Chapter – 9

Cell Line studies

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INTRODUCTION

Colloidal, vesicle based nanometric novel drug delivery systems such as nanoparticles; liposomes etc. can be fabricated to target the cytotoxic drug to the exact cancerous site. (Jain et al, 2008, Gupta et al, 2007,). Strategies have been designed to take the advantage of the receptors and moieties that are largely over expressed in cancer cells and under expressed or nearly absent in normal, healthy tissues. Designing the colloidal vesicular drug delivery systems such as liposomes with sizes lesser than tumour size can be easily taken up and retained by tumours by virtue of Enhanced permeability and Retention (EPR) phenomenon and enhanced cellular uptake of cytotoxic drug via receptor mediated mechanisms. (Akima, 1996). Efforts are being done to investigate and develop the ligands that can be directed towards such over expressed receptors and thereby achieve a highly target specific drug delivery. Attachment of ligand on liposomal surface will coax the liposomal delivery system only to tumours and not the adjacent healthy, non cancerous tissues.

CD44 is generally found in low levels in epithelial, haemopoeitic and neuronal cells, however, their levels are very high in breast (Bourguingon, 2000) colorectal (Bel, 2004) lung cancers (Zoller, 1995, Smadja-Joffe,1996, Rudzki,1997, Sneath, 1998,Lesley, 1998, Borland, 1998, Herrera-Gayol,1999, Tran, 1997,Fasano, 1997) These cell surface receptors exhibit inherent high affinity towards hyaluronic acid. Hyaluronic acid is a linear polysaccharide comprising of alternatively arranged D-glucoronic acid and N-acetyl glucosamine. Hyaluronic acid has got diverse physiological functions in body ranging from their effects on cell proliferation to triggering of inflammatory response. Hyaluronic acid (HA) is a major component of extracellular fluid and CD44 has an important role in metabolism of dissolved HA. CD44 regulates lymphocyte adhesion to cells of high endothelial venules during lymphocytic migration (Gallatin, 1986, Jalkanen, 1986) as well as metastatic dissemination of solid tumours. (Nemec, 1987, Sher et al, 1988, Toole, 1979). CD44 role has been elucidated in proliferation of various carcinogenic and metastatic growths. (Bartolazzi, 1994, Birch et al, 1991) have also delineated vital role of CD44 in determining the profile of disseminated melanoma cells.

Most malignant tumours exhibit high levels of HA (Lokeshwar, 2000) including lung carcinomas. (Penno, 1994). Though CD44 is expressed on various normal host cells, it has been found that these cell types are either not in contact with main bloodstream or require activation before binding to HA. Approaches to manipulate this CD44-HA interaction usually include of delivery of high molecular weight HA (Zeng, 1998), anti CD44 antibody (Zawadzki, 1998), CD44 receptor globulin (Zawadzki, 1998, Sy et al., 1992) etc. Due to EPR phenomenon and highly leaky vasculature of tumours, ligand grafted liposomes can easily extravasate and can gain access to the innermost layers of tumour. (Netti et al, 1999) CD44 may be a potential targeting moiety for treatment of cancers that over express these receptors.

CD44 is a cell surface receptor for hyaluronate, a major glycosaminoglycan component of extracellular matrix. Adhesion to extracellular matrix, a critical initial step in the metastatic process, has been found to be CD44-dependent in several malignancies. In concert with CD44, matrix metalloproteinase (MMP) expression is important for tumor invasion. Cell surface CD44 induces co-clustering of MMPs, thereby promoting MMP activity, tumor invasion, and angiogenesis. High molecular weight HA-drug conjugates have been studied to achieve the same. (Luo, 1999). HA grafted liposomes/lipoplexes can be targeted to cancer cell lines (such as A549) over expressing CD44. A549 cell lines derived from carcinoma cells of Type II pneumocytes behave like lung epithelial carcinoma and this cell line is widely used as an alternative model for studying human bronchial as well as lung cancer. (Taetz, 2009, Dohadwala, 2002).

5-dimethylthiazol-2yl]-2. 5-diphenyl tetrazolium bromide (MTT) belongs to the group of fluorochromes which fluoresce as a result of cell metabolism. MTT test is based of measurement of metabolic activity of viable cells. The assay is nonradioactive and can be performed entirely in a microplate using an ELISA plate reader. It is suitable for measuring cell proliferation, cell viability or cytotoxicity. The assay is based on the reduction of the tetrazolium salt MTT by viable cells. The reaction produces a water-insoluble formazan salt which can be solubilized using Dimethyl Sulphoxide (DMSO) or similar organic solvent.

Cell Cycle

In order to facilitate survival and sustenance of a living organism, individual cells need to grow in size, replicate their stored genetic material and undergo a process of cell division and cell proliferation. Eukaryotic cells of higher organisms possess DNA in the form of genetic material, stored in chromatin network of nucleus in central zone of cell. The entire process of cell growth and proliferation in eukaryotic organisms can be characterized by four distinct phases.

Quiescence: Cells that are not proliferating or in active stage of multiplication are said to be quiscient or in "G0" phase.

G1 Phase: During the first phase (G1) cells grow in size in-response to mitogenic signals such as soluble extracellular growth factors and intracellular contact, triggering a commitment to enter the next phase of the cell cycle.

The Restriction point: Late in G1, cell types enter the next phase of the cell cycle termed as restriction point. Existence of a restriction point can be demonstrated by depriving cells from growth factors in their medium. Many types of cells will continue to complete a single cell cycle, if they are in S-phase, G2 or mitosis, but will be then arrested in G1 phase.

S-Phase: Soon after the restriction point a cell begins to replicate its genetic material through the activity of DNA polymerase enzymes which are capable of synthesizing an exact replica of the entire DNA genome. This phase of the cell cycle is biochemically distinct from the other phases of cell cycle and can easily be differentiated from other cells by their ability to stably incorporate fluorescent or radio labeled tagged nucleotides-building blocks of DNA into their chromosomes.

G2 phase: At the completion of S phase, DNA replication stops and cells enter the G2 phase of the cell cycle.

Mitosis: In this phase of cell cycle, cells physically divide into two separates daughter cells initially by dissolving nuclear membrane which will later reform once cell division is complete. The DNA-containing chromosomes then condense into structures that can be visualized using light microscopy. The chromosomes then segregate to two sides of the cell so that each half of the cell gets exactly one copy of each chromosome. On completion of mitosis, cells undergo cytokinesis or separation into two equal halves.

Cell cycle analysis based on measurements of DNA content generates a clear pattern of distribution: G0/G1 phase (one set of paired chromosomes per cell), S phase (DNA synthesis with variable amount of DNA) and G2/M phase (two sets of paired chromosomes per cell, prior to cell division) DNA content can be determined using fluorescent DNA selective stains that exhibit emission signals proportional to DNA mass. Flow cytometric analysis of stained cell populations is used to produce a frequency histogram that reveals the phases of the cell cycle. This analysis is typically performed on permeabilized or fixed cells using a cell impermeable nucleic acid stain.

Nuclear DNA is one of the parameters measured by flow cytometry. This measurement calculates the percentage of a cell population in each phase of the cell cycle. The percentage of cells in S-phase indicates proliferative activity of the given cell population.

Flow cytometer can indicate relative cell size and density by measuring forward and side scattered laser light respectively. In addition, flow cytometer can measure relative fluorescence from fluorescent probes which bind to cell associated molecules. These fluorescent probes are often fluorochrome labeled antibodies specific for cell surface molecules, but also may be nucleic acid probes (e.g. Propidium iodide), a cell function probe or fluorescent proteins (e.g. GFP). As the labeled cells flow past a laser beam, probes fluoresce and the emitted light is directed to detectors which translate light signals into information concerning the relative fluorescence intensity associated with cell under observation. Flow cytometry measures the percentage of cells in a population with each fluorescent probe attached. The cell sorter can sort out specific cell population from a mixture of cells based on fluorescence profile.
The most commonly used dye for DNA content/cell cycle analysis is Propidium Iodide (PI). It can stain whole cell or isolated nuclei. The PI intercalates into the major groove of double stranded DNA and produces a highly fluorescent adduct that can be excited to 488 nm with a broad emission around 600 nm. Since, PI can also bind to double stranded RNA, it is necessary to treat cells with RNase for fair DNA resolution.

Confocal Laser Scanning Microscopy (CLSM)

It is relatively a newer light microscopic and imaging technique that has now found wide array of applications in biological sciences. The primary importance of CLSM to a biologist is its ability to produce optical sections through a 3-dimensional (3-D) specimen e.g. an entire cell or a piece of tissue contains information from only one focal plane. Thus, by moving the focal plane of the instrument stepwise through the depth of the specimen, series of optical sections can be recorded. This property of CLSM is fundamental for solving problems related to 3-D biological specimens where information from regions distant from plane of focus can obscure the image. Usually, epi fluorescence or epi reflection mode is preferred with biological samples. The computer controlled CLSM produces digital images which are amenable to image analysis and processing and can be used to compute surface or volume rendered 3-D reconstructions of the specimen.

9.1 MATERIALS AND EQUIPMENTS

Material	**Source**
Alveolar Epithelial adenocarcinoma cells (A549 Cell Line)	National Centre for
Dulbecco's Modified Eagle Medium (DMEM), sodium bicarbonate, foetal bovine serum and streptomycin, penicillin solution, MTT (3-(4,5-dimethyl thiazol-2-yl)-2,5 diphenyl tetrazolium bromide), Hank's Balanced Salt solution (HBSS), Nonidet P40 (NP40)	HiMedia, Mumbai
Propidium iodide	Sigma Aldrich Corporation, Mumbai.
RNase(Ribonuclease)	Sisco Research Laboratory, Mumbai.
6-coumarin	Gift Sample from Neelikon Dyes, Mumbai.
Hydrogenated soya phosphatidyl choline (HSPC),Dipalmitoylphosphatidylethanolamine (DPPE)	Lipoid Gmbh, Germany.

Cholesterol, potassium dihydrogen phosphate, potassium chloride, potassium hydroxide, glacial acetic acid, methanol, Acetonitrile, Chloroform, ammonium hydroxide, methylene chloride	S.D.Fine Chemicals Ltd., Mumbai,India.
HPLC grade methanol, chloroform, acetonitirile	Loba Chemicals, India.
Nuclepore Polycarbonate membrane 0.2, 0.5 and 2 μm, 25 mm	Whatman, USA
Bichinconinic acid protein (BCA) Assay Kit	Genei, Bengaluroo,India.
Glassware	**Source**
Calibrated volumetric pipettes of 1, 5 and 10 ml, serological pipettes, 100 ml. volumetric flasks, 10 ml, 100 ml and 250 ml volumetric flasks, funnels (i.d. 5.0 cm) and necessary glassware	Schott and Corning(India) Ltd., Mumbai.
Equipments	**Source**
Analytical Balance	Precisa 205A SCS, Switzerland.
pH meter	Systronics, 335,India
Rotary Flash Evaporator	Superfit Equipments, Mumbai,India.
Probe Sonicator	RR120, Ralsonics,Mumbai.
Bath Sonicator	INCO, Ambala
Cooling Centrifuge	Osterode, Germany
Vacuum pump F16	Bharat Vacuum Pumps, Bengaluroo.
ELISA Reader	Synergy HT and Powerwave XS, Biotek-vermont,

	USA
Lyophilizer	Heto Dry Winner, Denmark.
Flow Cytometer (FACSVantage)	Vantage (Becton Dickinson, Braintree, USA
Confocal Microscope LSM 10	Carl Zeiss, U.K.
UV Visible Spectrophotometer (UV 1601)	Shimadzu, Japan.
HPLC system	Dionex HPLC with Chromelon 6.5 Data processing software.
Plasticware (T-75, T-150 Tissue Culture Flasks, petri dishes, disposable pipettes, centrifuge tubes, cryovials, syringes and requisite plasticware)	Tarsons Ltd., Kolkatta,India.

9.2 METHODOLOGY

Cell culture

Alveolar epithelial cells (A549) were maintained on Dulbecco's modified eagle medium, supplemented with sodium bicarbonate, fetal bovine serum and streptomycin–penicillin solution, in T-250 flasks. All the incubations were performed at 37°C and 5% CO_2.

9.2.1 *In Vitro* Cell Uptake Studies

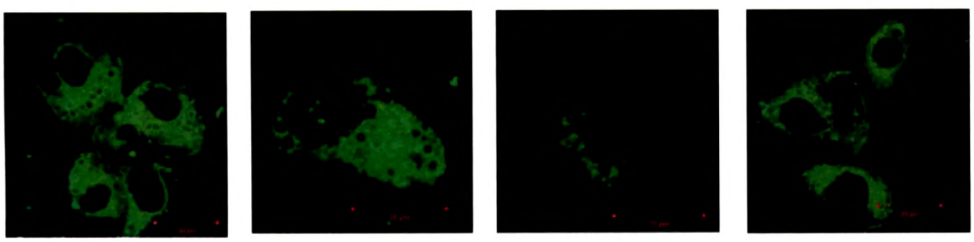
9.2.1.1 Preparation of 6-coumarin loaded liposomes

Liposomes containing 6-coumarin were prepared by Thin Film Hydration technique. 6-Coumarin was dissolved along with lipids, in 2:1 Chloroform: Methanol solvent mixture. The dry film was prepared under controlled temperature and pressure conditions followed by hydration of dried lipid film using double distilled water. The liposomes obtained were sonicated so as to obtain nanometric size range.

9.2.1.2 Cell Uptake studies

A549 cells were seeded on a cover slip placed in 35 mm petri dishes at a cell density of 10^5 cells per petri dish and allowed to attach overnight. The cells were treated with HA grafted and non grafted Etoposide/Docetaxel liposomes loaded with 6-coumarin. At different time intervals, media was aspirated, the monolayers were washed twice with PBS to remove uninternalized liposomes followed by observing the cells under laser scanning confocal microscope Laser Scanning Microscope-LSM 10 (Carl Zeiss, UK) and fluorescent microscope and results for non grafted and HA grafted ETP and DOC liposomes for different time intervals ranging from 15-45 min. are shown in Fig. 9.1(a), (b), (c). The control experiment was performed by incubating cells with 6-coumarin solution released from liposomes in vitro in 2 hours.[Fig. 9.1(d)]. Fig.9.2(a), (b),(c) and (d) show jet stacked images at different cellular depths.

Fig 9.1 (a) Cell Uptake of non grafted and HA grafted ETP/DOC liposomes in A549 cell lines by Confocal Microscopy in 15 minutes



ETPLIP HAETPLIP DOCLIP HADOCLIP

Fig 9.1 (b)-Cell Uptake of non grafted and HA grafted ETP/DOC liposomes in A549 cell lines by Confocal Microscopy in 30 minutes

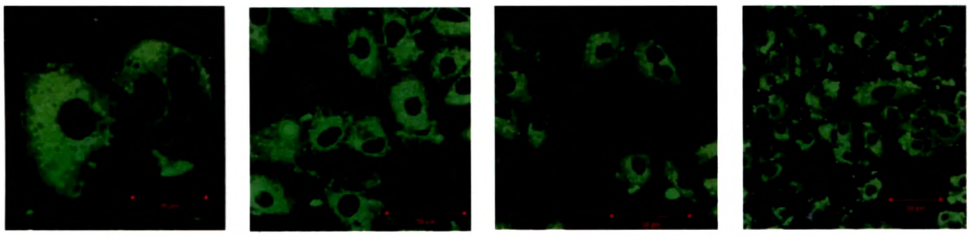


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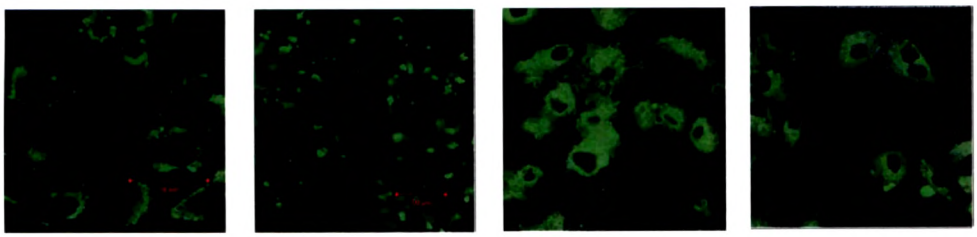
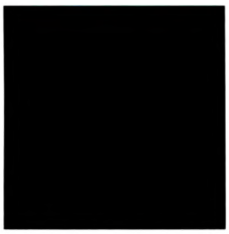
Fig.9.1 (c)-Cell Uptake of non grafted and HA grafted ETP/DOC liposomes in A549 cell lines by Confocal Microscopy in 45 minutes



ETPLIP HAETPLIP DOCLIP HADOCLIP

Fig. 9.1 (d)-Confocal Microscopy of A549 cells incubated with 6-coumarin solution



Insignificant Fluorescence was observed after 2 h.

Fig 9.2 (a) - Jet Stacked Images of ETPLIP

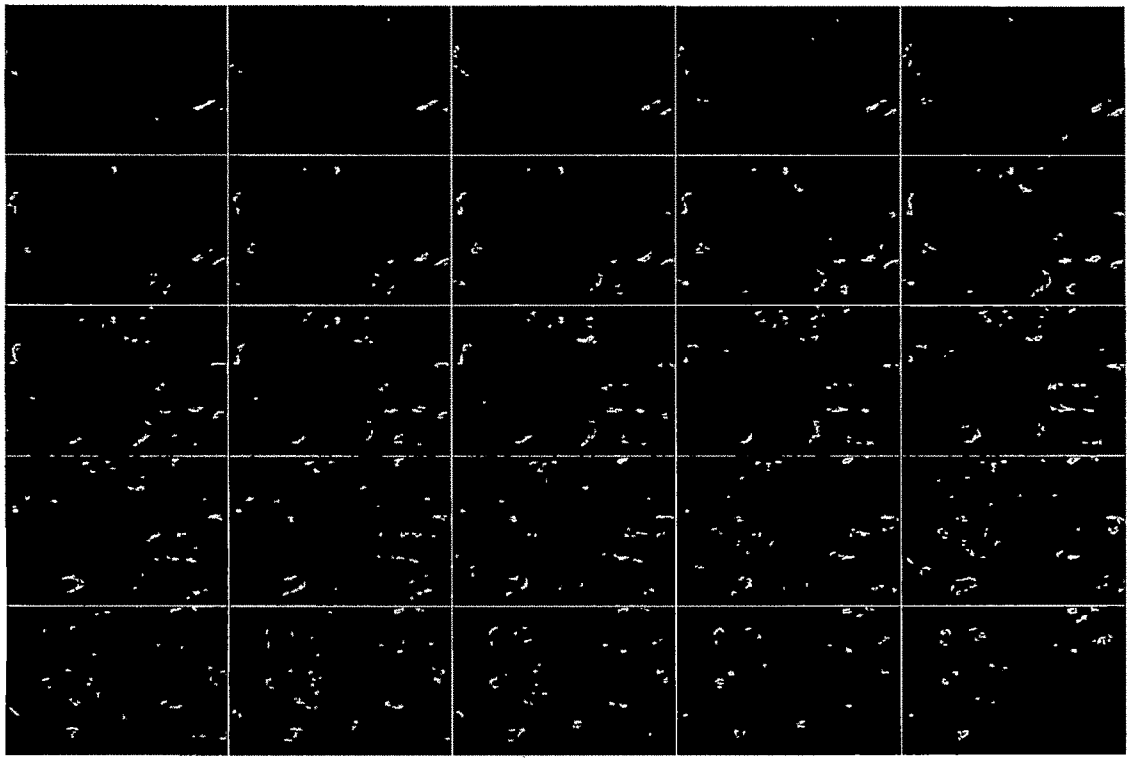


Fig. 9.2 (b) - Jet Stacked Images of HAETPLIP

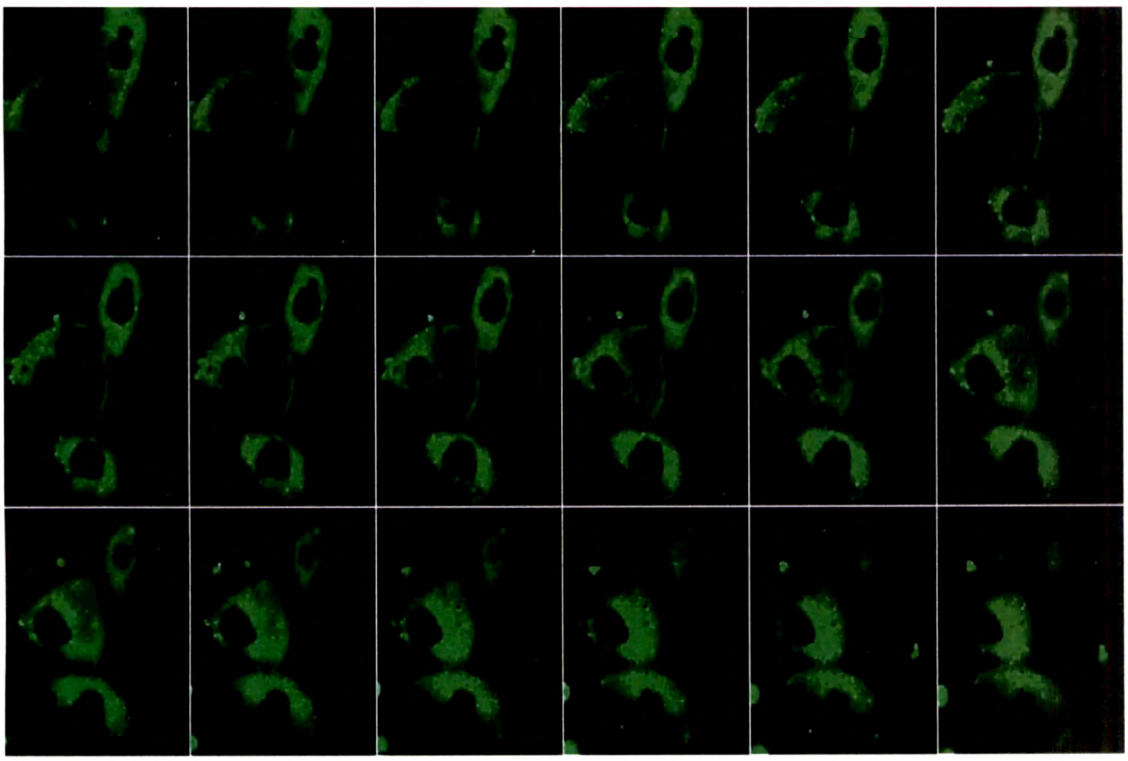


Fig. 9.2 (c) - Jet Stacked Images of DOCLIP

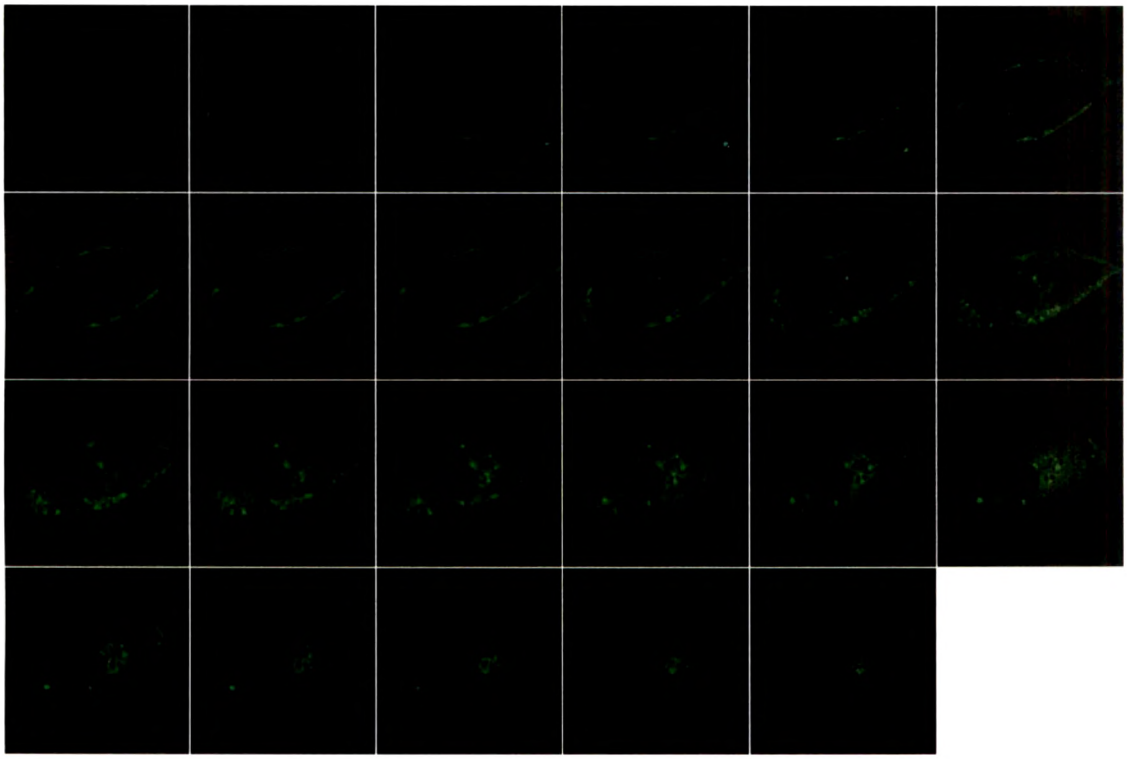
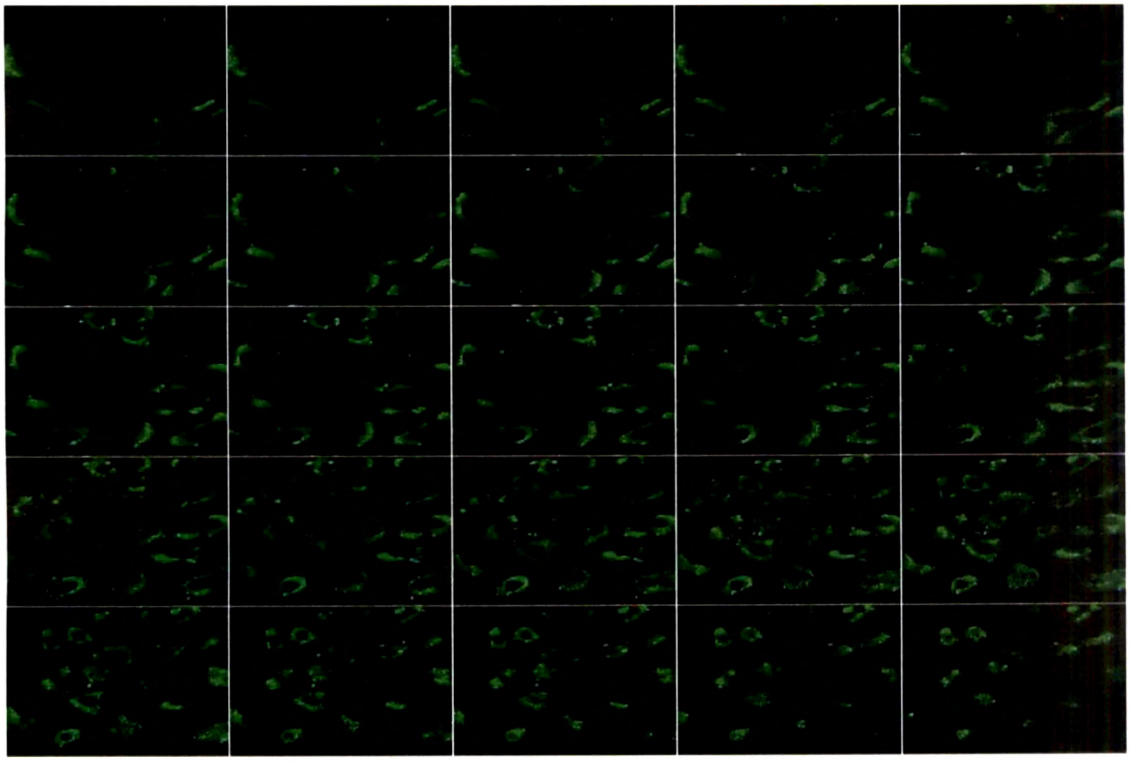


Fig. 9.2 (d) - Jet Stacked Images of HADOCLIP



To study the quantitative cellular uptake, HA grafted and non grafted Etoposide/Docetaxel liposomes loaded with coumarin (2 μM) were added to monolayers of A549 cells (10^5 cells) grown in 45 mm petri dishes. The cells were incubated for 30, 60, 90 and 120 minutes at 37 C. The cell monolayers were washed thrice with PBS, scrapped off from petri dish and lysed in 1 ml of PBS containing 0.1% Nonidet P40 (NP 40). Total number of cells was estimated by estimating total cellular protein using BCA protein assay method.

9.2.2 *In Vitro* Cytotoxicity Studies by MTT Assay

A549 cell lines (Passage 90) in exponential phase of their growth were used for this purpose. A549 cells were seeded in 96 well plates in Dulbecco's Modified Eagle medium, supplemented with sodium bi-carbonate and fetal bovine serum, at a density of 1.0×10^5 cells/well. The cells were allowed to attach overnight.

The cells were then treated with different formulations of Etoposide and Docetaxel (non grafted and HA grafted liposomal formulations of both the drugs) in various concentrations and with free drug solution in triplicate. Untreated cells and blank liposomes treated cells were used as controls. The plates were incubated at 37^0C and 5% CO_2. After 24 hours of drug addition, the media was removed and the cells washed twice with PBS. The cells were then treated with 20 μl of MTT. The plates were incubated for 4 hours to facilitate metabolism of MTT. The media was then discarded and the concentration of formazan (reduced metabolite of MTT) was determined using an Elisa plate reader at the absorbance of 560 nm and 670 nm. (Synergy HT and Powerwave XS, Biotek-vermont, USA) All the determinations were performed in triplicates.

Cytotoxic concentrations (IC_{50}) for developed formulations were determined using the following formula (Equation No. 2) and recorded in Fig. 9.3 and Fig. 9.4.

$$\% \text{ Cell Viability} = \frac{Abs\ (Test\ Sample)}{Abs\ (Control)} \times 100 \qquad \text{---------(1)}$$

Abs test sample- absorbance of cells subjected to treatment with various concentrations of drug formulations.

Abs control- is the absorbance of control cells (Cells incubated with culture medium only).

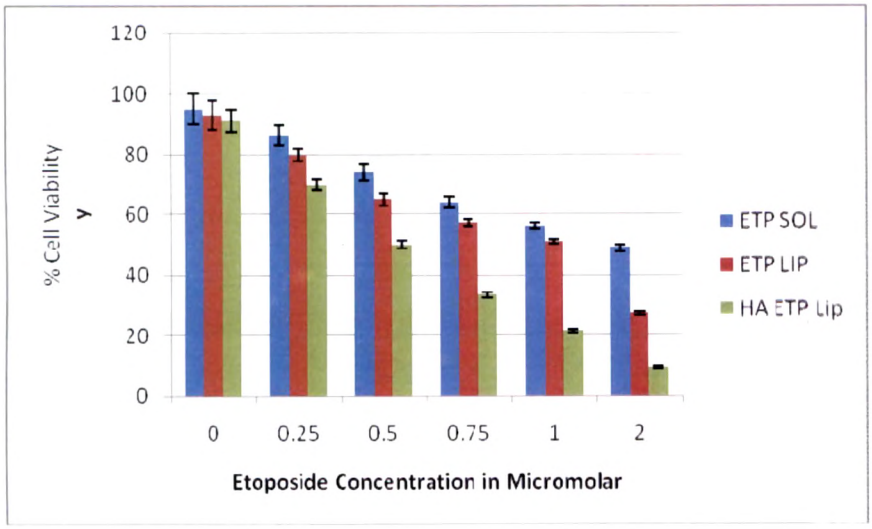


Fig.9.3-Cytotoxicity of Etoposide Liposomal formulations and free Etoposide Drug solution after 48 hour incubation time in A549 alveolar epithelial lung cancer cell line, (Results are expressed as Mean ± S.D., n=3).

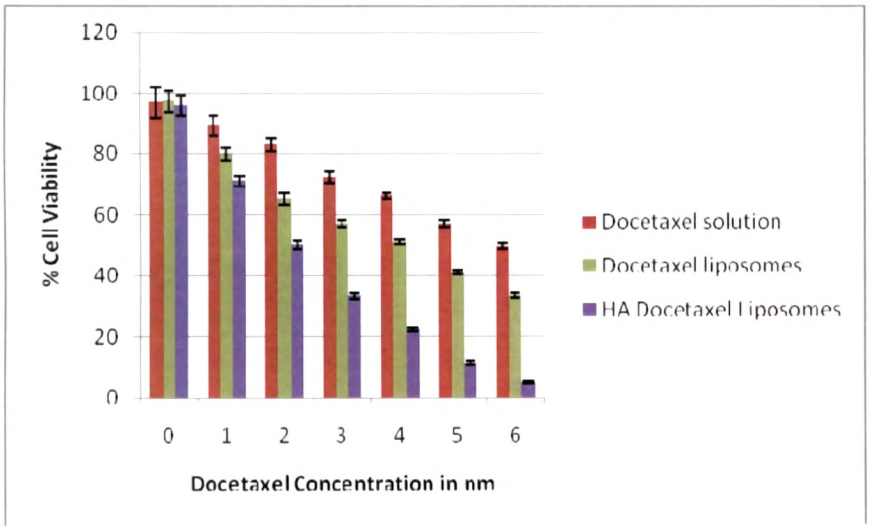


Fig .9.4- Cytotoxicity of Docetaxel Liposomal formulations and free Docetaxel Drug solution after 48 h incubation time in A549 alveolar epithelial lung cancer cell line, (Results are expressed as Mean ± S.D., n=3).

9.2.3 Pharmacokinetic (Intracellular Drug Determination) Assessment

A549 cells (Passage 90) were seeded in T75 flasks at density of 10^5 cells in DMEM supplemented with sodium bicarbonate and 10 % foetal bovine serum and allowed to attach overnight. ETP, DOC and non grafted and HA grafted liposomal formulations equivalent to their IC_{50} concentrations (determined previously by MTT assay) were added to the labeled wells. Untreated cells and blank liposomes treated cells were used as controls. The medium was changed on subsequent day and no further dose of the drug was added. The media was aspirated at different time intervals and the cells washed twice with PBS to remove uninternalized liposomes and free drug. The cells were then were lysed by incubating them with distilled water for 30 minutes. A part of lysed cells was used for estimation of total amount of cell protein using BCA (Bichinconinic acid) protein assay method. The total amount of drug (free and entrapped in liposomes) was extracted from cell lysates with methanolic PBS, centrifuged and injected into HPLC column for drug estimation. Pharmacokinetic assessment results and intracellular

concentrations achieved with different formulations are shown in Fig.9.5 and Fig. 9.6 and various pharmacokinetic parameters calculated are depicted in Table 9.1 and Table 9.2.

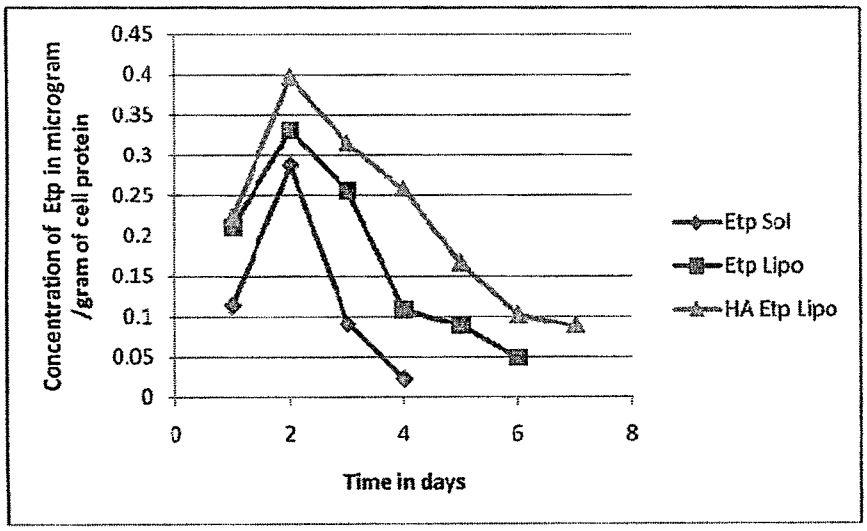


Fig. 9.5-Cellular Etoposide levels following treatment with Etoposide in solution and in liposomal formulation obtained by intracellular pharmacokinetic assessment by BCA protein assay method.

Table 9.1: Cellular Pharmacokinetic parameters following administration of Etoposide Drug solution (ETPSOL), Etoposide Liposomes (ETPLIP) and HA grafted Etoposide liposomes (HAETPLIP)

Sr. No.	Parameter Assessed	ETPSOL	ETPLIP	HAETPLIP
1	Elimination Constant (Kel)	0.608	0.343	0.243
2	Half Life ($t_{1/2}$) in days	1.13	2.01	2.84
3	Area Under the Curve (AUC for 0-8 days)	0.447	0.893	1.46
4	AUC for Infinite Time Interval (0-∞)	0.486	1.033	1.71

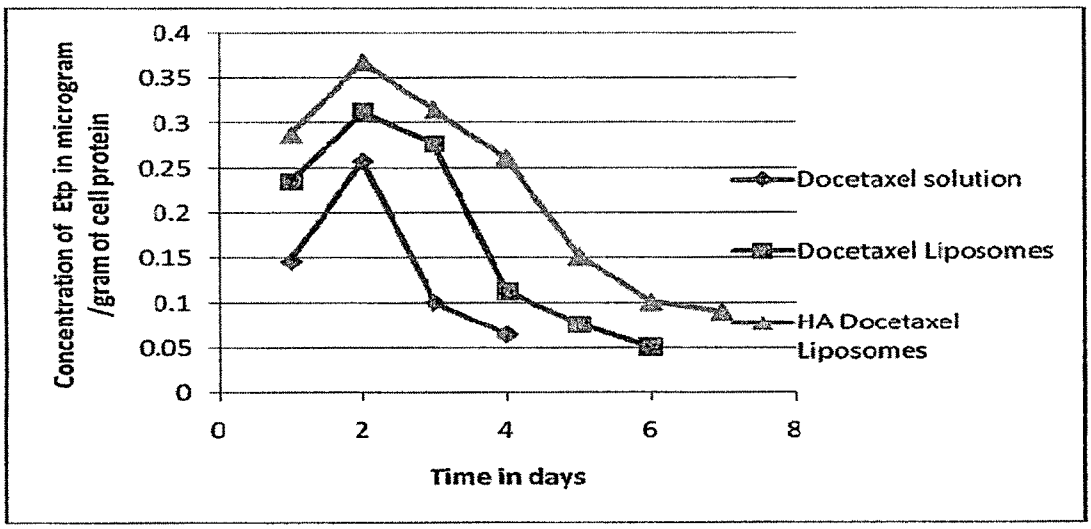


Fig 9.6-Cellular Docetaxel levels following treatment with Docetaxel in solution and in liposomal formulation obtained by intracellular pharmacokinetic assessment by BCA protein assay method.

Table 9.2: Cellular Pharmacokinetic parameters following administration of Docetaxel Drug solution (DOCSOL), Docetaxel Liposomes (DOCLIP) and HA grafted Docetaxel liposomes (HADOCLIP)

Sr. No.	Parameter Assessed	DOCSOL	DOCLIP	HADOCLIP
1	Kel	0.338	0.315	0.239
2	$t_{1/2}$	2.04 days	2.19 days	2.89 days
3	AUC (0-8 days)	0.465	0.905	1.58
4	AUC (0-∞)	0.657	1.09	1.91

9.2.3.1 Effect of temperature and time of exposure on in vitro cell uptake

To study the effect of temperature and time of exposure on cellular uptake of non grafted and HA grafted ETP and DOC liposomes in A549 cell lines, cell uptake experiments in were conducted at different temperatures for different time periods. The results recorded in Fig. 9.7 and Fig. 9.8.

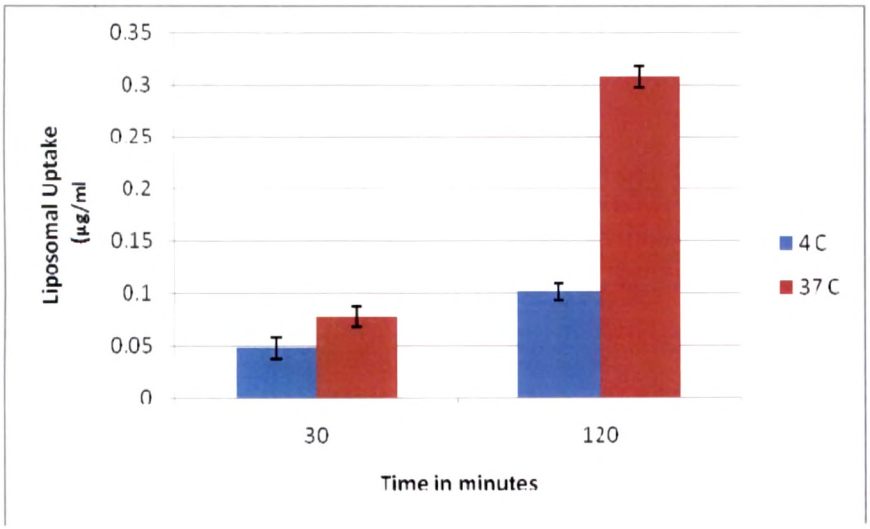


Fig. 9.7- Liposomal uptake of ETPLIP by A549 cell line following incubation of cells at 4^0C and 37^0C for 30 and 120 minutes.(Results expressed as mean ± S.D., n=3)

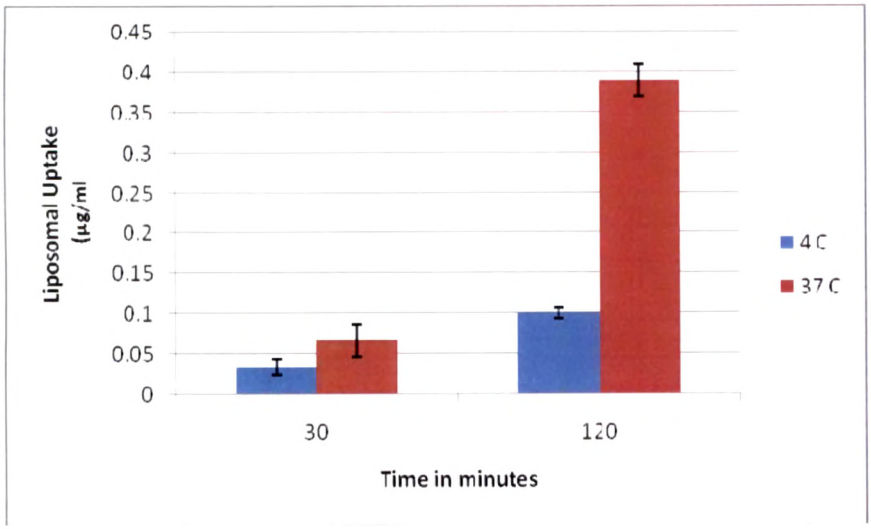


Fig 9.8- Liposomal uptake of Docetaxel liposomal formulations by A549 cell line following incubation of cells 4^0C and 37^0C for 30 and 120 minutes.(Results expressed as mean ± S.D., n=3)

9.2.4 Cell Cycle Analysis by Flow Cytometry

Flow cytometry using Propidium Iodide staining was undertaken to determine the phase of cell cycle in which the cell division was arrested by drug solution and developed drug formulations under question. Briefly, A549 (Passage 92) cells were grown in DMEM medium supplemented with 10% foetal bovine serum. The cells were seeded at density of $1x10^6$ in 35 mm petri dishes containing 2 ml of DMEM media in each one and allowed to attach overnight. ETP, DOC and HA grafted and non grafted liposomal dispersions were added in concentrations equivalent to their IC_{50}. Blank liposomes treated cells; untreated cells and methanol treated cells were taken as controls. The medium was changed after 24 hours and no further dose of the drug was added. After 24 hours, the cell monolayer was washed twice with PBS, trypsinized and cells were suspended in 500 µl PBS. This cell suspension was fixed with 1.5 ml ethanol at 4^0C till further analyzed. After washing the cells thrice with PBS and incubating with RNase, the cells were then stained with Propidium iodide (2 µl of 5 µg/ml solution) and incubated for 20 minutes at room temperature in dark. The Propidium iodide (PI) stained cell aliquots were filtered through 70 µm filters just before flow cytometric analysis followed by measurements made with a laser based (488 nm) flow cytometer FACSVantage (Becton Dickinson, Braintree, USA) and data were acquired using the Cell Quest software. Results were recorded in Fig. 9.9, 9.10 Table 9.3 and Table 9.4.

Table 9.3: Cell Cycle Analysis of Etoposide in A549 cell lines by Propidium Iodide staining using Flow Cytometry A549 cells were incubated with Etoposide Liposomes (ETPLIP) and HA grafted Etoposide liposomes (HAETPLIP).

Formulations	% Cells in G1 phase	% cells in S phase	% cells in G2/M phase
Etoposide Liposomes(ETPLIP)	66.00	10.95	12.42
HA grafted Etoposide Liposomes (HAETPLIP)	28.03	23.46	44.79

Table 9.4: Cell Cycle Analysis of Docetaxel in A549 cell lines by Propidium Iodide Staining using Flow Cytometry A549 cells were incubated with Docetaxel Liposomes (DOCLIP) and HA grafted Docetaxel liposomes (HADOCLIP)

Formulation with Formulation code	% Cells in G1	% cells in S phase	% cells in G2/M	% Apoptosis
Docetaxel Liposomes(DOCLIP)	18.67	11.60	63.57	3.11
HA grafted Docetaxel Liposomes (HADOCLIP)	16.50	40.17	35.44	4.75

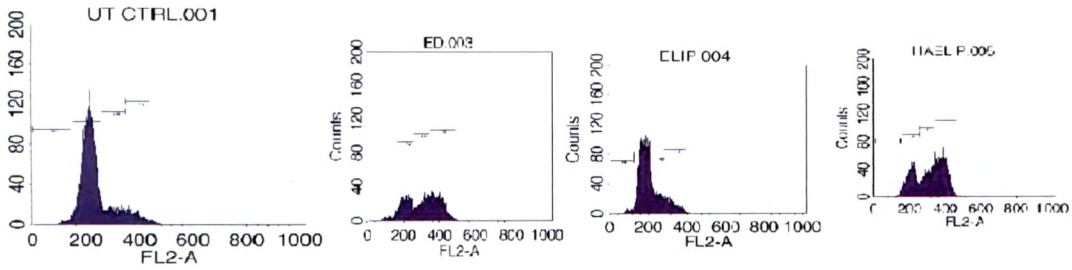


Fig. 9.9- Cell Cycle Analysis by Flow Cytometry (using PI staining technique) in A549 cells incubated with non grafted and HA grafted Etoposide liposomes. (Blank denotes Untreated cells – cells not treated with drug)

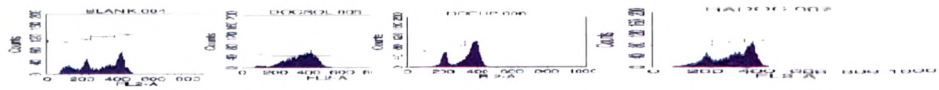


Fig. 9.10- Cell Cycle Analysis by Flow Cytometry (using PI staining technique) in A549 cells incubated with non grafted and HA grafted Docetaxel liposomes. (Blank denotes Untreated cells-cells not treated with drug)

9.3 DISCUSSION

6-coumarin has been characterized as a lipophilic marker for confocal microscopy to study cellular uptake of vesicular systems like nanoparticles owing to its high fluorescence activity at very low concentrations. Apart from that, 6-coumarin exhibits high fluorescent intensity even at low dye loading. The particle size and zeta potential of 6-coumarin loaded non grafted and HA grafted liposomes were similar to drug loaded liposomes. Hence, it was expected that their cellular uptake profile would also be similar to that of drug loaded liposomes.

As shown in Figure 9.1(d) there was very little or no fluorescence in case of cells incubated with dye solution indicating that dye or simple drug solution itself does not get internalized in the cells significantly. Fluorescence in cells (Fig.9.1) was observed to increase with passage of time from 15 to 45 minutes indicating corresponding increase in cell uptake of non grafted and HA grafted drug loaded liposomes. Significantly higher fluorescence was associated with cells treated with liposomes and more with HA grafted liposomes than with non-grafted liposomes. Hence, one can conclude that fluorescence observed inside the cells was only due to liposomal uptake and higher cell uptake in case of HA grafted ETP and DOC liposomes can be attributed to the fact that CD44 receptors-the target moiety is over expressed in A549 alveolar epithelial lung cancer cell line which resulted in highly intense fluorescence in case of cells incubated with HA grafted drug loaded liposomes.

A control experiment was performed by incubating cells with 6-coumarin solution released from liposomes in vitro for 2 hours. The intracellular fluorescence of the control was insignificant as compared to that in case of cells incubated with liposomes. Hence it can be inferred that the dye does not leak out from the liposomes during the course of experimental study.

The cytotoxicity of optimized non grafted and HA grafted liposomal formulations of ETP and DOC was assessed using A549 alveolar epithelial lung cancer cell line by MTT assay method. The principle underlying MTT assay (Mossman, 1983 and Lee, 1994) for cell cytotoxicity is reduction of tetrazolium dye (MTT) to formazan by mitochondrial dehydrogenase enzyme in physiologically viable cells. MTT is thus, reduced to violet coloured formazan crystals by viable, proliferative cells. Colour intensity of formazan crystals reflects the number of physiologically

active cells (live/viable) and thus, gives an idea about overall viable cell population. Thus, reduced absorbance indicated either reduction or total aversion in conversion of MTT to formazan leading to inference that cells are dead.

Etoposide concentration in liposomes was adjusted to same as that of free drug. IC_{50} (Minimum Concentration of drug needed to inhibit 50% of viable cell population) was determined for free drug solution, non grafted liposomal formulations (ETPLIP) and HA grafted Etoposide liposomes (HAETPLIP) and has been summarized in Fig.9.3

The results recorded in Fig.9.3 indicate that the concentration of Etoposide inhibiting 50 % of experimental cell population of A549 cell lines (IC_{50}) was significantly lesser in case of HA grafted liposomes as compared to non grafted liposomes and etoposide solution. The IC_{50} values for HAETPLIP, ETPLIP and ETPSOL were found to be 0.5 μM, 1 μM and 2 μM respectively. Cytotoxicity of HA grafted Etoposide liposomes was four times higher as compared to Etoposide drug solution (ETPSOL) in A549 cell lines. Blank liposomes (not loaded with Etoposide) did not exhibit any cytotoxicity against A549 cell line and were used as controls. Similarly, it was observed that IC_{50} in case of DOCSOL was 6 nM, while in case of DOCLIP it reduced to 4 nM and 2nM in case of HADOCLIP. There was nearly threefold increase in cytotoxicity of HA grafted Docetaxel liposomes as compared to Docetaxel drug solution (DOCSOL) in A549 cell lines. (Fig. 9.4)

Rapid internalization and higher retention of cytotoxic drugs when delivered through liposomal delivery systems can be attributed to rapid uptake of colloidal, vesicular systems by endocytosis and reduced effects of efflux pumps like p-glycoprotein. (Couvreur and Vauthier, 1991). The results of these studies are in corroboration with those obtained by Szoka et al, 2001. In the studies carried out by Szoka et al, 2001. it was observed that liposomally entrapped Doxorubicin and HA grafted Doxorubicin loaded liposomes exhibited a significantly high cytotoxicity in B16F10 cell lines in contrast to simple Doxorubicin drug solution. Hence, it can be concluded that colloidal, vesicular drug delivery systems like liposomes can be used to target drug to specific site and its site specificity can be further augmented by coupling with suitable ligands such as HA.

Cellular drug levels for different formulations were estimated at different time intervals after treating A549 cells with plain drug, non grafted and HA grafted liposomes for 24 hours at 37^0C. The peak intracellular concentration of Etoposide was found to be 0.311 μg/mg and 0.397 μg/mg in case of ETPLIP and HAETPLIP respectively, which were significantly higher than those achieved with ETPSOL (0.288 μg/mg). The drug levels declined rapidly and Etoposide was detectable till day 4 (0.022 μg/mg) in the cells treated with Etoposide drug solution. For cells treated with ETPLIP and HAETPLIP, intracellular drug levels were detectable till day 6 and day 8 respectively.(Fig.9.5)On the same track, A549 cells treated with DOCLIP and HADOCLIP exhibited higher peak intracellular concentrations of Docetaxel (0.308 μg/mg and 0.393 μg/mg respectively) as compared to DOCSOL (peak intracellular concentration was 0.258 μg/mg) (Fig 9.6). The cells treated with DOCSOL exhibited rapid decline in intracellular drug levels as compared to cells treated with DOCLIP and HADOCLIP where intracellular drug levels were detectable till day 6 and day 8 respectively. (Fig. 9.6)

Based on cellular drug concentration-time plot, pharmacokinetic parameters were calculated for conventional and developed Etoposide formulations and are shown in Table 9.1. Liposomal Etoposide formulations exhibited higher Area Under the Curve (AUC) than ETPSOL. Among liposomal formulations of Etoposide, HA grafted Etoposide liposomes exhibited higher AUC as compared to non grafted Etoposide liposomes. AUCs were found to be 0.447, 0.893 and 1.46 for ETPSOL, ETPLIP and HAETPLIP respectively. Kel for ETPSOL was found to be 0.608, which is 1.77 times and 2.45 times higher than ETPLIP and HAETPLIP respectively.

Higher AUC and lower elimination rate constants observed in case of liposomal formulations (ETPLIP and HAETPLIP) as compared to Etoposide drug solution (ETPSOL) is a testimonial to sustained release of liposomally entrapped Etoposide followed by its enhanced retention.

Similarly, higher AUC values were observed for liposomally entrapped Docetaxel as compared to Docetaxel plain drug solution (DOCSOL). Among liposomal formulations of Docetaxel, HADOCLIP exhibited higher AUC as compared to non grafted Docetaxel liposomes. AUCs were found to be 0.465, 0.905 and 1.58 for DOCSOL, DOCLIP and HADOCLIP respectively. It was observed that drug elimination slowed down in case of liposomal formulations as compared

to plain drug solution. Elimination rate constant for DOCSOL was found to be 0.338, which is 1.07 times and 1.41 times higher than DOCLIP and HADOCLIP respectively. (Fig.9.6, Table 9.2)

Higher AUC and lower elimination rate constants in case of liposomal formulations of ETP and DOC as compared to ETP/DOC solution respectively indicated that liposomal formulations of Docetaxel exhibited sustained drug release accompanied with enhanced drug retention.

The cell uptake studies and intracellular pharmacokinetic determinations of liposomal formulations clearly indicated that HA grafted liposomal ETP/DOC treated A549 cells not only showed enhanced cell uptake but also resulted in retention of higher intracellular concentrations of HA grafted ETP/DOC liposomes in A549 cells. Higher drug retention for prolonged time intervals in cells may be attributed to cytoadhesive nature of HA. HA is reported to bind specifically to CD44 receptors and N-acetyl D-glucosamine residues located at the surface of alveolar epithelium (Margalit R., 2004, Szoka, 2001) and hence, HA grafted liposomes (HADOCLIP) resulted in enhanced cell uptake followed by prolonged intracellular retention in A549 cells. Hence, apart from its well documented role as a targeting moiety for CD44 over expressing cell lines, cytoadhesive nature of HA also significantly retarded drug release leading to sustained drug release along with prolonged intracellular drug retention.

Effect of temperature and time of exposure on cellular uptake of vesicular systems was studied by conducting cell uptake experiments at different temperatures for different time periods. The results recorded in Fig.9.7 show that after 30 minutes of exposure, uptake of ETPLIP in cells incubated at 37°C was 1.62 times higher than when incubated 4°C and 3 fold higher when incubated for 120 minutes. From Fig. 9.8, it is clear that accumulation of Docetaxel loaded liposomes in cells incubated at 37^0C was 1.91 times higher than 4^0C when incubated for 30 min and 3.85 fold higher at 37^0C than 4^0C when incubated for 120 min. Thus, from above results it may be concluded that temperature and time of exposure are rate limiting steps for cellular uptake, since, incubation of cells at 37^0C for 120 minutes showed higher uptake of liposomes. Secondly, the temperature dependent cell uptake is an evidence to the fact that endocytic cell

uptake mechanism was involved in cell uptake of Docetaxel liposomes (HA grafted and non grafted).

Cell cycle analysis was done to determine the phase of cell cycle arrest. Etoposide is a topoisomerase inhibitor and arrests the cell cycle mainly at G2/M phase leading to accumulation of cells in this phase (Reddy, 2006, Zhu, 2005).

Flow cytometric analysis of the effects of Etoposide on the cell cycle of the A549 cells showed that both Etoposide liposomes (ETPLIP) and HA grafted Etoposide liposomes (HAETPLIP) were able to accumulate in A549 cells and were able to arrest cells in G2/M phase. The results recorded in Table 9.3 and Fig. 9.9 reveal higher proportion of cells in the G2/M arrest phase in case of treatment with HAETPLIP as compared to ETPLIP. The reason for low cell arrest in G2/M phase in case of ETPLIP may be attributed to the fact that non ligand grafted liposomes act as simple, non selective vesicular systems leading to slow cellular uptake of encapsulated drug. However, HA grafted Etoposide (HAETPLIP) showed enhanced rate and extent of cellular uptake which supports the hypothesis that grafting liposomes with HA rendered the liposomes more selective leading to increased Etoposide induced cell cycle arrest at G2/M phase in contrast to ETPLIP.

Docetaxel mainly targets the mitotic spindle and has higher affinity for Beta tubulin sub unit and inhibits depolymerization of microtubules leading to cell arrest. The drug mainly inhibits the cell cycle during S Phase resulting in cell accumulation in S Phase. (Hennequin, 1995) However, like any other cytotoxic drug, Docetaxel is also non selective, cytotoxic drug that acts on actively replicating cells. Failure to differentiate among cancer cells and fast replicating normal host cells limits the advantages of Docetaxel. Mere encapsulation of Docetaxel in liposomes does not increase the selectivity of Docetaxel against cancer cells. However, grafting the Docetaxel containing liposomes with targeting moieties like Hylauronic acid (HA) may help achieve the highly desired cancer cell selectivity.

Flow cytometric analysis of the effects of Docetaxel on the cell cycle of the A549 cells showed that both Docetaxel liposomes (DOCLIP) and HA grafted Docetaxel liposomes (HADOCLIP) were able to accumulate in A549 cells and were able to arrest cells in S phase. The results shown

in Table 9.4 and Fig.9.10 reveal that in case of Docetaxel liposomes (DOCLIP), 18.67 % cells were in G0/ G1 phase, 11.6 % in S phase and 63.57 % in G2/M phase. Whereas, in case of HA grafted Docetaxel liposomes (HADOCLIP), there are 16.50 % inG0/ G1, 40.17 % in S phase and 35.44 % in G2/M phase. The reason for low cell arrest in S phase in case of DOCLIP may be attributed to the fact that non ligand grafted liposomes act as simple, non selective vesicular systems and cell uptake of non grafted liposomally encapsulated drug would have been relatively slower. The results can be further justified by the fact that there was relatively higher cell arrest in G2/M which supports the fact that those cells that could escape the cytotoxic effect of Docetaxel due to relatively slower drug release from liposomes, could be successfully arrested at G2/M phase. However, HA grafted liposomes showed enhanced uptake of Docetaxel (40.17% cell arrest in S phase as compared to 11.6 % in case of Docetaxel liposomes- DOCLIP) which may be attributed to the hypothesis that grafting liposomes with HA rendered HADOCLIP more selective than DOCLIP and could easily target the A549 cells that over expressed CD44 receptors, leading to increased Docetaxel induced cell cycle arrest at S phase in contrast to DOCLIP. Flow cytometric results for DOCLIP and HADOCLIP are consistent with MTT assay data which corroborates the hypothesis that HA renders the Docetaxel loaded liposomes more site specific leading to enhanced cell uptake and thereby cell cytotoxicity in A549 cells as compared to DOCSOL and DOCLIP. Blank liposomes had no effect on cell cycle distribution and the results were similar to those obtained with untreated control.

The results clearly demonstrated significantly higher cell uptake, achievement and sustenance of higher intracellular drug levels for prolonged time period, enhanced cell bioavailability and hence, greater cytotoxicity with Hyaluronic acid grafted liposomes as compared to non grafted liposomes and plain drug solutions.

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