pathway (Chen et al., 2006). Curcumin is involved in the induction of apoptosis through down-regulating the expression

of c-myc,Bcl-2, and mutant-type p53, and up-regulating the expression-of Fas (Wu et al., 2002).

II.11 Effect of Curcumin on Signal Transducer and Activator of Transcription 3 (STAT3).

The Signal Transducer and Activator of Transcription 3 (STAT3) protein is one of the important members of the STAT family of transcription factors.STA3 plays a role in the cancer development and progression and over expression or high level of STAT3 has been observed in various types of cancers. Curcumin inhibits constitutive STAT3 phosphorylation (Glienke et al., 2010). Other results also show that the curcumin significantly suppressed STAT3 phosphorylation in broncho-epithelial cells and lung cancer derived cells, indicative of STAT3 pathway suppression, and finally inhibits the proliferative capacity of broncho-epithelialcells and lung cancer cells (Alexandrow et al., 2012).

II.12 Effect of Curcumin in Peroxisome Proliferator-Activated Receptors (PPARs).

PPARs belong to the super family of nuclear receptors, containing three genes that give different proliferation of activated HSCs (Zhou et al., 2013). A study of curcumin effects on colon cancer cells confirmed that growth inhibition and stimulation of the trans-activating activity of peroxisome proliferator-activated receptor c (PPAR-c),which appears to mediate the suppression of gene expression of cyclin D1 and the epidermal growth factor receptor (EGFR) (Chen et al., 2008). Subtypes such as PPAR-α and PPAR-elor a dewohs nimucruC .ɣ in the up regulation of PPAR- detalumits ,gnilangis FGE dna FGDP htiw detpurretni dnaɣ gene expression of PPARɣ and thereby playing a role in the inhibition of cells.

II.13 Effect of Curcumin on Matrix Metalloproteinases-9 (MMP-9*)*.

Matrix metalloproteinases (MMPs) have been considered as one of the important vital molecules assisting tumor cells during metastasis (Fingleton et al., 2006). Amember of the matrix metalloproteinases (MMPs)-MMP9, shows a major role in the breakdown of extracellular matrix in normal physiological processes, including embryonic development,reproduction, and tissue remodelling, as well as in disease processes such astumor metastasis(Fridman et al., 1995). Altered expression of MMP-9 has been observed in various types of tumors. However, curcumin shows a vital role in the inhibition of MMP-9 activities and finally plays a role in the management of cancer. A study showed that curcumin inhibits TPA induced MMP-9 expression and cell invasion through suppressing NF-ƙB and AP-1 activation (Kim et al., 2007).Another study showed that curcumin

significantly inhibited the MMP-9 enzymatic activity and protein expression that was induced by PMA (Woo et al.,2005). An important study has shown in a human breast cancer xenograft model that administration of curcumin noticeably decreased metastasis to lung and suppressed the expression of NF-kB, MMP-9,COX-2, VEGF, and intercellular adhesion molecule-1 (Aggarwal et al., 2005).Earlier results suggest that curcumin plays a role in regulating cell metastasis by inhibiting MMP-2 and MMP-9 in breast cancer cell line (Hassan et al., 2012). Curcumin showed inhibition of phorbolester-induced up-regulation of cyclooxygenase-2 and matrixmetalloproteinase-9 inMCF10A human breast epithelial cells study (Lee et al., 2011).

II.14 Anti cancerous activity of curcumin

Apoptosis, a form of programmed cell death, is required to maintain the integrity and homeostasis of multicellular organisms. It is an important biological process, impairment of which may lead to cancer. The ability of curcumin to induce apoptosis in cancer cells without cytotoxic effects on healthy cells contributes to the understanding of the anti-cancer potential of curcumin. Curcumin efficiently induces apoptosis in various cell lines like HL-60, K562, MCF-7 and HeLa(Roy et al, 2002).

II.15 Curcumin's Mechanisms of Action: The Role of STAT3 and NF-κB

The nuclear factor (NF)-κB, is a ubiquitous transcription factor that regulates many genes implicated in growth regulation, inflammation, carcinogenesis, and apoptosis (Gupta et al., 2010, Sen et al., 1986). STAT3 is one of the major mediators of carcinogenesis. The oncogenic significance of activated STAT3 molecules is due to their effects on various parameters, such as apoptosis, cell proliferation, angiogenesis, and immune system evasion. Constitutively active STAT3 has been involved in the induction of resistance to apoptosis, probably through the expression of Bcl-xL and cyclin D1 (Catlett-Falcone et al., 1999, Zushi et al., 1998). Its role in tumorigenesis is mediated through the expression of genes that suppress apoptosis, mediate proliferation, invasion, and angiogenesis. Constitutive activation of STAT3 has been implicated in a variety of cancers, including breast cancer, prostate cancer, head and neck squamous cell carcinoma, multiple myeloma, lymphomas and leukemia, brain cancer, colon, gastric, esophageal, ovarian, nasopharyngeal and pancreatic cancer (Huang et al., 2000). Constitutive activation of the STAT3 and NF-κB signaling pathways has been demonstrated in prostate cancer cell lines and clinical samples of prostate cancer (Chen et al., 2008).

Curcumin has also been suggested to induce apoptosis and cause down-regulation of EGFR, Akt, cMETcyclin D1, in CL-5 xenograft tumors (Lee et al., 2011). In addition, it has been documented to inhibit lung cell invasion and metastasis through up-regulation of HLJ1 expression in cancer cells (Shishodia et al., 2007). Apart from its action on STAT3 and NF-κBpathways,curcumin has been shown to inhibit cell proliferation, cell cycle arrest and stimulate apoptosis *via* modulation of other transcription factors, such as AP-1, Erg-1, p53, β-catenin, Notch-1, Hif-1, and PPAR-α (Davie et al., 2008).

II.16 Curcumin and Sp-1

Sp-1, a transcription factor highly expressed in breast, gastric and thyroid tumor cells compared to normal cells, has been demonstrated to interact with co-activators and co-repressors and, thereby, activate multiple biological functions, including cell cycle and carcinogenesis. Inhibition of Sp-1 and its housekeeping gene expressions may serve as an important hypothesis to prevent cancer formation, migration, and invasion (Cho et al., 2009, Chadalapaka et al., 2008). Curcumin may act by suppressing the Sp-1 activation and its downstream genes, including *ADEM10*, calmodulin (*CALM*), *EPHB2, HDAC4*, and *SEPP1* in a concentration dependent manner in colorectal cancer cell lines; curcumin could suppress the Sp-1 activity in bladder cancer and could decrease DNA binding activity of Sp-1 in non-small cell lung carcinoma (NSCLC) cells (Aigner et al., 1998, Lou et al., 2006). Curcumin has significantly reduced colony formation in colorectal cancer cells.

II.17 Curcumin and AdhesionMolecules

Curcumin has been shown to enhance cell adhesion ability, through induction of extracellular matrix components collagen I, collagen III, collagen IV, collagen IX, laminin, and fibronectin in a concentration dependent manner. Curcumin suppresses FAK activity by means of inhibition of its phosphorylation sites and also induces extra-cellular matrix components to enhance cell adhesion ability, thus, preventing detachment of cancer cells and cell migration. Inhibition of FAK expression leads to increased cell adhesion, which may be the potential mechanism of thenon-invasive effect of curcumin.Curcumin could exert its function against metastasis, through downregulation of Sp-1, FAK, and CD24 and by promoting E-cadherin expression in colorectal cancer cells (Zhou et al., 2013).

II.18 Anti-oxidant activity of curcumin

Curcumin is a powerful scavenger of many free radicals such as anion, hydroxyl radical and nitric oxide (Elizabeth and Rao, 1990). Curcumin inhibits the generation of NO from

activated macrophage. Curcumin has a protective effect against H2O2-induced cell damage in NG108-15 cells when added concentrations are 12.5 – 100 μM with or without FK506 as a reference drug, H2O2 alone causes a decrease in cells viability. Pulla Reddy and Lokesh (1992) observed that curcumin is capable of scavenging oxygen free radicals, such as superoxide anions and hydroxyl radicals, which are the initiators of lipid peroxidation. The lipid peroxidation has a main role in the inflammation, in heart diseases, and in cancer (Jayaprakasha *et al*., 2005). Curcumin is reported to be a powerful antioxidant to repair both oxidative and reductive damage caused to protein by radiation (Priyadarsini, 1997). In gastric mucosal damage induced by indomethacin-ROS, curcumin stopped or reduced lipid peroxidation in rats (Chattopadyay *et al.,* 2006).A single oral dose of curcumin inhibited the carrageenan-induced inflammation by 15 – 52%, also lowered the carrageenan-induced edema in the foot pads of rats in a dose dependent manner. Curcumin when fed through the diet, reduced the incidence of carrageenan induced paw edema, delayed the onset of arthritis, and reduced the severity of paw inflammation in arthritic rats.The mechanism of curcumin anti-inflammatory effects may involve inhibition of the induction of COX-2, iNOS and production of cytokines such as interferon-γ, at least in part due to its suppression of the Janus kinase (JAK)-STAT signaling cascade via its effect on the Src homology 2 domain-containing protein tyrosine phosphatases (SHP)-2 (Kim et al., 2007; Sharma et al., 2005).

In myeloma cells, curcumin has also been shown to inhibit STAT3 phosphorylation and thus suppress interleukin 6 production (Bharti *et al.,* 2003). Curcumin suppress activation of nuclear factor kappa B (NF-κB), which hamper subsequent nuclear translocation of the functionally active subunit of NF-κB. Curcumin is reported as a lead candidate for anti-inflammatory agent as it inhibits protease activated receptors (PAR2 and PAR4)-mediated mast cell activation through a block of extra cellular signal regulated kinase (ERK) pathway (Jayaprakasha*et al.,* 2005). The experimental data suggested that curcumin inhibited the formation of arachidonate metabolites (pgE2-leukotrienes) and the secretion of lysosomal enzymes-elastase, collagenase and hyaluronidase by macrophages (reviewed by Srinivasan, 2005).

Because of its antioxidant activity, curcumin has been found to exhibit anti-mutagenic and anti-carcinogenic (Soudamin et al., 1989) properties. Moreover, curcumin has been known to inhibit phorbol ester-induced tumour promotion in mouse skin (Huang et al., 1989). It is an inhibitor of arachidonic acid metabolism and is a good anti-inflammatory agent (Mukhopadyay et al., 1982). It is being evaluated as a chemopreventive agent by the National Cancer Institute(Kelloff et al., 1994).

Recent studies indicate its possible use as an antiviral agent against human immunodeficiency virus Ill. Natural curcumin isolated from Curcuma lunga contains curcumin I (diferuloyl methane) as the major component but also contains curcumin II (6%) and curcumin III (0.3%). It has been reported that the presence of curcumin II and III have a synergistic effect on the anti-inflammatory activity of curcumin I indicating that other curcumins could have higher biological activity compared to curcumin I.

II.19 Role in Detoxification.

Curcumin exerts its chemoprotective and chemopreventive effects via multiple mechanisms. It has been reported to induce expression of the antioxidant enzymes in various cell lines. Curcumin significantly increased expression of Nrf2 as well as phase II detoxifying and antioxidant enzymes. The redox-sensitive transcription factor, nuclear factor erythroid 2 p45 (NF-E2)-related factor (Nrf2) plays a key role in regulating induction of phase II detoxifying or antioxidant enzymes. Thus, activation of Nrf2 is considered to be an important molecular target of many chemopreventive and chemoprotective agents. Curcumin has also been seen to raise the levels of the significant antioxidant, glutathione 71. A low dose administration of curcumin elicited the dual adaptive response of an instant enhancement in GSH and the tendency for producing an increased amount of glutathione 71.

II.20 Anti-inflammatory Mechanisms

Research shows curcumin is a highly pleiotropic molecule capable of interacting with numerous molecular targets involved in inflammation. Curcumin modulates the inflammatory response by down-regulating the activity of cyclooxygenase-2 (COX-2), lipoxygenase, and inducible nitric oxide synthase (iNOS) enzymes; inhibits the production of the inflammatory cytokines tumor necrosis factor-alpha (TNF-a), interleukin (IL) -1, -2, -6, -8, and -12, monocyte chemoattractant protein (MCP), and migration inhibitory protein; and down-regulates mitogen-activated and Janus kinases(Goel et al., 2008, Abbe et al., 1999).COX-2 and iNOS inhibition are likely accomplished via suppression of nuclear factor kappa B (NF-κB) activation. NF-κB, a ubiquitous eukaryotic transcription factor, is involved in regulation of inflammation, cellular proliferation, transformation, and tumorigenesis. Inhibition of inflammatory cytokines by curcumin is achieved through a number of mechanisms. *In vitro* studies indicate that curcumin regulates activation of certain transcription factors such as activating protein-1 (AP-1) and NF-κB in stimulated monocytes and alveolar macrophages, thereby blocking expression of cytokine gene expression. Down-

regulation of intercellular signaling proteins, such as protein kinase C, may be another way in which curcumin inhibits cytokine production.

II.21 Cardioprotective Effects. Curcumin has been effective against atherosclerosis and myocardial infarction(Aggarwal et al., 2005). The proliferation of peripheral blood mononuclear cells (PBMCs) and vascular smooth muscle cells (VSMCs), which are hallmarks of atherosclerosis, is inhibited by curcumin. Curcumin prevents the oxidation of low density lipoproteins (LDLs), inhibits platelet aggregation, and reduces the incidence of myocardial infarction.

II.22 Skin Diseases.Curcumin has been shown to be effective against different skin diseases including skin carcinogenesis, psoriasis,(Heng et al., 2000) scleroderma,(Tourkina et al., 2004) and dermatitis. Numerous reports suggest that curcumin accelerates wound healing. In addition, curcumin also prevents the formation of scars and plays a role in muscle regeneration following trauma(Aggarwal et al., 2005)

II.23 Diabetes. In type II diabetes, administration of curcumin reduced the blood sugar, hemoglobin, and glycosylated hemoglobin levels significantly in an alloxan-induced diabetic rat model. Diabetic rats maintained on a curcumin diet for 8 weeks excreted less albumin, urea, creatinine, and inorganic phosphorus. Dietary curcumin also partially reversed the abnormalities in plasma albumin, urea, creatine, and inorganic phosphorus in diabetic animals(Aggarwal et al., 2005)

II.24 Rheumatoid Arthritis.Curcumin has also been shown to possess anti-rheumatic and anti-arthritic effects, most likely through the down regulation of COX2, tumor necrosis factor (TNF), and other inflammatory cytokines(Aggarwal et al., 2005)

II.25 Multiple Sclerosis. Multiple sclerosis is characterized by the destruction of oligodendrocytes and myelin sheath in the CNS. Curcumin inhibits experimental allergic encephalomyelitis by blocking interleukin (IL)-12 signaling in T cells, suggesting it would be effective in the treatment of multiple sclerosis(Aggarwal et al., 2005)

II.26 Alzheimer'sDisease.Curcumin can suppress oxidative damage, inflammation,cognitive deficits, and amyloid accumulation in Alzheimer's disease.

II.27 Inflammatory Bowel Disease. Aggarwal al.2003,recently investigated the protective effects of curcumin on inflammatory bowel disease induced in a mouse model. Pretreatment of mice with curcumin for 10 days significantly ameliorated the appearance of diarrhoea and the disruption of the colonic architecture.

II.28 Cystic Fibrosis.Cystic fibrosis, the most common lethal hereditary disease in the white population, is caused by mutations in the cystic fibrosis transmembrane conductance regulator gene. In a recent report, curcumin corrected the cystic fibrosis defects in DeltaF508 CF mice.

II.29 Others. Curcumin was found to be a potent and selective inhibitor of human immunodeficiency virus (HIV-1) long-terminal repeat-directed gene expression, which governs the transcription of type 1 HIV-1 provirus. It has also been shown to prevent cataractogenesis in an *in vitro* rat model. Treatment with curcumin also prevented experimental alcoholic liver disease. Curcumin has a protective effect on cyclophosphamide-induced early lung injury. Nephrotoxicity, a problem observed in patients who are administered chemotherapeutic agents, can be prevented with curcumin(Aggarwal et al., 2005).

Overall, curcumin is a molecule which has multiple activity on regulating cellular and molecular mechanisms in human pathology. There is lot of studies which proves its action scientifically using *in vitro* cell culture and analysis methods. However, its potential has not been proven much in animal models to the extent to prove its therapeutic value, mainly due to poor aqueous solubility and bioavailability. Even then research continues to explore molecular mechanism by *in vitro* experimentation.

II.30 Molecular targets of curcumin

Various studies have shown that curcumin modulates numerous targets. These include the growth factors, growth factor receptors, transcription factors, cytokines, enzymes, and genes regulating apoptosis.

II.30.a Cytokines and Growth Factors. Numerous growth factors have been implicated in the growth and promotion of tumors. Curcumin has been shown to down-regulate the expression of several cytokines including TNF, IL-6, IL-8, IL-12, and fibroblast growth factor-2 (Aggarwal et al., 2005)

II.30.b Receptors.Curcumin has been shown to down-regulate both epithelial growth factor receptor (EGFR) and HER2/neu receptors. It also modulates androgen receptors(Aggarwal et al., 2005)

II.30.c Transcription Factors.Curcumin may also operate through suppression of various transcription factors including NF-κB, STAT3, Egr-1, AP-1, PPAR-γ, and beta catenin activation(Aggarwal et al., 2005). These transcription factors play an essential role in various diseases. The constitutively active form of NF-κB has been reported in a wide variety of cancers. NF-κB is required for the expression of genes involved in cell proliferation, cell invasion, metastasis, angiogenesis, and resistance to chemotherapy. Bharti et al.,2012 demonstrated that curcumin inhibited IL-6–induced STAT3 phosphorylation and consequent STAT3 nuclear translocation. Activation of PPAR-γ inhibits the proliferation of non-adipocytes. Xu et al.,2013 demonstrated that curcumin dramatically induced the gene expression of PPAR-γ and activated PPAR-γ. AP-1, another transcription factor that has been closely linked with proliferation and transformation of tumor cells, has been shown to be suppressed by curcumin. Studies also suggest that curcumin has a potential therapeutic effect on prostate cancer cells through downregulation of AR and AR-related cofactors(Aggarwal et al., 2005)

II.30.d Pro-inflammatory Enzymes.Curcumin has been shown to suppress the expression of COX2, 5-LOX, and iNOS, most likely through the downregulation of NF-κB activation(Aggarwal et al., 2005).

II.30.e Protein Kinases. Curcumin suppresses a number of protein kinases including mitogen-activated protein kinases, JNK, PKA, PKC, src tyrosine kinase, phosphorylase kinase, IκBα kinase, JAK kinase, and the growth factor receptor protein tyrosine kinases(Aggarwal et al., 2005).

II.30.f Cell Cycle.Curcumin modulates cell-cycle–related gene expression. Specifically,curcumin induced G0/G1 and/or G2/M phase cell cycle arrest, upregulated CDKIs, p21WAF1/CIP1, p27KIP1, and p53, and slightly down regulated cyclin B1 and cdc2. Curcumin can indeed down regulate cyclin D1 expression (Mukhopadhyay et al., 2001) at the transcriptional and posttranscriptional levels.

II.30.g Adhesion Molecules. Curcumin inhibits inflammation by blocking the adhesion of monocytes to endothelial cells by inhibiting the activation of the cell adhesion molecules ICAM-1, VCAM-1, and ELAM-1(Aggarwal et al., 2005).

II.30.hAntiapoptotic Proteins.Curcumin induces apoptosis by inducing cytochromecrelease, Bid cleavage, and caspase 9 and 3 activation and by downregulating the anti-apoptotic proteins Bcl-2 and BclXL(Aggarwal et al., 2003).

II.30.i Multidrug Resistance Gene.Multidrug resistance is associated with decreased drug accumulation in tumor cells due to increased drug efflux. Curcumin down-regulates drug resistance by inhibiting the expression of the mdr gene, which is responsible for this phenomenon.

II.31 Clinical Use of Curcumin as a Drug

Curcumin is has gained much interest as a potential anticancer agent. Many *in vitro* experiments have confirmed that curcumin is effective against cancer growth, inflammation and infection. However, *in vivo* experiments have reported poor results, which may be associated with low aqueous solubility, bioavailability and metabolic breakdown of curcumin (Gupta et al., 2010). It is therefore a challenge to harness the vast knowledge available about curcumin and administer the drug to treat cancer patients effectively. The development of an effective drug delivery system may improve both the bioavailability and stability of curcumin.

II.32 Overview of turmeric in treating diseases

Traditionally, turmeric has been used for many ailments, particularly as an anti-inflammatory agent, and curcumin has been identified as the active principle of turmeric. Curcumin has been shown to exhibit antioxidant, anti-inflammatory, antimicrobial, and anti-carcinogenic activities. Additionally, the hepato- and nephro-protective, thrombosis suppressing, myocardial infarction protective, hypoglycemic, and anti-rheumatic effects of curcumin are also well established.

Various animal models(Shankar et al., 1980) or human studies (Lao et al., 2006, Chen et al., 2008)proved that curcumin administered orally is extremely safe even at very high doses. For example, three different phase I clinical trials indicated that curcumin, when taken as high as 12 g per day, is well tolerated (Lao et al.,2006). Similarly the efficacy of curcumin in various diseases including cancer has been well established. Several clinical studies dealing with the efficacy of curcumin in humans can also be cited (Aggarwal et al., 2003). The pharmacological safety and efficacy of curcumin makes it a potential compound for treatment and prevention of a wide variety of human diseases. In spite of its efficacy and safety, curcumin has not yet been approved as a therapeutic agent, and the relative

bioavailability of curcumin has been highlighted as a major problem for this(Gupta etal.,2010)

II.33 Problems of Curcumin Bioavailability

The reasons for reduced bioavailability of any agent within the body are low intrinsic activity, poor absorption, and high rate of metabolism, inactivity of metabolic products and/or rapid elimination and clearance from the body. Studies to date have suggested a strong intrinsic activity and, hence, efficacy of curcumin as a therapeutic agent for various ailments. However, studies over the past three decades related to absorption, distribution, metabolism and excretion of curcumin have revealed poor absorption and rapid metabolism of curcumin that severely curtails its bioavailability.

II.34 Low Serum Concentration

One of the major observations related to curcumin studies involves the observation of extremely low concentration in serum. The first reported study to examine the uptake, distribution, and excretion of curcumin was by Wahlstrom and Blennow in 1978 using Sprague–Dawley rats. Negligible amounts of curcumin in blood plasma of rats after oral administration of 1g/kg body weight of curcumin showed that curcumin was poorly absorbed from the gut (Wahlstrom et al., 1978). In 1980, Ravindranath et al. showed that after oral administration of 400 mg of curcumin to rats, no curcumin was found in heart blood, whereas a trace amount (less than 5 μg/mL) was found in the portal blood from 15 min to 24 h after administration of curcumin(Ravindranath et al., 1980).

There are several reports of attempting various carrier/deliver vehicles that might promote stability, solubility and bioavailability. It is widely known that the enhanced permeability and retention effect of nano-materials may improve the accumulation of chemotherapeutic agents at tumor sites.

II.35 Albumin as a drug carrier

Albumin is a multifaceted, highly soluble, stable, biocompatible and biodegradable plasma protein. Because of its versatile nature, it can be used for the delivery of the drugs, hormones, metals and fatty acids by binding to its specific binding sites. The structure, location, size, charge and hydrophobicity of these drug binding sites are very important to optimize the interaction of drugs with albumin**.**

II.35.a. Physiological Functions of Albumin

Albumin is important for various physiological processes like maintenance of colloidal osmotic pressure, immunomodulation, endothelial stabilization, solubilizing long chain fatty acid, delivery of nutrients to cells, and balancing plasma pH. It is responsible for the antioxidant property of human serum, either directly or by binding to radical scavengers, or by sequestering transition metal ions with pro-oxidant activity. Albumin acts as a NO depot and carrier, leading to covalent modification of molecules. Moreover, heme-HSA mutants have been proposed as O_2 carriers not only for red blood cell substitutes but also for O_2 therapeutic reagents (Theodore P.,1995.,Fasano M ., 2005).

II.35.b. Merits of Albumin as a Drug Carrier

Albumin is best known for its remarkable ligand binding capacity. It is a versatile protein with anti-oxidant, immune-modulatory, detoxifying properties and can act as a potent drug carrier. Albumin can be used as an exogenous or endogenous protein for the treatment of various diseases like cancer, rheumatoid arthritis, diabetes and hepatitis. Albumin-based drug delivery systems include albumin drug nanoparticles, albumin fusion protein, pro-drugs and peptide derivatives that bind covalently to albumin as well as physically bind to the antibody fragments and therapeutically active peptides. During the last decade, the use of albumin has been a source of the debate focusing on whether albumin can act as a better drug carrier for delivery of macro-molecules or not(FasanoM ., 2005., Cistola DP.,1991).Human albumin has the capacity to bind an extraordinarily diverse range of molecules. Following are the main features which explain its specificity as drug carrier-

- Albumin provides a depository for a wide variety of compounds that may be available in quantities well beyond their bioavailability in plasma. This is feasible because the negative charge of HSA (Human Serum Albumin) facilitates electrostatic binding of various ligands with albumin, acting as a depot and carrier for many drug compounds.
- Albumin also performs transport functions through the binding sites which are present in its tertiary structure. The substances transported by albumin includes large number of drugs, bilirubin, bile acids, hormones, metals, anions, long-chain fatty acids, L-thyroxine, nitric oxide, endotoxins and other bacterial products such as the protein G-like albumin-binding molecule (Lejon S,2000).

The methodology, selectivity and capacities of ligand binding to albumin are as assorted as the compounds, which has the capacity to bound (Theodore P.,1995). The modes of binding include complex formation with metals, hydrophobic and electrostatic interactions and covalent binding. Several binding sites are present on albumin molecules. For fatty acids, seven binding sites have been described. The most eminent sites for drug binding are the site I and site II located on the sub-domains IIA and IIIA, respectively (Simard et al., 2006., Sudlow G, 1976).

- Complex formation and high-affinity binding are principally reversible, whereas covalent binding to albumin may occur in a reversible or irreversible manner.
- The effectiveness of a drug is dependent on accumulation at the site of action at therapeutic levels, however,challenges such as rapid renal clearance, degradation or non-specific accumulation requires drug delivery enabling technologies.
- Albumin is a natural transport protein with multiple ligand binding sites, cellular receptor engagement, and a long circulatory half-life due to interaction with the recycling neonatal Fc receptor.
- Exploitation of these properties promotes albumin as an attractive candidate for half-life extension and targeted intracellular delivery of drugs attached by covalent conjugation, genetic fusions, association or ligand-mediated association.

II.35.c. Characteristics of albumin

Albumin is the most abundant plasma protein in human blood (35–50 g/L human serum) with a molecular weight of 66.5 kDa(Kratz F, 2008). It is synthesized in the liver by hepatocytes with 10–15 g of albumin produced and released into the vascular space daily. Circulation in the blood proceeds for an extended period of ~ 19 days (Kim J, 2007). This long half-life is thought to be mainly due to neonatal Fc receptor (FcRn)-mediated recycling, and the Megalin/Cubilin-complex rescue from renal clearance. Termination of the circulation is typically caused by catabolism of albumin in organs such as the skin and muscles. Modifications of albumin, for instance by non-enzymatic glycosylation, is thought to trigger lysosomal degradation.Albumin contains multiple hydrophobic binding pockets and naturally serves as a transporter of a variety of different ligands such as fatty acids and steroids as well as different drugs. Furthermore, the surface of albumin is negatively charged (Peters T.,1985) making it highly water-soluble. .Drugs and drug metabolites can also bind covalently to albumin. Glucuronidation of drugs as part of metabolism, often occurs to drugs having a carboxylic acid group resulting in acid glucuronides (Kragh-Hansen et al., 1981).

Albumin contains 35 cysteine residues of which 34 form disulfide bridges internally in the structure. These contribute to the high stability of albumin. The availability of a free cysteine residue at position 34 (cys34) for covalent attachment of drugs is an attractive feature for drug delivery as it holds a free thiol group (−SH) accounting for 80 % of thiols in the plasma. Cys34 is located on the outer surface of albumin distant from the main interior drug binding sites and has, therefore, been a focus for covalent conjugation of drugs.The natural transport function, multiple ligand binding sites, and cellular interactions provides rational for the exploitation of albumin for drug delivery.

II.35.d.Albumin as a life-saving exogenous protein:

Fractionation of human plasma collected in blood bank has been popular from 1950s when Cohn standardized ethanol precipitation technique to isolate albumin and since then technology has been improved by different scientists to obtain >90% homogenous albumin as biotherapeutic. Currently available pharmacopoeia grade commercial albumin preparation is sterile and viral safe for infusion and is considered as a life-saving drug in many disease conditions. It is used for treating a variety of conditions, including shock due to excessive blood loss, burns, low protein levels surgery or liver failure, and as additional medicine in bypass surgery. Albumin accumulates in malignant and inflamed tissue due to leaky capillaries. Therefore, prospect for using albumin as drug carrier has high potential.

II.35.e.Photophysical Studies on Binding of Curcumin to Bovine Serum Albumin

Serum albumins such as bovine serum albumin (BSA) and human serum albumins (HSA) are plasma proteins contributing significantly to physiological functions and act as carrier proteins (Kragh-Hansen et al., 1981, Carter et al., 1994). They aid in the transport, distribution and metabolism of many exogenous ligands. The ligands include fatty acids, amino acids, metals, drugs and pharmaceuticals (Jacobsen et al., 1983, Gelamo et al., 2002). There are a number of reports in the literature, where binding of metabolites, drugs, dyes, fatty acids, etc. have been studied in detail. BSA has conformational adaptability while binding to the ligands of great varieties. X-ray diffraction studies have shown that the principal binding sites in serum albumin are located in the hydrophobic cavities. The tertiary structure of the protein is composed of three domains, and at least six binding sites (Mikusinska-Planner et al., 2000) are available for the binding of the ligands. Hence, the nature of binding of a ligand with BSA is different for different ligands (Kragh-Hansen et al., 1981, Carter et al., 1994).

Previous work in our laboratory demonstrated that conjugation of curcumin to serum albumin (Alb) increases aqueous solubility of the former without affecting its *in vitro* drug action on both cancer cell lines and primary cells in culture(Thomas et al.,2014). Conjugation of curcumin (Curc) to albumin (Alb) has been found to increase the aqueous solubility of the drug. Dalton's lymphoma ascites (DLA) cell viability was inhibited by the Curc-Alb conjugate in a dose dependent manner *in vitro*, as evidenced by the MTT assay. Administration of up to 11.4 mg of conjugated curcumin per kg body weight to healthy animals was non-toxic both in terms of lethality and weight loss. Histological analysis of vital organs (kidney, liver and spleen) also did not show toxic effects. Favorable immuno-modulatory activity was observed after continuous administration of sub-acute doses of the conjugate which caused increase in total leukocyte count, platelet count, and viable cell count in bone marrow, and enhanced proliferation of lymphocyte *in vitro* upon culture. *In vivo* studies in the DLA tumor modelin mice demonstrated that conjugated drug induces tumor reduction and prevention(Aravind et al., 2016).

In this background it is important to study if the action of free curcumin and albuminated curcumin is comparable at the molecular level in cancer cell and in normal fibroblast. The current study aimed to prove the possible safe use of the Curc-Alb conjugate in animals and to demonstrate that it retains drug action. The purpose of this study is to evaluate if Curc-Alb is internalized as active as free curcumin in both cancer cells and in normal cells.

II.36. Gap area

Albuminated curcumin that showed 100-fold higher solubility than free Curc was stable and inhibitory to proliferation, it induced cell cycle arrest and apoptosis. The conjugate showed apoptotic effects on endothelial cells indicating its anti angiogenic property. Primary fibroblast growth was also inhibited but when a higher dose was used. The *in vitro* results suggest that Alb-Curc which is free of insoluble native drug may find application in cancer therapy after appropriate *in vivo* evaluations.In spite of all these studies showing positive outcome to use the conjugate as drug delivery method, it has not been proven if the entry of Cruc-Alb to cancer cells is similar to that of other cells. Also it is not known if the mechanism of action of Curc-Alb on cell apoptosis is similar to that has been established in the case of free curcumin. So there is a need to further explore to establish if mitochondrial pathway is involved for the apoptotic action of Curc-Alb.

II.37. Hypothesis:

1. Curcumin conjugated to albumin (Curc-Alb) may be tagged with FITC and endocytosis into various cells may be analyzed qualitatively and quantitatively.
2. Dose response of Curc-Alb for inducing apoptosis may be demonstrated using simple apoptosis assays.
3. Caspase actvation by Curc-Alb may be understood by live cell imaging using FRET based probes.

II.38. Goal and Objectives.

The objectives of the study are:

(1) To prepare fluorescent tagged Curcumin-Albumin conjugate;

(2)To establish the Curc-Alb concentrations that cause cytotoxic response in lung cancer cell line A549;

(3)To establish endocytosis of the conjugate into cancer cells and normal cells using fluorescent Curc-Alb;

(4) To establish induction of apoptosis and death of cancer cells and normal cells in a dose - dependent manner using Annexin V binding assay; and

(5) To confirm caspase activation by Alb-Curc using FRET based probe for live cell imaging.

Chapter II

Materials and Methods

In order to test the hypothesis of this study, experiments were carried out based on different set objectives. Various materials and methods used for the experiments are listed in this chapter. Also the experimental protocol is described in detail. Where the standard method is used, reference is cited with a brief description. Where new experiment is standardized for meeting the objectives the procedure is written in detail. To establish that curcumin in the conjugated form is active, tests carried out are the endocytosis of conjugate into the cells using fluorescent probe tagged to the conjugate, detection of cytotoxic concentration using standard MTT assay, quantitative apoptotic assay using Annexin V/PI and finally the pathway analysis using specific molecular probes were done.

2.1 Materials

Curcumin, Bovine serum albumin,(BSA, Sigma-Aldrich, USA), Sephadex G-25 beads(Sigma), DMEM/F12(Gibco®,USA), Antibiotic-Antimycotic (Gibco,USA), fetal bovine serum (FBS) (Gibco,USA), Trypsin , Fluorescein iso thio cynate (FITC) (Sigma) Copper sulphate (Merck), Sodiumchloride, Sodiumazide, Sodium carbonate, Sodium bicarbonate, Sephadex G-25 beads(Sigma), culture dishes(Thermo scientific), Dulbecco's Modified Eagle's Medium (DMEM) (Gibco®,USA), HP 8543 UV-Vis Spectrophotometer (Hewlett-Packard, USA), 37% Formaldehyde (SDFCL, Mumbai), Leica IM-DRB Fluorescent microscope (Leica, Germany), PBS (pH 7.4),Apoptosis kit (Vibrant,USA), 0.1N NaOH,0.5 % $CuSO_4$ in 1% N Sodium potassium tartrate ,Folin's Reagent, 3-(4, 5 dimethy thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) (Sigma), DMSO(Merck), LipofectamineTM 2000 (Invitrogen, Carlsbad, CA), in chambered cover glass (Lab-TekTM, Nunc, NY) were all employed in this study.

2.2.Methods

2.2.1. Culture and maintenance of cells

Lung cancer cell lines (A549) and control lung fibroblasts (W138) obtained as kind gift from Dr.Santhosh Kumar T.R., Scientist E, RGCB, Trivandrum was used for the study. Cell lines received were sub cultured and sufficient stock vials were stored in −80˚C deep freezer. Revived cells were maintained in DMEM supplemented with 10% heat-inactivated FBS, antibiotics at 37°C in a 5% CO_2/95% air humidified atmosphere. For each Experiment, A549

cells were seeded in 6 well plates and 4 well plates, incubated for 24 hours at 37°C and used for treating with Curc-Alb. To test the effect of Alb-Curc an additional control used was primary adipose derived mesenchymal stem cells (ADMSCs) isolated and maintained from lipoaspirates (with ethical committee approval) and kindly gifted by senior Ph.D students.For harvesting cells for experiments and for subculture, trypsinization was done once the cells became 100% confluent. For trypsinization the medium was removed and cells were washed with HBSS buffer. 0.25% Trypsin EDTA was added to the culture flask and the cell were incubated for 2 minutes at 37 C in 5% CO2 incubator. The trypsin activity was arrested after incubation by adding serum containing media and the cells were collected into a sterile centrifuge tube. After centrifugation at 400*g* for 5 minute the cells were re-suspended in complete medium and seeded into tissue culture dish.

2.2.2. Preparation of Curcumin albumin conjugate

Five hundred mg BSA was added into 10ml Tris HCl buffer (pH 7.0) and allowed good dissolution by keeping the mixture at 4^{o}C overnight. 250 µl curcumin stock was added to the albumin to make 50mM for 50 mg albumin (1 ml) and incubated at room temperature for 2 h. Curcumin albumin conjugate was loaded on Sephadex G-25 columns for removing un reacted curcumin from the conjugate. 10 ml was the bed volume of Sephadex G25 beads used for loading 1 ml reaction mixture; which is equilibrated with Tris HCl buffer and 1 ml fractions were collected. The Curc-Alb conjugate was identified by measuring the molar ratio (A280:A420) of 1 ml fractions eluted from the column. The protein fractions with minimum spectral peak ratio were considered to have maximum conjugated Curc and such fractions were pooled and 1 ml aliquots of the pooled conjugate was lyophilized (Edwards Modulyo, Edwards, UK) and stored at 4˚C.

2.2.3. Preparation of Curcumin-Albumin- FITC conjugate

For tagging FITC to Curc-Alb conjugate, after conjugation of Curcuimin with albumin dissolved in Tris-HCl, pH 7.0 buffer, the reaction mixture was loaded on a 10 ml Sephadex G25 column equilibrated in carbonate buffer, pH 9.6.The fractions with bestA280:A420 ratio was selected for adding 50 µl of FITC dissolved in DMSO was added to Curc-alb conjugate and incubated in room temperature for 4 h. The mixture was gel filtered a second time using a Sephadex G25 column equilibrated with Tris-HCL, pH 7.0 buffer and Curc-Alb-FITC conjugate was identified by measuring the A280:A420:A495 peaks of 1 ml fractions eluted from the column and pooled conjugate was dispensed in1ml volumes followed by their

lyophilisation and were sealed and stored at 4°C. The lyophilized fluorchrome –tagged conjugate (Curc-Alb-FITC) was dissolved in water for endocytosis detection experiments and sterile filtered through 0.22 μm syringe filter (Millipore, USA).

2.2.4. Estimation of Bound curcumin and bound FITC

First step was to prepare standard calibration curve.To prepare standard (known concentrations), graded concentrations 100 mg curcumin was dissolved in 1 ml DMSO. For making known graded concentrations of FITC, 11 mg was dissolved in 1ml DMSO. Graded concentrations of curcumin (1mg, 1.5 mg, 2mg,2.5 mg,3.0 and 3.5 mg) and FITC (0.11 mg, 0.165 mg, 0.22 mg, 0.275μl, 0.33 mg and 0.385 mg) from stock solution were made up in 1 ml DMSO, by addingrequired volumes from the stock. The calibration curve was plotted by measuring absorbance at 420 nm for curcumin and 495nm for FITC using 8053 Hewlett Packard Diode Array spectrophotometer (USA).

For estimation of bound curcumin and bound FITC in the conjugate, initially 50 μl of water was added to the lyophilized conjugate (original 1 ml). After mixing well to a homogenous mixture, 950μl of DMSO was added and mixed thoroughly. The absorbance of curcumin molecules in DMSO extract was measured at the characteristic wavelength of 420 nm and 495 nm for the extracted FITC, respectively. Based on the absorbance of the DMSO extract, the concentration was estimated using the respective calibration curve for each.

2.2.5. Lowry's method of protein quantification in conjugate

The concentration of protein (albumin) present in each lyophilized aliquot was estimated using Lowry's method (Lowry et al,1951).Lyophilized protein was dissolved in 1 ml water. The samples were diluted using 10 times buffer prior to the assay. Assay was done by the standard method and a calibration curve was prepared using known concentration of standard BSA obtained from commercial source. Reagent A was prepared by dissolving 1% sodium carbonate in 0.1 N NaOH. Reagent B was prepared by dissolving 0.5 % $CuSO_4$ in 1% N Sodium potassium tartarate. Reagent C was prepared from Reagent A and Reagent B by mixing it in a ratio of 50:1 1ml of this solution was added to 100 μl of the sample and was incubated for ten minutes. 100 μl of Folin's Reagent was added and incubated in dark for half an hour. Absorbance at 600 nm was taken using UV-Visible Spectrophotometer (Hewlett Packard Diode array 8453, Germany).

2.2.6. Cytotoxic activity of Curcumin

Cytotoxicity was assayed by employing the standard MTT assay. This is a colorimetric assay that measures the percentage of metabolically active cells. The test is based on the principle that MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, coloured (dark purple) formazan product. Reduction of yellow MTT in the reagent by mitochondrial succinate dehydrogenase is the first step. This formazan production is directly proportional to the viable cell numbers and inversely proportional to the degree of cytotoxicity. The cells are then solubilized with an organic solvent (eg. isopropanol) and the released, solubilised formazan reagent is measured spectrophotometrically. Since reduction of MTT can only occur in metabolically active cells the level of activity is a measure of the viable cells. Thus the metabolic activity of each culture treated with different dose of Curc-Alb was determined by the standard MTT assay and compared to those of untreated cells.

Cells were cultured in 96-well plates containing 100 µl medium, kept this plate in CO_2 incubator (5% CO_2) at 37°C for 24 h. Diluted curcumin conjugate solutions (25 µM, 50 µM , 100 µM) were freshly prepared by dissolving the lyophilized conjugate in DMEM F12 medium and added in 96 well plate for 24 h. Commercial MTT (.5 mg/ml) reagent was dissolved in medium. This solution was filtered through a 0.2 µm filter and stored at 2 - 8°C. After removal of 100 µl medium, MTT dye solution was added and the plates were incubated at 37°C for 4 h in a humidified 5% CO2incubator, followed by 100µl of DMSO added to each well, and mixed thoroughly to dissolve the dye crystals. The absorbance was measured using an ELISA plate reader at 570 nm. High optical density readings corresponded to a high intensity of dye color. The fractional absorbance was calculated by the formula:

$$\% \text{ Cell survival} = \frac{\text{Mean absorbance in test wells x100}}{\text{Mean absorbance in control wells}}$$

Cytotoxicity of Curc-Alb conjugate on A549 cells was calculated as cell growth inhibition rate (IR),IR = 100−PR.

The average cell survival obtained from triplicate determinations at each concentration was plotted as a dose response curve.

2.2.7. Estimation of cellular uptake of Curc-Alb

For qualitative analysis of endocytosis of Curc-Alb by the cells, FITC tagged conjugate was treated with cell cultures at different concentrations such as 25μM, 50 μM and 100 μM. After definite time periods such as 2h, 6h, 10h and 24h, the cells were viewed under the fluorescence microscope. Images were taken to see the frequency of green fluorescence in the cell cytoplasm. The cells in 6 well plates were subjected to serum-free medium and HBSS wash and then visualized by Fluorescence microscope thereafter cells were harvested by trypsinization, washed and fixed using 3.7% paraformaldehyde solution. The treated cells were analyzed by flow cytometer in order to quantify the percentage of cells positive for FITC and the mean fluorescence intensity was also analysed to determine if the number of molecules of conjugate getting endocytosed was influenced in a dose dependent and time-dependent manner.

2.2.8. Apotosis Assay using Annexin-V/Propidium Iodide staining

The phosphatidyl serine translocation in apoptotic cells was monitored by Annexin V-FITC/propidium Iodide (PI) staining using apoptosis detection kit (Invitrogen, USA). Apoptosis is a carefully regulated process of cell death that occurs as a normal part of development. In normal live cells, phosphatidylserine (PS) is located on the cytoplasmic surface of the cell membrane. However, in apoptotic cells, PS is translocated from the inner to the outer leaflet of the plasma membrane, thus exposing PS to the external cellular environment. The FITC Annexin V/Dead Cell Apoptosis Kit with FITC annexin V and PI for flowcytometry provides a rapid and convenient assay for apoptosis. The kit contains recombinant annexin V conjugated to fluorescein (FITC annexin V), as well as a ready-to-use solution of the red-fluorescent propidium iodide (PI) nucleic acid binding dye. PI is nonpermeating in to live cells and apoptotic cells, but stains dead cells with red fluorescence, binding tightly to the nucleic acids in the cell. After staining a cell population with FITC annexin V and PI in the provided binding buffer, apoptotic cells show green fluorescence, dead cells show red and green fluorescence, and live cells show little or no fluorescence . These populations can easily be distinguished using a flow cytometer with the 488 nm line of an argon-ion laser for excitation.

In brief, 5 x 10^5 cells were treated with graded concentrations of conjugated curcumin such as 25μM, 50 μM and 100 μM for 24h. Thereafter, cells were collected, washed and re-

suspended in 1X Annexin binding buffer followed by the addition of 1µl Annexin-V-FITC and 1µl Propidium Iodide solution (BD Biosciences, New Jersey, USA). Cells wereincubated in the dark for 15 min at room temperature and thereafter subjected to flow cytometric analysis. Data wasacquired by BD FACS Diva software (BD Biosciences, San Jose, CA, USA) using standard fluidics, optical and electronic configuration. The light source used was blue laser 488nm with filters, FITC (530/30) and PI (585/42). The FITC and PI channels were compensated with appropriate controls. The gating on the cell population was set up by FSC/SSC scatter plot. 10,000 events were recorded and analysed for Annexin-V/Propidium Iodide stain.

2.2.9. Live Cell Imaging to detect Caspase activation

An image based high-throughput method for detecting caspase activation and for anticancer drug screening using stable cells expressing FRET based genetically encoded sensor was used.

Expression vectors and generation of stable cell lines

This experiment was conducted by the technical staff at RGCB and validated cells were provided for analysing the effect of Curc-Alb. Briefly, SCAT3,SCAT8& SCAT9 vectors were used. The SCAT3 is linked with preferred caspase 3/7-specific DEVD recognition site,SCAT 9 with caspase 9-specific recognition site LEHD in between donor and acceptor. SCAT 8 is interconnected with short linker of preferred caspase-8/-10 recognition site IETD between donor and acceptor. Cell transfection was performed using LipofectamineTM 2000 (Invitrogen, Carlsbad, CA) as per the manufacturer's instructions. Stably expressing clones were generated by selecting the cells in 800 mg/ml of G418 (Invitrogen, Carlsbad, CA) containing medium for 30–45 days. Following clonal propagation, the multiple clones with different levels of transgene expression were expanded and further sorted based on quantitative FRET, analysed by FACS Aria (Becton Dickinson) so as to obtain an enriched population of SCAT3, SCAT8 and SCAT9 expressing cells with optimum default FRET. Briefly, cells were excited with 407 nm laser and the FRET-high cells were identified from the scatter plot of ECFP emission collected using 450640 nm filter against EYFP emission collected using 530/630 nm filter. (Joseph etal.,2011).

2.2.10.Detection of Caspase activity.

An image based high-throughput method was adopted for detecting caspase activation using stable cells expressing FRET based genetically encoded sensor. This probe consists of donor fluorophore ECFP- and acceptor fluorophore EFYP joined with an activated caspase specific

amino acid linker DEVD. In the present work, we used Lung cancer cell line stably-expressing caspase sensor at the nucleus (with nuclear localization signal). For easy and uniform imaging purposes, single cell colonies with homogenous expression of the probe were expanded and used for the current study.

For imaging caspase activation, wide field microscopy with single excitation for ECFP and dual emission of ECFP and EYFP was collected in ratio mode using an automated fluorescence microscope (BD Pathway). For ratio imaging the cells were grown in chambered cover glass (Lab-TekTM, Nunc, NY) at desired density and were treated with the drugs. For microscopic ECFP/EFYP FRET ratio imaging, cells were excited with excitation filter of 438±24 nm and dual emission was collected with 483±32 nm and 542±27 nm using the dichroic 458LP.

For determining mitochondrial membrane potential, lung fibroblasts cells (W138) were grown on glass bottom 96 well plates and stained with TMRM for 15 minutes at a concentration of 100nm. The cells were further stained with Hoechst for nuclear imaging. The cells were treated with indicated concentration of drug with 20 nm of TMRM for 24h. The imaging was carried out using pathway Bioimager for TMRM and Hoechst fluorescence using specific filter sets. The cells with mitochondrial membrane potential loss were identified with loss of red mitochondrial fluorescence.

All quantitative data is acquired at least in 3 replicates using separate culture of cell. The mean ± SD (standard deviation) of three independent experiments are calculated and wherever feasible, is compared with different sets of data acquired on dose effect or exposure time effect to understand the effect of Curc-Alb on cellular activities.

Chapter III

RESULTS AND DISCUSSION

When curcumin is modified to improve the aqueous solubility and bio-availability for achieving clinical use as anti-cancer drug, it is important to establish that the modified molecule retains the expected biological property. If the conjugation of albumin to curcumin affects the functional binding sites, we may not get the same apoptotic effect. The experiments in this study main focus was on understanding the mechanism of action of Curc-Alb conjugate on cancer cell line and on primary human cells such as mesenchymal stem cells. At the first instance, it is important to prove that the conjugated Curc-Alb enters the cell cytoplasm. Only if it is accessible and interacts, molecular level action can be expected. The entry into the cell can be established by tagging the conjugate with fluorochrome and the dose effect and the time effect on endocytosis can be studied qualitatively and quantitatively. After entry into cytoplasm how the cytotoxicity and apoptosis induction has taken place is compared between different cell types.The results are illustrated and described below.

3.1 Conjugation efficiency

Conjugation efficiency was estimated by determining the protein content, curcumin content and FITC content in the lyophilized conjugate in a vial. In a solution of Curc-Alb prepared by dissolving lyophilized conjugate in 1 mL distilled water, the concentration of albumin was 1.9 mg/ml. On to this 1.9 mg albumin, 0.25 mg curcumin and 0.6 mg of FITC was bound. The calibration graphs used for estimating Curcumin and FITC extracted from the lyophilized conjugate is shown in Fig1 (A&B). For all studies in cell culture, the volume required for getting final concentration of 25 μM, 50 μM and 100 μM of curcumin in the medium was calculated and added to the culture from the stock of 1 ml Curc-Alb conjugate. When curcumin concentration is increased both the FITC concentration and albumin concentration also increased proportionately.

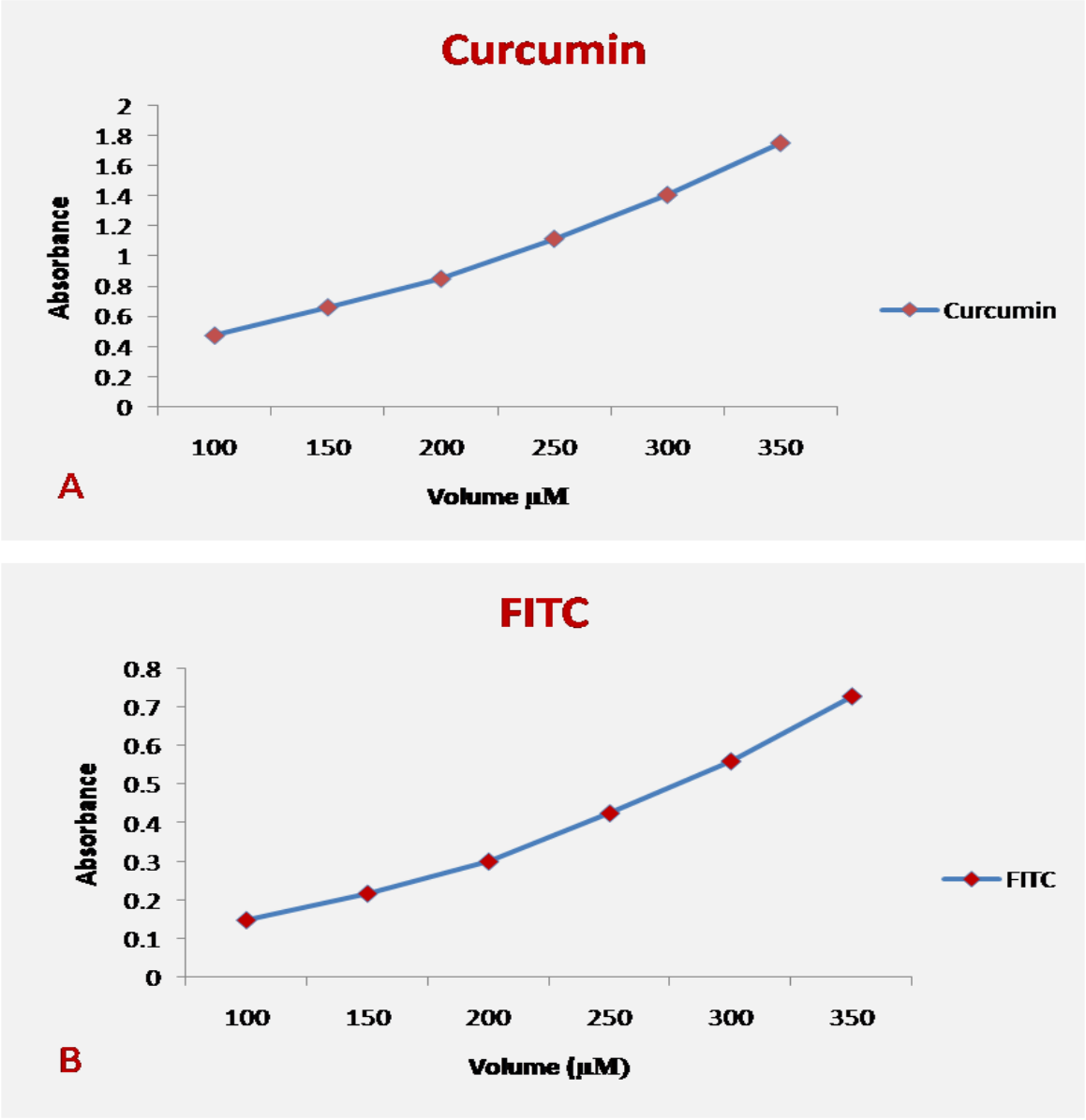
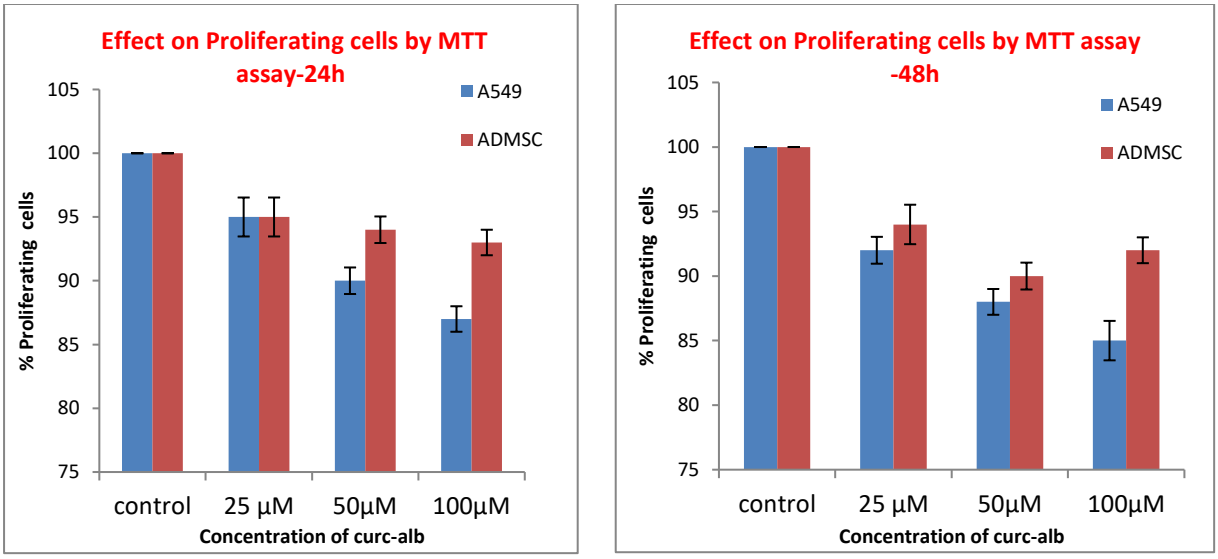


Fig: 1. A & B shows Standard plot for curcumin and FITC, respectively, dissolved in DMSO.The absorbance of DMSO extract from 1 vial (aliquot) was measured at both 420 nm and 495 nm, to estimate the concentration of Curcumin and FITC respectively.

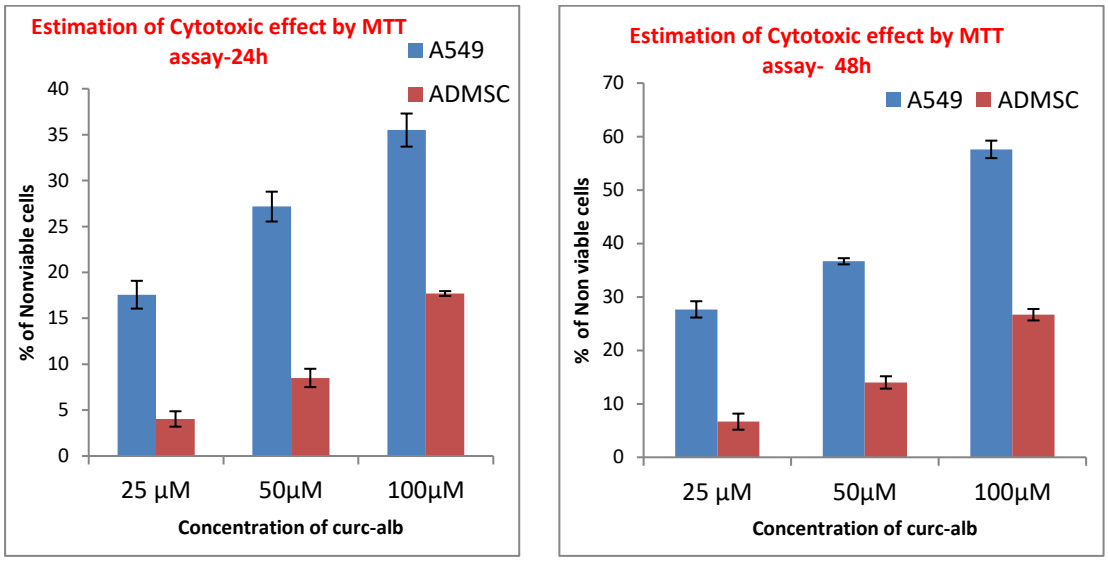
The estimation of bound concentrations enabled addition of required concentrations of drug molecule to study the dose response.

3.2.In vitro cytotoxicity profile

Cytotoxic concentrations of conjugate tothe A549 cells and ADMSC cells were estimated using standard MTT assay. Three time periods and 3 concentrations of Curc-Alb were tested. Both proliferating cells and cytotoxic cells were estimated from the same reading of product O.D. A dose-dependent inhibition of A549 cell viability was observed. At all concentrations, cell death was significantly increased as compared to control culture without any added conjugate.However, when incubated for more time, i.e. 72 h and 120 h no cells were viable. It clearly suggest that conjugate exhibits maximum cytotoxicity by 72h itself (data not shown).



Fg:2. Graphical representation of Proliferating rate of A549 & ADMSC cells in the presence of Curcumin albumin conjugate by 24 h and 48 h. Same number of cultures was exposed to graded concentrations of curcumin added into medium and assay was done using a blank reaction mixture without cells.



Fg:3. Graphical representation of percentage of non vialble A549 and ADMSC in presence of different concentrations of Curc-Alb conjugates after 24h and 48h.Same number of cultures was exposed to graded concentrations of Curc-Alb added into medium and assay was done after 24h.

From the graphs in fig:2 it is obvious that after 24 h and 48 h the conjugate reduces the proliferation potential of A549 cells in the presence of 25µM, 50 µM & 100µM by 24 h to 82%,72% and 64% ,respectively. But by 48 h, treatment of 25µM, 50 µM & 100µM Curc-Alb reduced the numbers of proliferating cells to 72%,63% & 42% respectively. From these it may be summarised that there is a time dependent deterioration of cells ability to proliferate. The cytotoxic response is also dependent on both dose and time. The response of Curc-Alb to cancer cells is therefore, promising and confirms that as reported for free curcumin, the conjugate is also effective in inducing cytotoxicity.

In order to estimate the cytotoxicity to normal cells ADMSCs were exposed to similar concentrations of the Cur-Alb conjugate for similar time periods. Interestingly, the proliferative potential was better retained in these cells and the effect was less intense. With 25µM, 50 µM & 100µM, after24 h, about 96%, 90% & 83% cells retained proliferation potential, respectively. Even after longer (48h) exposure with same concentrations such as 25µM, 50 µM & 100µM proliferative ability was much more stable as compared to A549 cells, showing 93%,86% & 73%, respectively. Upon calculating cytotoxicity, as compared to A549 cells, ADMSC shows much less cell death. At the highest concentration of 100 µM, after 48h, 55% A549 cells were nonviable whereas only 20% ADMSC were nonviable.

These results suggest that both low and high concentrations of Curc-Alb conjugate prevent cell proliferation and exhibit cytotoxicity in a time dependent manner in cancer cell lines. It is also evidenced that A549 responded more intensely than that of ADMSC.This could be due to some specific mechanism that promote cancer cells to internalize more conjugate. Therefore, experiments focused on understanding if the internalization of the conjugate is dose dependent, time dependent and cell-specific.

3.3. Assays for Curc-Alb Endocytosis.

Interestingly, the internalization of Curc-Alb conjugate by A549 cells was evident very quickly; the fluorescence signal was weak when 25 µM conjugate was added and more evident in 50µM treated cultures which suggest that when more conjugate is added more of it are internalized showing a dose dependency on the endocytosis of conjugate.

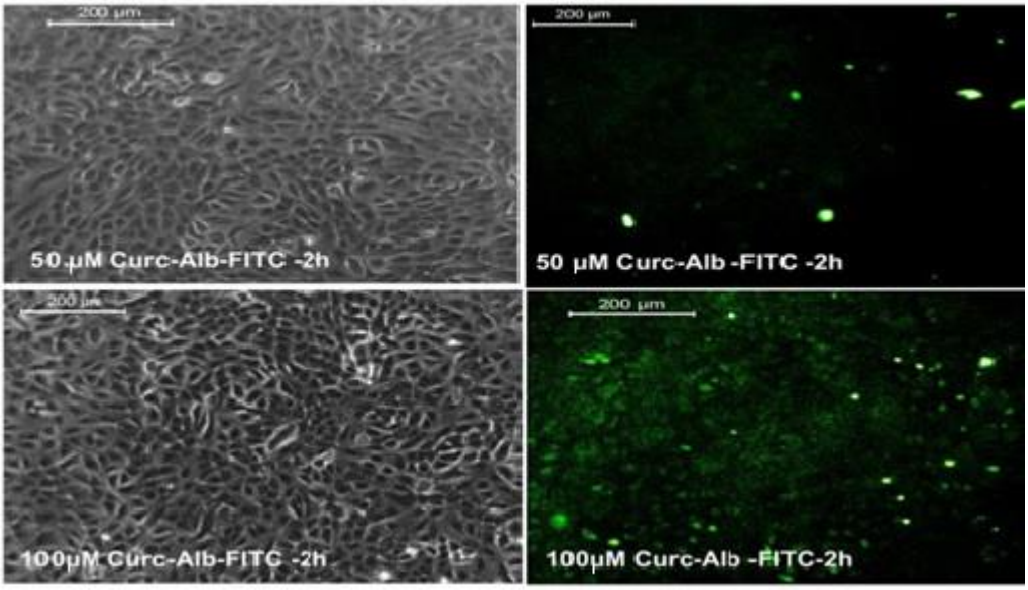


Fig:4. Representative photomicrographs showing uptake of Curc-Alb by A549 cells within2h of adding fluorochrome tagged Curc-Alb into the medium. Phase contrast microscopic images and corresponding fluorescent microscopic images of cultures treated with 50µM and 100µM are shown side by side.

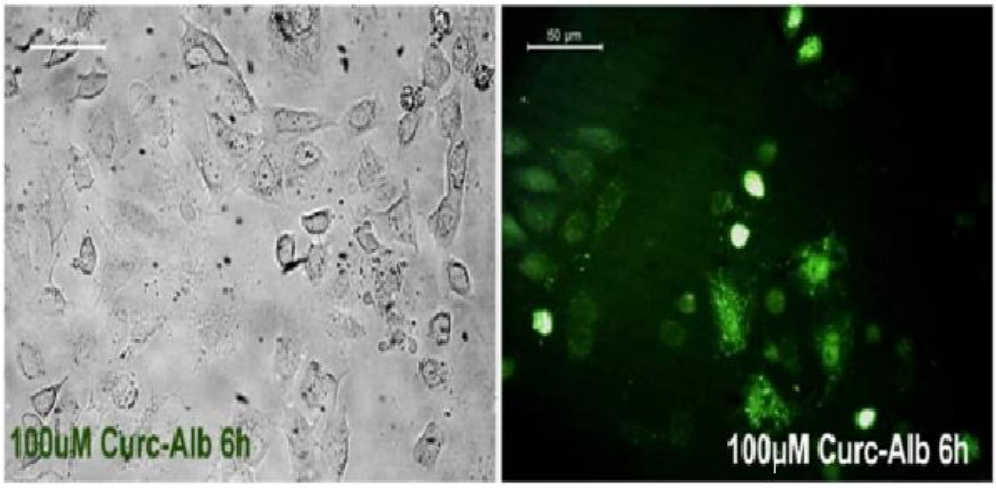


Fig:5. Representative photomicrographs showing uptake of Curc-Alb conjugate into A549cells after 6 h. Phase contrast and Fluorescent microscopic images of 100µM conjugate treated cultures are shown side by side.

Within 2h, all cells showed mild fluorescence upon treating with 50µM conjugate; at the same period, 100µM conjugate incubation resulted in very bright FITC-positive cells. Most of the cells appeared attached and live but more rounded and dead cells were seen in cultures treated with 100µM Curc-Alb (Fig 4).

After 6h, the numbers of attached cells reduced significantly as seen in the fluorescence micrographs (Fig 5). Fluorescence microscopic images confirmed that cellular internalization of the Curc- Alb conjugate by A549 cells is both dose dependent and time dependent. Conjugate showed intracellular green fluorescence. The observation indicates that within 2h itself most of the cells internalize the conjugate, irrespective of the dose. If the distribution of positive cells are considered, at 50µM, there is no significant difference between 2h to 6h or 6h to 10 h or 10h to 24h incubations. However, even when the lower dose is incubated, the amount of conjugate internalized seemed higher after 24h, so there is a time dependent internalization also.

In order to estimate homogeneity of cells in a given culture that internalized the conjugate, same graded concentrations which was used for qualitative assay were incubated with A549 and ADMSCs for different time periods. The harvested and fixed cells upon flowcytometric analysis indicated that more than 80% of the cells were positive for FITC by 2h, irrespective of the concentration in each culture. Representative dot plot is shown in fig: 6&7 showing the percentage of positive cells and a histogram of fluorescence intensity for A549 & hADMSC, respectively. Homogenous population with internalized Curc-Alb is evident in both types of cell culture but fluorescence intensity much high in A549 as compared to ADMSC. More the

intensity of fluorchrome, more the numbers of conjugate internalized. So there is a cell-specific mechanism which allows more conjugate to enter in A549.

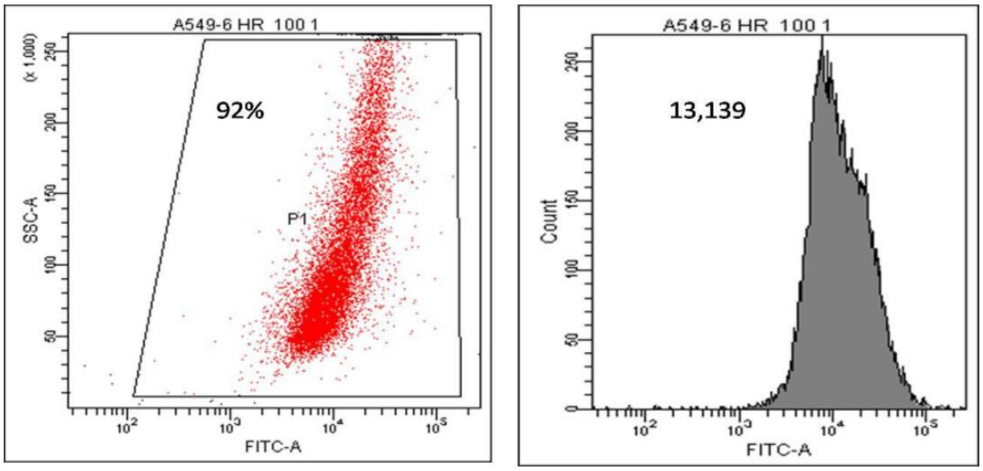


Fig:6. Representative Flow cytometric profile showing percentage of FITC positive cells & mean fluorescence intensity ofcurcumin albumin conjugate on A549 cells.

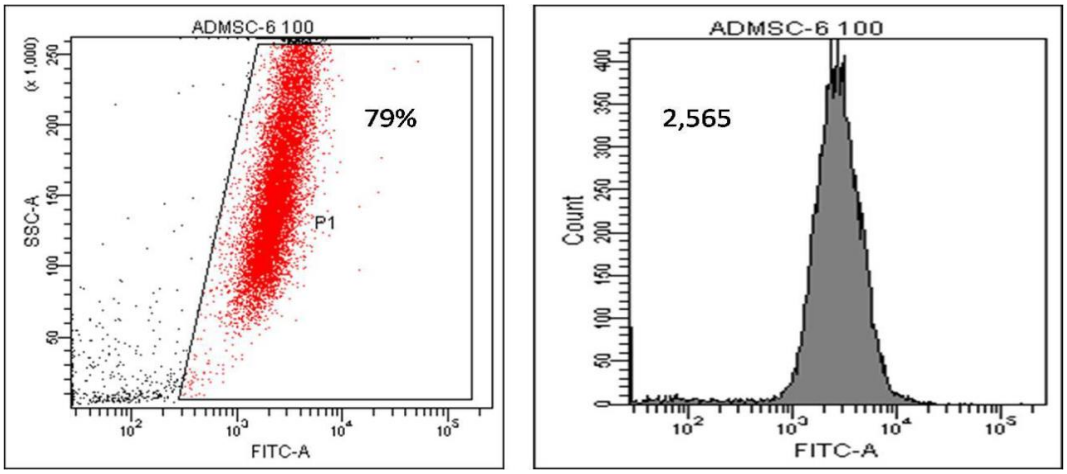


Fig:7. Representative Flow cytometric profile showing percentage of FITC positive cells and mean fluorescence intensity ofcurcumin albumin conjugate on ADMSC cells.

When the data from different time point and different concentrations are compiled and compared, there is no significant increase in number of cells that have endocytosed the conjugate (Fig:8).

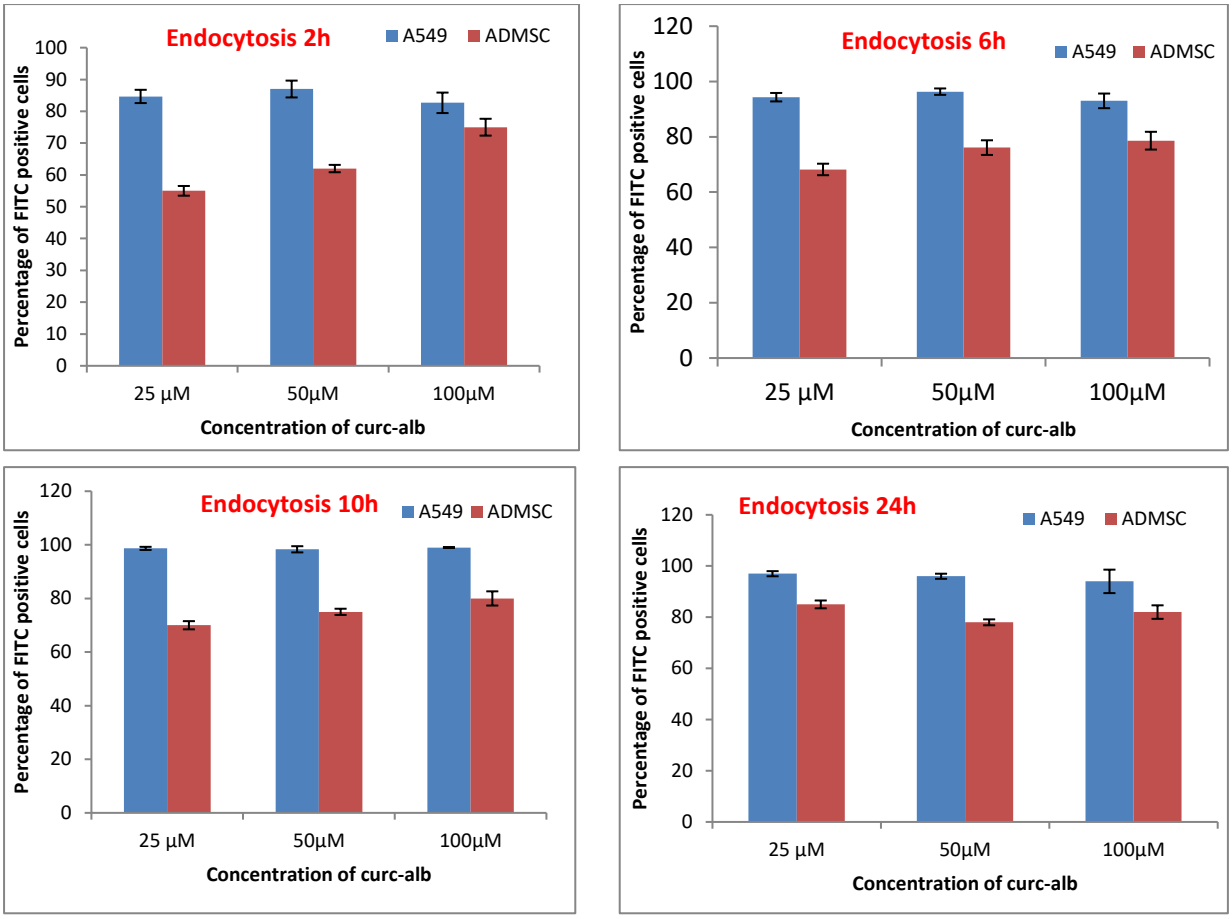


Fig: 8. Graphical representation of percentage of FITC positive cells indicating homogeneity for internalizing Curc-Alb into A549 & ADMSC cell after 2 h, 6h,10 h and 24h treatment with conjugate.

The compiled data shows internalization after 2h, 6h, 10h and 24h.The percentage of ADMSC which internalized Curc-Alb is found to be close to that of A549. Irrespective of the dose and time of exposure, 70 to 80% cells have endocytosed the conjugate (Fig 8). Even though fluorescence intensity was weak to capture in micrograph at lower dose but it is well detected in flowcytometric analysis.

That means irrespective of the concentration of the Curc-Alb added, most of the cells evenly internalize the conjugate. Therefore, to determine if the conjugate was internalized continuously, and if molecular concentration of internalized conjugate increases with time, the mean fluorescence intensity (MFI) was considered. When the conjugate concentrations were kept constant, with increase in incubation time, MFI increased significantly. This is obvious from the compiled MFI from different experiments with varying concentrations and time periods seen in Fig:9. The Y-axis scale of MFI increases remarkably with time. At all time periods, the MFI in ADMSC remained lower than 4000.

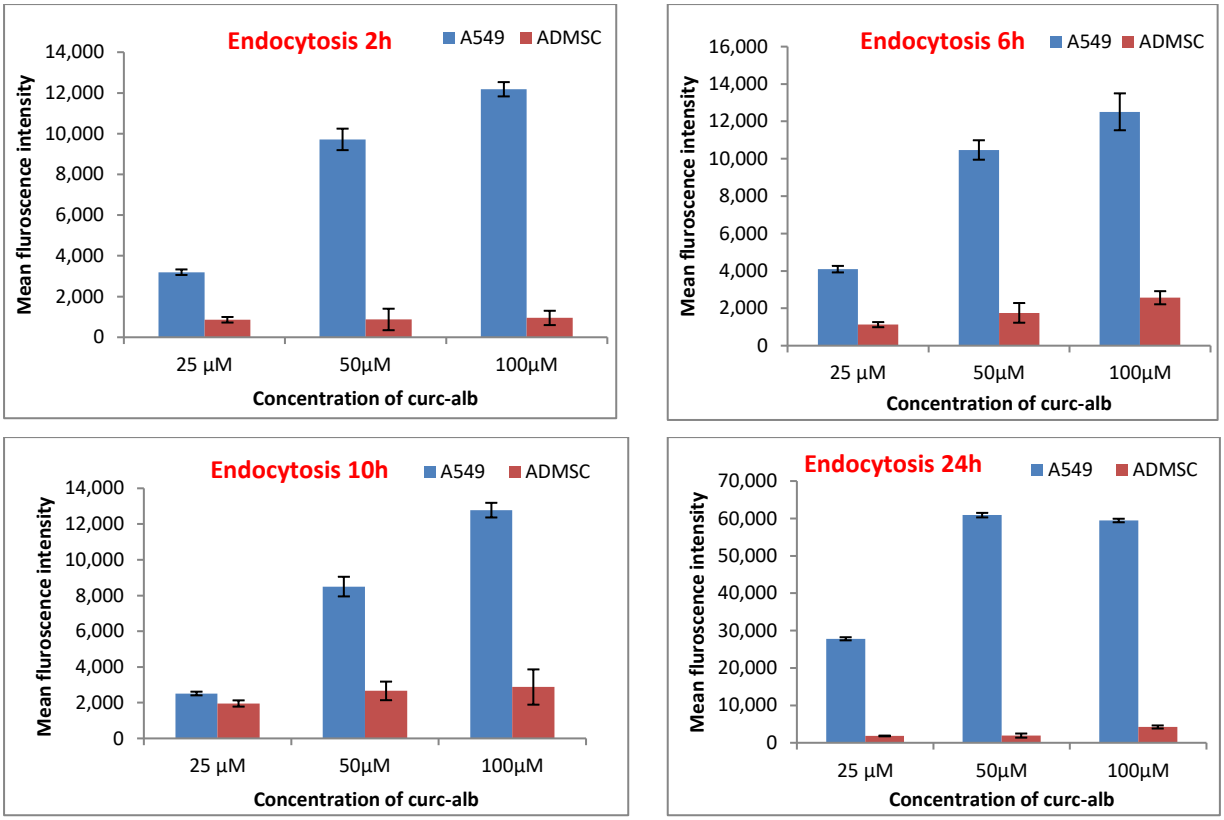


Fig:9.Graphical representation of MFI of FITC positive cells indicating higher dose-dependent and cell-specific intake of Curc-Alb into A549 as compared to ADMSC after 2 h, 6h,10 h and 24h treatment with conjugate.

About 15 fold higher quantity has got internalized in A549 as compared to ADMSCs at similar concentrations and time period. When ADMSCs are incubated with highest concentration of 100 µM of Curc-Alb, the MFI in 24h reached only 4000 (Fig 9). But in the case of A549, both at 50 µM and 100 µM concentrations, MFI reached 60000 (Fig.9). It indicates a 15 fold increase in the numbers of molecules of Cur-Alb conjugate entering the cancer cells as compared to normal primary cells in culture. Therefore, the difference in the cytotoxic effect which is seen between A549 and ADMSC may be a reflection of difference in endocytosis. The lesser the numbers of molecules endocytosed, more cells retain proliferation potential (80-90 % in ADMSC); when more molecules are endocytosedmore is the cytotoxic as well. Therefore, more A549cells were nonviable as compared to ADMSC (Fig3) and non-entry of Curc-Alb to normal cells may be expected to be a controlling mechanism that may limit death of normal cells.

It may be a specific property of cancer cells membrane vs normal cells which influence the differential internalization process to cause more toxic effect when exposed to the conjugate.

There is need evaluating if it true for normal fibroblasts also to add value to the pharmacologic use of Curc-Alb for anticancer therapy.

3.4.Effect of Curcumin- albumin conjugate on Apoptosis.

3.4.1 Detection of Apoptosis using AnnexinV binding

It may be interesting to study if apoptosis is also regulated and correlated to the differential endocytosis process in A549 and ADMSC. The representative dot plot shows (Fig 10 & 11) that cells responding to Curc-Alb conjugate is present in all 3 quadrants; dead cells (Q1), apoptotic cells (Q4) and both dead and apoptotic cells (Q2).

The total A549 cells which responded to Curc-Alb is compiled in fig:11, and it is clear that in 24 h >45% cells responded (apoptosis + necrosis) to 50 µM Curc-Alb and >90% cells responded (apoptosis +necrosis) to 100 µM Curc-Alb. On the contrary, ~35% ADMSCs responded (apoptosis+necrosis) to 50 µM Curc-Alb, whereas only < 50% cells responded (apoptosis+necrosis) to 100 µM Curc-Alb. This observation may have a positive impact when the conjugate is used for treatment of cancer. It may be expected that the drug will show selectivity for cancer cells and may exclude normal cells from causing death.

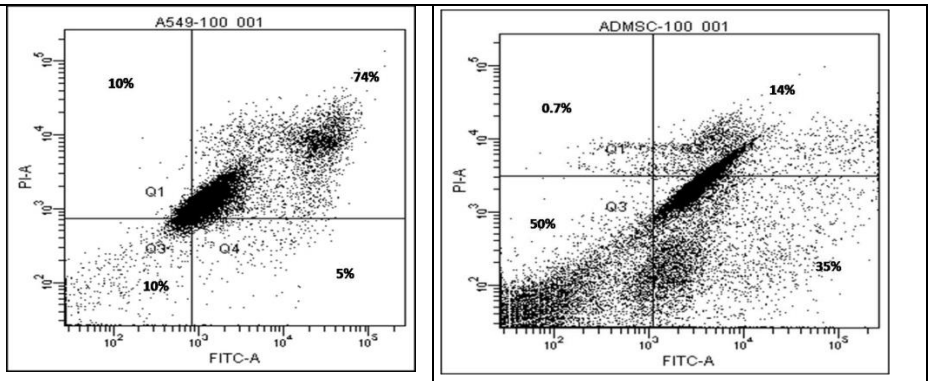


Fig: 10.Representative dot plot of Apoptosis assay.The data shown is of A549 and ADMSC cells and the concentration of drug added is shown in the graph.The assay was done after 24 h drug with culture. FITC conjugated Annexin V and PI staining was done as per the manufacturers' instruction.

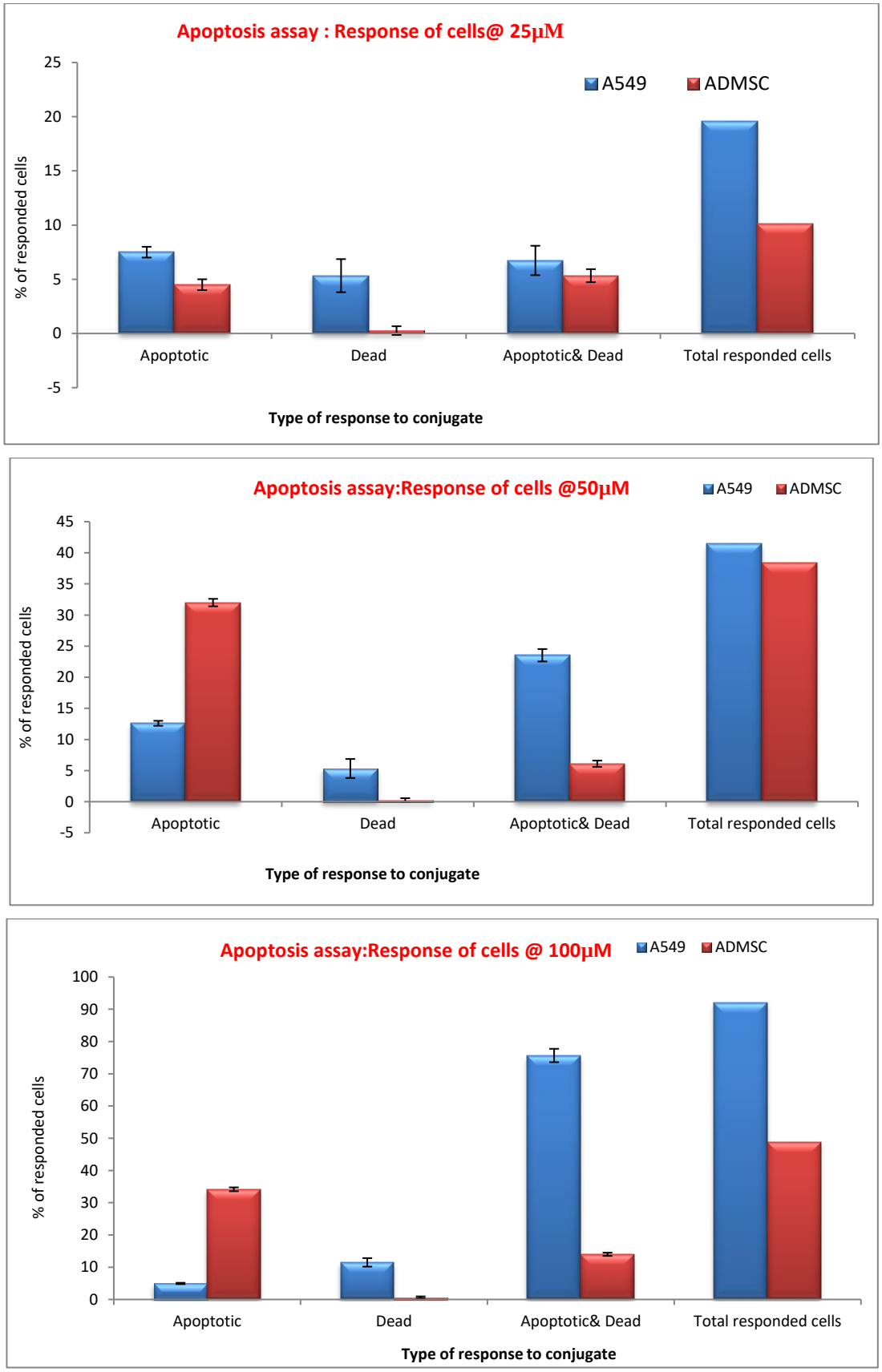


Fig:11. Graphical representation of percentage of Apoptotic and dead cells of A549 &ADMSC treated with different concentrations of Curc-Alb conjugate for 24 h.

Apoptosis, or programmed cell death, is a normal physiologic process for removal of unwanted cells. One of the earlier events of apoptosis includes translocation of membrane phosphatidylserine (PS) from the inner side of the plasma membrane to the surface. Annexin V, a Ca^{2+}-dependent phospholipid-binding protein, has high affinity for PS, and fluorochrome-labeled Annexin V can be used for the detection of exposed PS using flow cytometry.Curcumin albumin conjugate has been shown to induce apoptosis and cell death in A549 cell lines ,was used to demonstrate the Annexin V FITC assay in BD FACS Diva software.When compared with effect of 25μM, 50μM, 100μM conjugate on A549 cells, ADMSC retainedmore than 50% live cells and only 14% cells are dead and apoptotic compared to A549 which shows 74%.

Annexin V binding is a late process in cell death. Therefore, to pick up early response to Curc-Alb conjugate which may activate signalling pathways in apoptosis, caspase activation may be a better indicator of response of cells to the conjugate

3.4.2.Monitoring Caspase Activation using live cell imaging.

Apoptosis is characterized by a distinct series of morphological and biochemical changes that eventually result in the programmed death of cells. It plays an essential role in morphogenesis, host defence and homeostasis of all tissues, including the immune system. The degree of apoptosis induced plays a prognostic factor for therapy of a number of malignancies. Among the key biochemical mediators of apoptosis are a group of cysteine proteases, designated as the caspases. The caspases can be classified into three groups according to their substrate specificities.Group I (caspase-1, 4, and 5) prefers the tetrapeptide sequence WEHD, whereas Group II (caspase-2, 3, and 7), the primary apoptotic effector caspases, prefers the peptide recognition motif DEXD, while the caspases in Group III (6, 8, and 9) prefer the sequence (L/V)EXD. A genetic approach for directly monitoring caspase activity within living cells may provide a more rapid and accurate method and also afford the possibility of separating cells based on their caspase activity. With the advent of the GFP variants, CFP and YFP, the use of a CFP->YFP fluorescence resonance energy transfer (FRET) assay has become feasible to monitor many distance-sensitive cellular activities, such as protein-protein interaction, enzymatic activity, protein conformational change, kinase activity, etc. One application of this technology has been the development of assays to measure caspase activity in living cells. These methods make use of constructs of CFP and YFP with a linker between them. The linker harbors at least one caspase target sequence (LEVD, DEVD, or other caspase-sensitive cleavage sequence. If the length of the linker is

sufficiently short, FRET will occur between CFP and YFP. However, if the cell has been stimulated to express caspase activity, the linker will be cleaved resulting in two physically separated CFP and YFP molecules and a marked decrease in FRET activity. The measurement of FRET signals has largely been achieved using fluorescence microscopy (Hellwig et al.,2016).

Representative images of donor and acceptor fluorescence of control cells and cells treated with free Curcumin and Curc-Alb is shown in figures 14-16. As shown in the figures, cells with activated caspases are easily distinguished with an increase in donor fluorescence and decrease in acceptor florescence (measured with a change in CFP/YFP ratio).

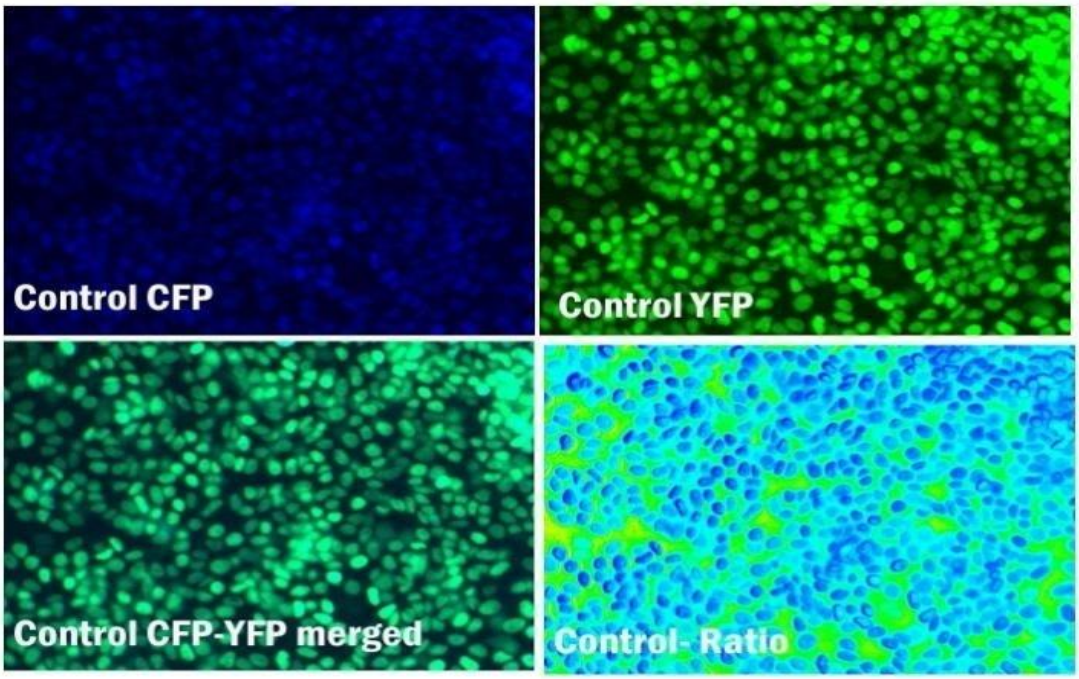


Fig:12. FRET ratio of control cancer cells showing presence of many live cells without caspase activation.

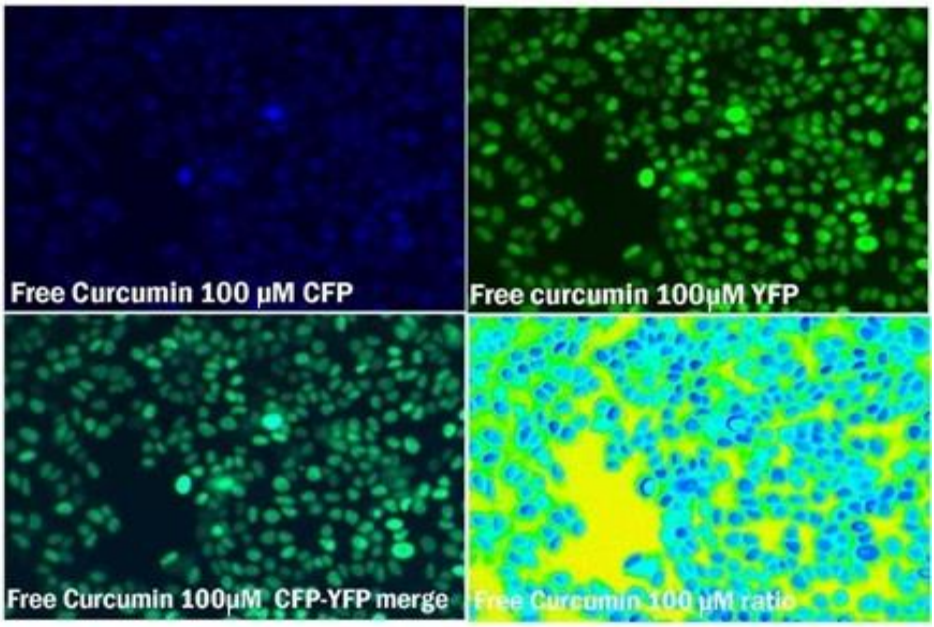


Fig:13. FRET activity of cancer cells treated with 100µM free curcumin shows moderate cell death with caspase activation. High molarity curcumin dissolved in DMSO was added into the culture in micro quantities to avoid DMSO-induced cell deathget but to get final concentration of 100µM in culture medium.

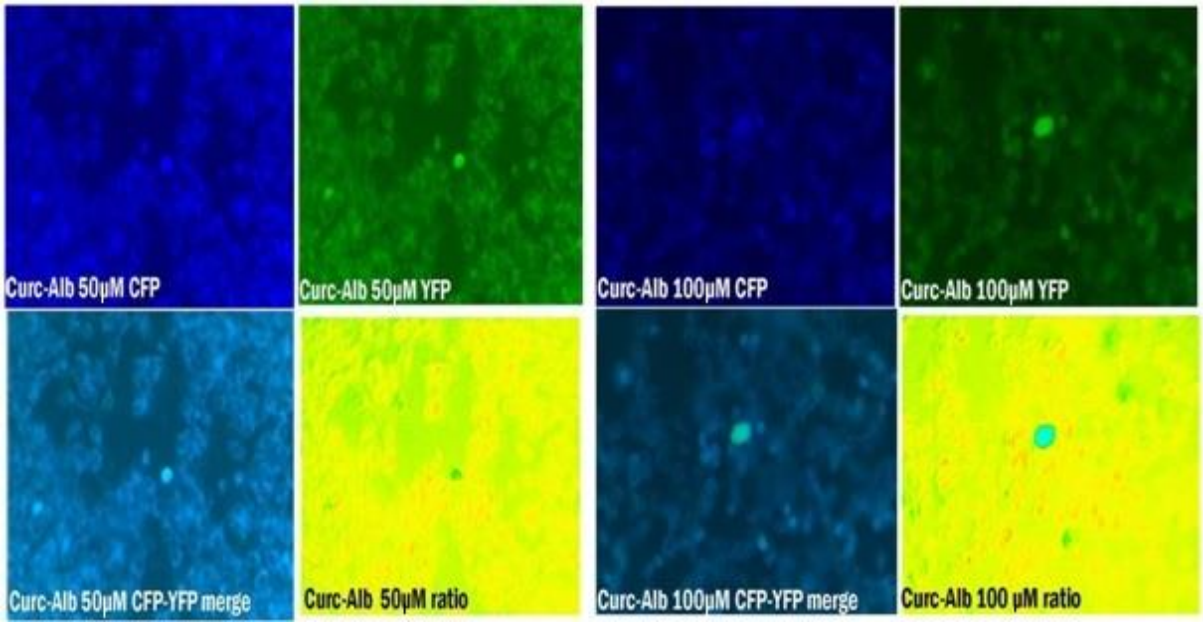


Fig:14. FRET ratio of cancer cells treated with 50µM and 100 µM Curc-Alb conjugate shows that in majority of cells undergo caspase activation.

Many of the anticancer drugs being developed are based on the anti-proliferative and cytotoxic effect. Not much effort is made to understand the molecular target and effect on apoptosis. Programmed cell death is likely to cause fewer side effects to the surrounding normal tissue. Therefore, one need to consider the link between cancer and apoptosis. Assays

should focus on understanding the role of the drug in inducing apoptosis. Accordingly, the present study adopted a FRET based assay using a very sensitive probe. The major highlight of this study is that the effect of same concentration Curc-Alb conjugate and free curcumin used seemed to show a significant difference in inducing apoptosis. The reason could be better internalization of the conjugate to induce caspase activation as compared to free curcmin due to albumin present on the conjugate. The internalization of free curcumin was not detected in this study due to lack of a fluorescent probe for tracking free curcumin.

Healthy mitochondrial membranes maintain a difference in electrical potential between the interior and exterior of the organelle, referred to as a membrane potential. Tetramethylrhodamine, methyl ester (TMRM) is a cell-permeating dye that accumulates in active mitochondria with intact membrane potentials. If the cells are healthy and have functioning mitochondria, the signal is bright. Upon loss of the mitochondrial membrane potential, TMRM accumulation ceases and the signal dims or disappears.

Representative image of TMRM stained fibroblasts treated with free Curcumin and Curc-Alb is shown in fig: 15. As shown in the figure, cells with activated caspases are easily distinguished with decreased fluorescence.

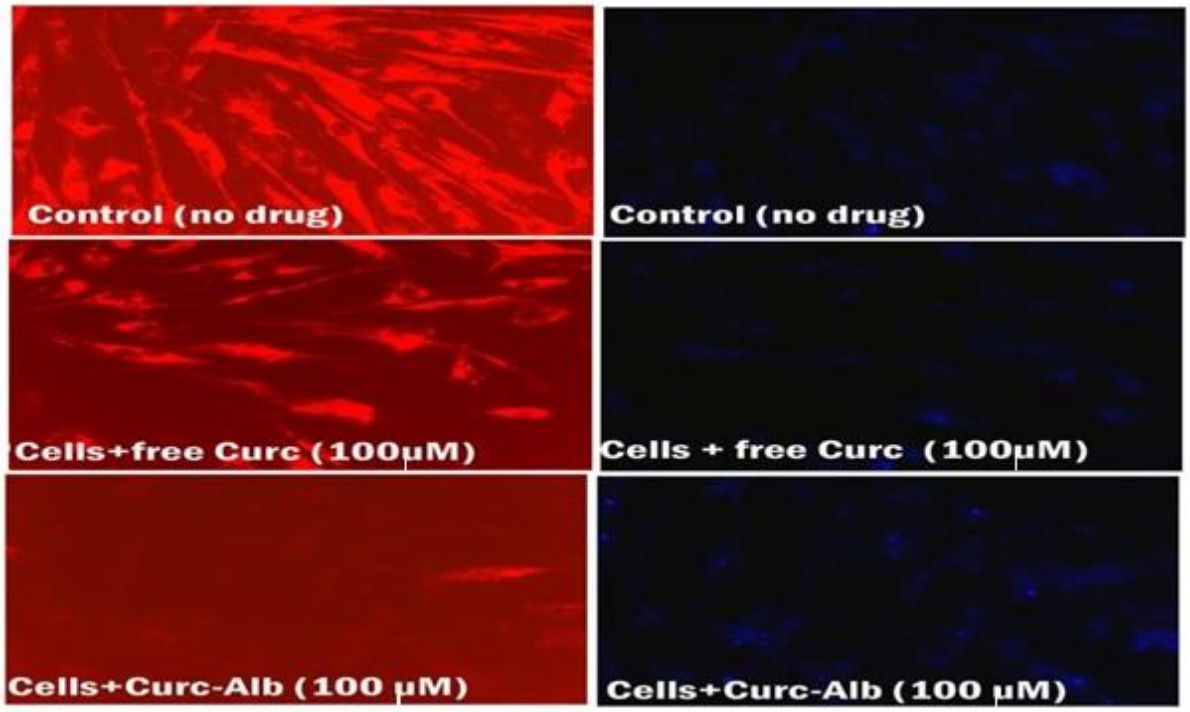


Fig :15. Representative images of TMRM stained, differently treated lung fibroblast cultures.

Absence of red fluorescence in cells when treated with Curc-Alb is more obvious as compared to cells treated with free curcumin. More experiments are being done to systematically analyse the dose effect of Curc-Alb. When same concentration of Curcumin is added either in the free form or in the conjugated form, the loss of red fluorescence was more obvious in the case of addition of Curc-Alb as compared to free curcumin addition. Therefore, conjugation has obviously improved bioavailability which could be the reason for more activity.

FRET ratio analysis in cancer cells showed that even when 50µM Curc-Alb conjugate is added complete cancer cell death occurs (Fig:14). Therefore, a similar study of cancer cells and fibroblasts with similar but lower concentration of Curc-Alb conjugate may be required to understand if there is any specificity for cancer cells without affecting fibroblasts. Thus the next objective is to determine the minimum concentration of Curc-Alb required to induce caspase activation and to what extent the same concentration of conjugate affects fibroblast.

Chapter IV

Summary and conclusion

4.1 Summary

Clinical application of curcumin has been limited due to poor aqueous solubility and low bioavailability.Albumin proves to be a multifaceted,highly soluble and versatile molecule capable of significantly improving curcumin delivery. The most attractive observation is that Curc was bound to Alb in simple reaction mixture and there was no requirement of any chemical modification.Conjugation efficiency was estimated by determining the protein content, curcumin content in the lyophilized conjugate and the lyophilized powder is stable and dissolved very quickly for further studies. The conjugate showed anti-proliferative and cytotoxic effect in dose- and time- dependent manner on the lung cancer cells A549. Since all anticancer drug has a tendency to be toxic to normal cells also, adipose derived mesenchymal stem cells (ADMSC) was also included in the cytotoxicity evaluation. Cytotoxic concentrations of conjugate on the A549 cells and ADMSC cells were estimated using standard MTT assay.The proliferative ability was better retained in ADMSC cells. Also the cytoxic effect significantly less in ADMSC, as compared to A549 when similar dose was used. This is a promising finding which can be a beneficial property of Curc-alb conjugate for use in patients. It is likely that patients undergoing treatment with the conjugate may escape severe side effects.

Further this study was focused on delineating the mechanism of action of Curc-Alb conjugate through caspase activation and cell death. To begin with fluorescent Curc-Alb conjugate was prepared and demonstrated that the conjugate enters the cytoplasm in a dose and time-dependent manner. The FITC concentration and albumin concentration also increased proportionately when curcumin content was increased. From internalization study it was confirmed that selectivity for the conjugate is dependent on the cell's ability to internalize the conjugate.The fluorescence signal guarantees that only cells that have taken up conjugate are considered and also provides a measure of uptake.It is seen that increase in dose is not proportional to additive response in the case of ADMSC. This observation explains why the conjugate was found less toxic to ADMSC. It may be expected that the drug will show selectivity for cancer cells and may exclude normal cells from inducing death.

The dose response for inducing apoptosis is also supporting the data on cytotoxicity assay and endocytosis assay. Clearly, the dose at which maximum Annexin binding is obtained by flow cytometric measurement after 24h treatment with A549 cells is less

responsive to ADMSC. Therefore, extent of cell death is also proportional to the internalized conjugate, proving that for the biochemical pathway of apoptosis to take place enough conjugate should enter the cytoplasm. So it may be concluded that the entry of drug into the cell is the limiting factor for induction of apoptosis.

Interestingly, the live cell imaging using FRET-based probe also demonstrated marked caspase activation even at 50μM concentration of Curc in the conjugate.When compared to 100 μM free curcumin added to the cell culture, the effect of 50 μMCurc-Alb appears to be much higher. Therefore, again it proves that the conjugate is more prone to enter into cytoplasm and a very low concentration only is necessary for inducing apoptosis.

4.2 Conclusion

- The study concludes that Curc-Alb conjugate exhibits potential anti cancerous activity.
- The preparation of soluble Curc was achieved by conjugating the drug with albumin. Solubility was extensively enhanced.
- Using FITC tagged Curc-Alb conjugate, endocytosis of the drug into cell in a dose-dependent and time-dependent manner was established.
- Conjugated curcumin affected cell proliferation and cytotoxic concentration for A549 is less toxic to ADMSC.
- Internalization of Curc-Alb into ADMSC was also less as compared to A549 and the reduced entry reflected on lower cells death as estimated by Annexin binding.
- Caspase activation by Curc-Alb is evident from FRET assay and the dose required is much less as compared to the dose of free curcumin required for activation.

4.3 Future prospects

- To study kinetics of drug action *in vitro* in other normal cells to establish the safety of the conjugate as compared to other clinically use drugs.
- To determine the minimum dose of conjugate required for caspase activation in cancer cells and compare the activation in normal cells at the detected active dose.
- To check action of the conjugate in lung cancer model to establish cancer reduction.

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Appendix

PBS(1000mL) pH 7.4

NaCl - 8g

KCl - 0.2g

HPO_4 - 1.44g

KH_2PO_4 -0.24g

Solution is filtered and stored at room temperature.

Reagents for Lowry's protein estimation

Reagent A : 2% Na_2Co_3 in 0.1N NaOH

Reagent B : 0.5% $CuSO_4.5H_2O$ in 1% sodium potassium tartarate

Reagent C : 50mL reagent A:1mL reagent B

Reagent D : 1mL Folin's reagent : 1m L deionised water.

10% DMEM F12(50 mL)

DMEMF12 : 45 mL

FBS : 5 mL

Antibiotics : 500µL

HBSS(1000 m L) pH – 7.4

KCL : 0.4g

KH_2PO_4 : 0.06g

NaCl : 8g

Na_2PO_4 : 0.0482g

The solution was filtered autoclaved and stored at $4^{\circ}C$.