CHAPTER 6
IN VITRO CELL LINE STUDIES

6. *IN VITRO* CELL LINE STUDIES

6.1Introduction

6.1.1Cell Cycle

Using cytotoxic agents to treat cancer is based on understanding several important concepts, including the cell life cycle, cell cycle time, growth fraction and tumor burden. All the tissues in our bodies are comprised of cells that grow and reproduce to replace cells that are lost during injury or normal wear and tear. The cell cycle is a five-step process that both normal cells and abnormal cancer cells go through in order to grow and reproduce to form new cells.

Phase 1 - Resting Phase (G-O) – In this phase, cells are not dividing and are temporarily out of the cell cycle. Depending on the type of cell, this phase can last for a few hours to several years. When the cell is signaled to reproduce, it moves into the G1 phase.

Phase 2 - Post-mitotic phase or interphase (G-1) – In this phase, the cell starts making more proteins in preparation for cell division. Enzymes needed for deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) synthesis are produced. DNA is an essential nucleic acid comprised of deoxyribose, a phosphate and 4 nitrogenous bases -adenine, guanine, cytosine and thymine. Chemical reactions occur between the bases leading to the formation of the double-stranded DNA helix, which serves as the genetic template for cell division. The duration of the G-1 phase can last from hours to days.

Phase 3 - Synthesis (S) – In this phase, the proteins containing the genetic code (DNA) are copied so that both of the new cells formed will have the right amount of DNA. The S phase lasts approximately 10 to 20 hours.

Phase 4 - Premitotic phase (G-2) – This phase occurs just before the cell starts splitting into two cells. Additional protein and RNA synthesis occurs. The G-2 phase lasts from 2 to 10 hours.

Phase 5 - Mitosis (M) – Cellular division occurs in this phase. When mitosis occurs, the cell then divides, creating two identical cells. The M phase lasts from one-half hour to one hour (Hartwell and Weinert, 1989; http://academy.d20.co.edu/, http://users.rcn.com/jkimball.ma.ultranet/BiologyPages/C/CellCycle.html, http:// en.wikipedia.org/wiki/ Cell_cycle).

State	Phase	Abbreviation	Description
quiescent/ senescent	Gap 0	G_0	A resting phase where the cell has left the cycle and has stopped dividing.
Interphase	Gap 1	G_1	Cells increase in size in Gap 1. The G_1 checkpoint control mechanism ensures that everything is ready for DNA synthesis.
	Synthesis	S	DNA replication occurs during this phase.
	Gap 2	G_2	During the gap between DNA synthesis and mitosis, the cell will continue to grow. TheG_2 checkpoint control mechanism ensures that everything is ready to enter the M (mitosis) phase and divide.
Cell division	Mitosis	M	Cell growth stops at this stage and cellular energy is focused on the orderly division into two daughter cells. A checkpoint in the middle of mitosis (Metaphase Checkpoint) ensures that the cell is ready to complete cell division.

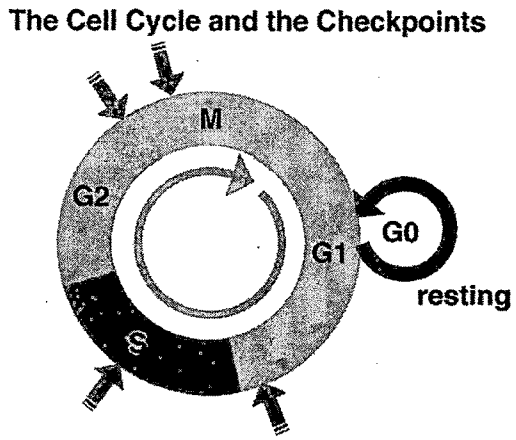


Figure 6.1 Cell Cycle with check points

6.2 Experimental

6.2.1Materials

Dubelcous Modified Eagle Media (DMEM), Trypsin-EDTA, MTT [3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide], Phosphate buffer saline (PBS) and Nonidet P-40 (NP-40) was purchased from Himedia, Mumbai, Hank's balanced salt solution, propidium iodide, Nonidet P-40 (NP-40), RNAase was purchased from Sigma Aldrich St. Louis, M.O. Fetal bovine serum (FBS), was purchased from Gibco (Life Technologies, AG, Switzerland). Non small cell lung cancer cell lines (A549) (92 passage), Human cervical cancer (HeLa) cell lines (105 passage) were purchased from the National Center for Cell Sciences, Pune, India, Tissue culture flasks (T 75, T 25), 96, 24, 12, 6-well plates was purchased from Tarsons, Ltd. Flow cytometer (FACS), Facs Calibur; Beckton & Dickenson, USA, Carbon dioxide incubator, Live fluorescence image, Olympus USA).

5.2.2 Solutions buffers and Media preparations

- **Lysis buffer:** 0.25 M Tris–HCl, pH 8.0 and 0.5% Nonidet P-40.
- **PI solution for 100mL:** sodium citrate 0.1%, PI 50µg/mL, Igepol 0.03%, RNASe 40µg/mL.
- **Running buffer/ Lit:** 3g Tris-HCl, 14.4g Glycine, 1g SDS.
- **Transfer buffer/ Lit:** 3g Tris-HCl, 14.4g Glycine, 200mL methanol.

- **BSA:** 5% of BSA solution in 1X TBST, 0.2% Tween-20
- **Chemiluminescent:** separately prepare 50 µl of peroxide in 500 µl and 50 µl of lumiglo in 500 µl mix it before addition.
- **10% Gel:** water 4mL, acrylamide 2.5mL, separating buffer 2.5 mL, 20%SDS 50 µL, APS 100 µL, TEMED 15 µL.
- **5% Stacking Gel:** water 3.175mL, acrylamide 0.5mL, stacking buffer 1.25 mL, 20%SDS 0.025mL, APS 50 µL, TEMED 0.015mL.
- **12% Separating Gel:** water 3.334mL, acrylamide 3.996mL, buffer 2.5 mL, 20%SDS 50 µL, APS 100 µL, TEMED 15 µL.

6.2.3 Cell Cultures

Both the HeLa, A549 cancer cells were cultured in complete growth media, Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% fetal bovine serum and streptomycin-penicillin solution at 37°C in humidified atmosphere of 5% CO_2 in air. Cultures of 85-90% confluency were used for all the experiments. The cells were trypsinized (Trypsin-EDTA), counted and subcultured in well plates for viability studies. The cells were allowed to adhere overnight before they were used for experiments.

6.3 Cytotoxicity

6.3.1 Introduction

While cytotoxicity testing is currently conducted using animals, studies published in recent years have shown a correlation between *in vivo* and *in vitro* acute toxicity. These studies suggest that *in vitro* methods may be helpful in predicting *in vivo* cytotoxicity. The use of cell culture *in vitro* as alternative to predict acute lethality *in vivo* has been under study for almost 50 years. Numerous demonstration of correlations between cytotoxicity *in vitro* and animal lethality *in vivo* exist. Recently, several major international *in vitro* initiatives have been directed toward reducing the use of laboratory animals for acute toxicity testing (Eagle and Foray, 1956; Phillips and Gibson, 1990).

6.3.2 Cell viability assay

The toxicity of GEM/ liposomal formulation toward HeLa and A549 cells was determined using 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay following literature procedures (Mosmann, 1983; Hansen et al., 1989). The cytotoxicity assay was performed in 96-well plates cells were seeded onto 96-well plates at 1×10^4 cells/well and incubated overnight at 37° C under a 5% CO_2 atmosphere. The medium in the wells was then replaced with fresh medium containing GEM/liposomal suspension (0.5–300μM) for comparison, and further incubated for 24 h. The effect of drugs on cancer cell proliferation was determined by using the MTT (3-(4,5-dimethylthiazol-2- yl)-2,5- diphenyltetrazolium bromide) assay. Then remove media, 100μL dilute MTT solution (5 mg/mL in stock) was added to each well, and the plate was incubated for 4 h at 37 °C. Medium was then removed, washed with PBS immediately, 200μL DMSO was added to dissolve the blue formazan crystal converted from MTT. Cell viability was assessed by absorbance at 565 nm, measured with a plate reader. Toxicity was expressed as % of viable cells. Statistical significance of differences in toxicity between different samples was analyzed using two-tailed unpaired student t-test.

6.3.3 Results and Discussion

Cytotoxicity of FT, SL and CL GEM liposomes and GEM was determined on both HeLa and A549 cells using an MTT assay. The results are summarized in Table 6.1. The results showed that enhancement in cytotoxicity exhibited by FT liposomal GEM had approximately two times lower IC_{50} value compared to that of GEM. The lack of a greater targeting ratio in CL and SL might be attributed to the gradual release of GEM into the media. These data suggested FR-dependence of the cytotoxicity exhibited by the FR-targeted liposomal GEM. The enhancement in cytotoxicity exhibited by FT liposomal GEM over CL and SL. The results nonetheless demonstrated a FR-dependence of the cytotoxicity. Since free GEM is more rapidly cleared *in vivo*, this factor might have a lesser effect on tumor cell targeting of the liposomes *in vivo*.

Formulation	IC_{50}(μM) in HeLa	IC_{50}(μM) in A549
CL	150	120
SL	120	100
FT	90	90
PD	190	160

Table 6.1 IC_{50} of Liposomal suspension with HeLa and A549 cell lines, CL (conventional liposomes), SL (stealth liposomes), FT (folate targeted liposomes), and PD (pure drug)

6.3.4 Conclusion

The cytotoxicity assay shows that the FT liposomal suspension shows an enhanced cytotoxicity compare to SL and CL liposomal suspension.

6.4 Cell Cycle Analysis

6.4.1 Introduction

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) (Zbigniew and Xuan, 2004; Shapiro, 2003; Ali and Ryan, 2009). DNA content can be measured using fluorescent, DNA-selective stains that exhibit emission signals proportional to DNA mass. Flow cytometric analysis of these stained populations is then used to produce a frequency histogram that reveals the various phases of the cell cycle. This analysis is typically performed on permeabilized or fixed cells using a cell-impermeant nucleic acid stain, but is also possible using live cells and a cell-permeant nucleic acid stain. While the choices for fixed-cell staining are varied, there are only a few examples of useful cell-permeant nucleic acid stains.

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 classic cell cycle. The percentage of cells in the S-phase gives an indication of the proliferative activity of that cell population.

The flow cytometer can indicate relative cell size and density or complexity by measuring forward and side-scattered laser light, respectively. In addition, the flow cytometer can measure relative fluorescence from fluorescent probes which bind to specific cell-associated molecules. These fluorescent probes are often fluorochrome-labeled antibodies specific for cell surface molecules, but may also be nucleic acid probes (e.g. Propidium Iodide), cell function probes (e.g. Indo-1), or fluorescent proteins (e.g. GFP). As the labeled cells flow past a laser beam, the probes fluoresce, and the emitted light is directed to detectors which translate the light signals into information concerning the relative fluorescent intensity associated with each cell.

Flow cytometry measures the percentage of cells in a population with each (or multiple) fluorescent probe(s) attached. The cell sorter is capable of sorting specific cell populations from a mixture of cells based on fluorescence profiles. The most

commonly used dye for DNA content/cell cycle analysis is propidium iodide (PI). It can be used to stain whole cells or isolated nuclei. The PI intercalates into the major groove of double-stranded DNA and produces a highly fluorescent adduct that can be excited at 488 nm with a broad emission centred around 600 nm. Since PI can also bind to double-stranded RNA, it is necessary to treat the cells with RNase for optimal DNA resolution (Zbigniew and Xuan, 2004; Shapiro, 2003; Ali and Ryan, 2009).

6.4.2 Materials and method

6.4.2.1 Cell Cycle Analysis

Cell cycle alterations induced by treatments were studied by flow cytometry analysis on both the HeLa and A549 cell line. Logarithmically proliferating cells were incubated with GEM/liposomal suspension after 4 hrs, washed with drug-free DMEM medium, released by trypsinization, and sedimented at 1500rpm for 5min. After a wash with ice-cold PBS, cells were fixed at 4°C in 50% (v/v) ethanol, digested with RNase A, PI was added to stain total DNA. Analysis was performed using a FACScan (Becton Dickinson, San Jose, CA, USA) and data analysis was carried out with CELLQuest software, while cell cycle distribution was determined using Modfit software (Verity Software House, Inc., Topsham, ME, USA).

6.4.3 Results and Discussion

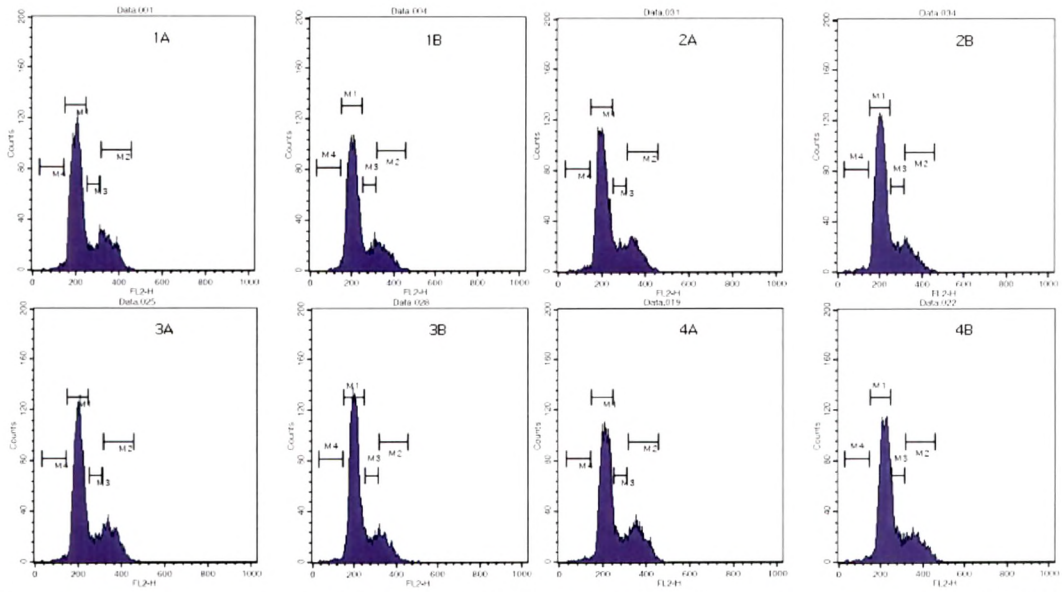


Figure 6.2. Cell cycle histograms of HeLa cells after treatment with GEM/ liposomal suspension for 4 hrs. Flow cytometry was performed at the end of treatment after DNA staining with propidium iodide, (M1 = G_1, M2 = G_2/M, M3 = S, M4 = early S,), A is control; B is treatment; 1=pure drug; 2=conventional liposomes; 3=stealth liposomes; 4=folate targeted liposomes

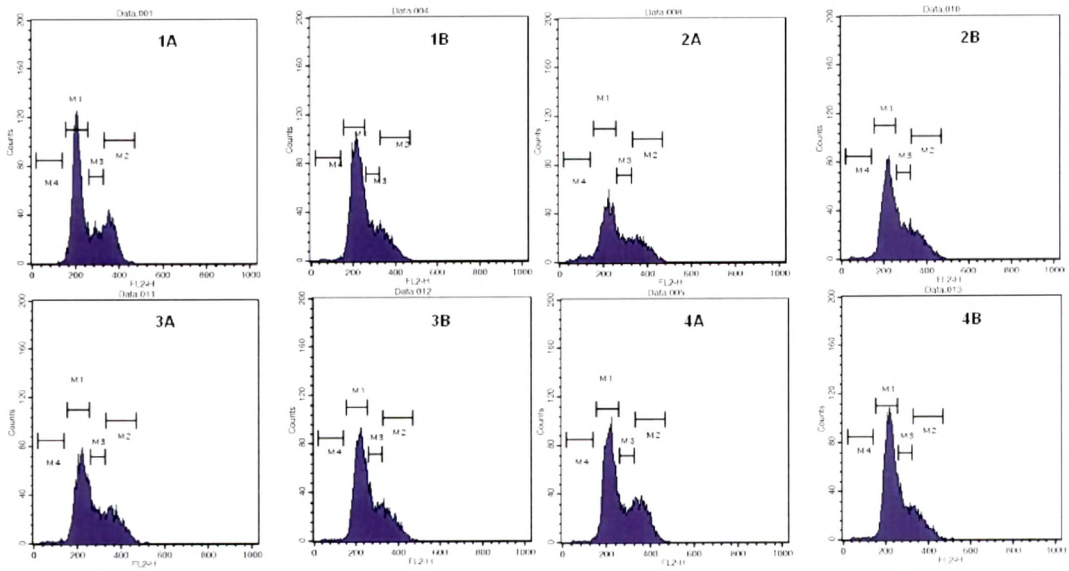


Figure 6.3 Cell cycle histograms of A549 cells after treatment with GEM/ liposomal suspension for 4 hrs. Flow cytometry was performed at the end of treatment after DNA staining with propidium iodide, (M1 = G_1, M2 = G_2/M, M3 = S, M4 = early S,), A is control; B is treatment; 1=pure drug; 2=conventional liposomes; 3=stealth liposomes; 4=folate targeted liposomes

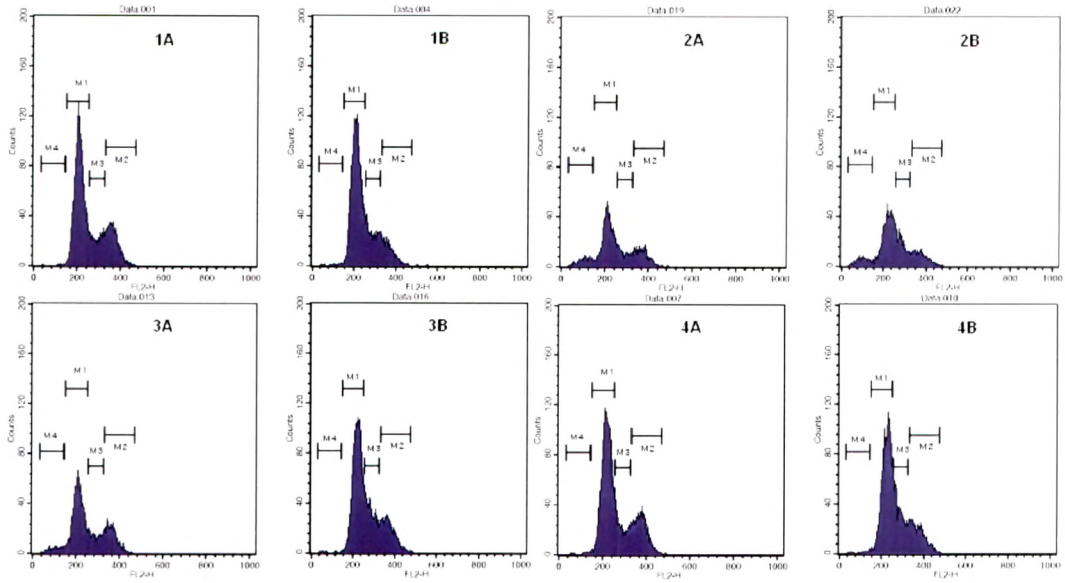


Figure 6.4. Cell cycle histograms of A549 cells after treatment with GEM/ liposomal suspension for 8 hrs. Flow cytometry was performed at the end of treatment after DNA staining with propidium iodide, (M1 = G_1, M2 = G_2/M, M3 = S, M4 = early S,), A is control; B is treatment; 1=pure drug; 2=conventional liposomes; 3=stealth liposomes; 4=folate targeted liposomes

Table 6.2 Cell cycle data of HeLa cells at 4 hrs

Formulation	G1	G2	S
PD control	68.09± 0.65	18.35± 0.48	11.38± 1.21
PD Treatment	71.89± 0.48	14.77± 1.18	11.07± 1.12
CL control	68.95± 1.37	16.52± 1.58	12.29± 1.32
CL Treatment	74.41± 1.66	11.28± 1.46	11.94± 1.17
SL control	67.29± 1.35	18.80± 1.50	11.75± 1.54
SL Treatment	74.13± 1.52	11.84± 1.57	11.82± 1.15
FT control	62.56± 1.06	23.18± 1.12	11.61± 1.04
FT Treatment	64.27± 1.11	17.42± 1.32	14.54± 1.74

Table 6.3 Cell cycle data of A549 cells at 4 hrs

Formulation	G1	G2	S
PD control	61.15± 2.12	21.52± 1.97	15.30± 0.87
PD Treatment	64.22± 1.69	14.68± 1.74	18.41± 1.48
CL control	50.69± 1.89	23.48± 1.31	19.81± 1.18
CL Treatment	58.20± 2.12	17.71± 0.98	21.13± 1.56
SL control	53.16± 1.46	24.80± 1.46	19.45± 1.98
SL Treatment	59.96± 1.78	17.05± 1.41	20.26± 1.08
FT control	57.42± 1.39	22.65± 1.74	17.45± 1.26
FT Treatment	63.47± 2.13	14.40± 1.16	19.47± 1.51

Table 6.4 Cell cycle data of A549 cells at 8 hrs

Formulation	G1	G2	S
PD control	62.51± 1.36	20.05± 1.14	14.18± 1.01
PD Treatment	68.94± 1.09	10.62± 1.48	17.75± 1.35
CL control	54.87± 1.16	19.84± 1.39	14.70± 1.49
CL Treatment	50.13± 1.44	16.42± 1.18	22.88± 2.21
SL control	56.44± 1.76	20.98± 1.79	15.85± 1.04
SL Treatment	56.73± 1.33	17.93± 1.44	22.48± 1.87
FT control	59.70± 1.39	22.29± 1.38	14.93± 1.51
FT Treatment	54.99± 1.24	17.01± 1.91	23.56± 1.61

Gemcitabine was able to affect the cell cycle of cervical (HeLa) and NSCL (A549) cancer cells (Fig.6.2-6.4). In particular, after 4 h treatment flow cytometric studies demonstrated that gemcitabine blocked cells in the G_1 boundary in HeLa cells. In particular, in HeLa cells liposomal suspension caused an increase in the population of cells in G1 and S phase by this it shows apoptosis in later G1 and S phase arrest compare to pure drug among this FT liposomes will be more effective as shown in Fig. 6.2 and Table 6.2. In case of A549 cell line the liposomal suspension shows greater arrest in the latter G1 and S phase compare to pure drug at 4 hrs (Fig. 6.3, Table 6.3), but after 8hrs (Fig. 6.4, Table 6.4) treatment it shows that exponential increase of cell population in S phase thereby it shows greater arrest of S phase after 8hrs in A549 cell lines, FT shows more arrest then CL and SL liposomes. The

increase in its activity in the schedule gemcitabine may be the result of modulation of cell cycle, potentially facilitating dFdCTP incorporation in DNA. By comparing the both the cell lines the FT liposomal suspension shows more specific targeting to HeLa cervical cell line by arresting at latter G1 and S phase at 4hrs compare to PD, CL and SL as shown in figure, this may be because of folate receptor is a tumor marker that is consistently over expressed HeLa then A549 because of folate receptor expression in A549 cell line is lesser then HeLa cell line.

6.4.4 Conclusion

The FT liposomal suspension shows an improvement in apoptosis in both the HeLa and A549 cell line compare to SL and CL. The FT liposomal suspension shows more specific arrest in HeLa cells after 4 hrs incubation.

6.5 Cell Uptake

6.5.1 Introduction

The cellular uptake facilitates penetration of various molecular cargo (from small chemical molecules to nanosize particles and large fragments of DNA). The "cargo" is associated with the peptides either through chemical linkage via covalent bonds or through non-covalent interactions. The function of the uptake are to deliver the cargo into cells, a process that commonly occurs through endocytosis with the cargo delivered to the endosomes of living mammalian cells. A series of surface modifications were first explored to increase efficiency uptake by cells and to elucidate possible mechanisms of cell internalization. There is a large body of literature indicating the use of pegylation of proteins, dendrimers, aptamers, polymers, anticancer drugs and immunoliposomes to create "stealth particles" that can evade uptake by immune cells and enhance the duration of particle persistence *in vivo* (Steven et al., 2007).

6.5.2 Materials and methods

6.5.2.1 Cellular uptake by Flow Cytometry Analysis

Both the HeLa and A549 cells (1.5×10^5 cells /well) were grown overnight on 6 well plates using DMEM cell culture medium supplemented with 10% FCS and 1% penicillin streptomycin. Next day, the medium was removed and replaced with serum free medium, followed by treatment with GEM/Liposomal-6Coumarin conjugates for 30 min. The cells were washed with phosphate buffered saline (PBS), trypsinized and centrifuged at 1500 rpm for 5 min to obtain a cell pellet. The cells were then rinsed with PBS, spun down twice, resuspended in PBS. Analysis was carried out in flow cytometer (FACS caliber, Becton Dickinson, San Jose, CA, USA). In addition to green fluorescence detection, GFP intensity (FL1) was determined by getting cells at an excitation wavelength of 488 nm laser. The presence of GFP was detected by emission at a wavelength of 508 nm. For each cell sample, 10,000 events were collected using the high-speed mode (200-300 cells/s). The data was expressed as histogram plots with number of counts vs. fluorescent intensity and the mean fluorescence intensity was calculated at each time point. During FACs analysis, only

those cells seen both in forward and side scatter were used for counting, hence eliminating artifacts.

6.5.3 Results and Discussion

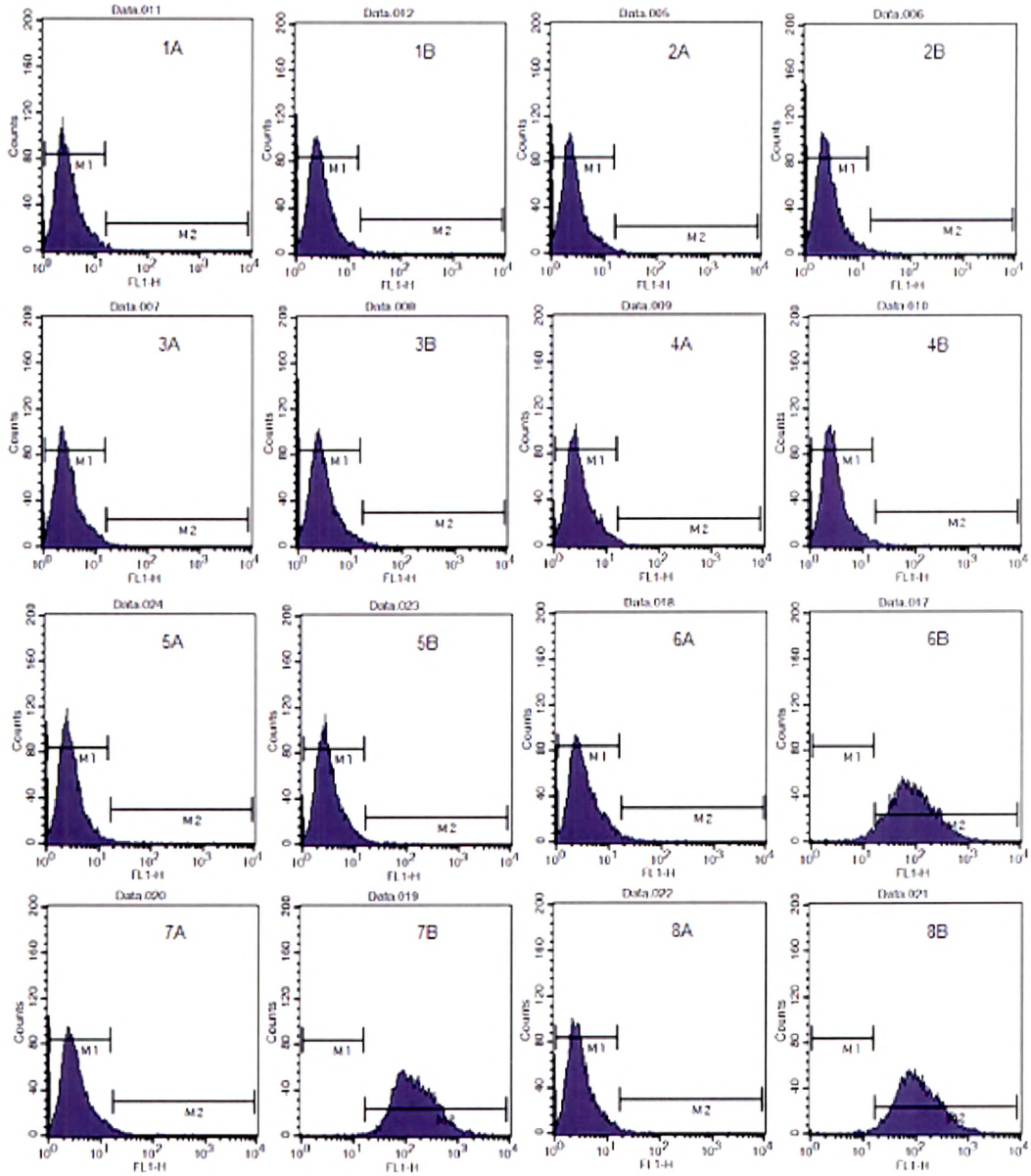


Figure 6.5 Flow cytometry of the cell entry dynamics of GEM/liposomal suspension in HeLa cervical cell line. The log of 6-coumarin absorption intensity (FL1-H on x-axis) is plotted against the number of cells (counts on y-axis). The exponential increase is the cellular uptake of liposomes at 30 minutes is evident. A is control; B is treatment; 1,5=pure drug; 2,6=conventional liposomes; 3,7=stealth liposomes; 4,8=folate targeted liposomes; 1-4 is at 15min and 5-8 is at 30min.

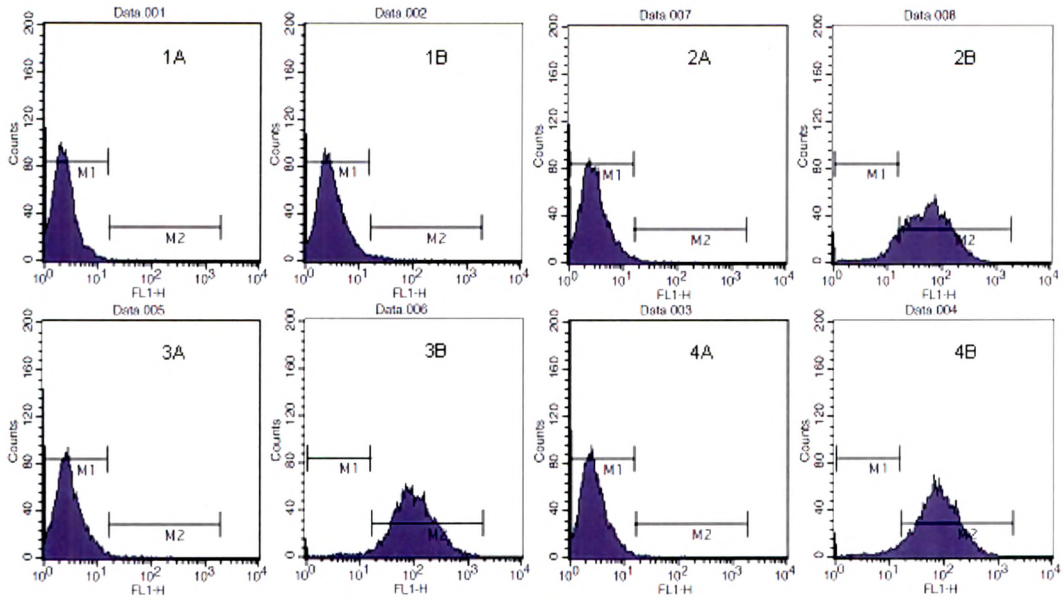


Figure 6.6 Flow cytometry of the cell entry dynamics of GEM/liposomal suspension in A549 cell line. The log of 6-coumarin absorption intensity (FL1-H on x-axis) is plotted against the number of cells (counts on y-axis). The exponential increase is the cellular uptake of liposomes at 30 minutes is evident. A is control; B is treatment; 1=pure drug; 2=conventional liposomes; 3=stealth liposomes; 4=folate targeted liposomes

Cell entry of conjugates in both the HeLa and A549 Cells, entry of 6-Coumarin-labeled liposomes-drug conjugates were evaluated using flow cytometry. Results are represented as the log of fluorescence intensity plotted against the number of events. In HeLa cells liposomal suspension shows small increase in uptake within 15 min, after 15 min it shows an "exponential" increase in fluorescence intensity at 30 min, indicating rapid entry of liposomal suspension in the cell compare to free drug as shown in Figure 6.5. In A549 cells also it shows exponential increase in uptake by liposomal suspension at 30 min shown in figure 6.6. However, for GEM -6coumarin conjugates increase in intensity was gradual over a period of 30 min, with a gradual shift in peak along the fluorescence intensity axis in both the HeLa and A549 cell lines. From flow cytometry it was observed that the cell uptake peaked at 30 min for liposomal conjugates. There was statistical difference ($p > 0.05$) in the cell entry dynamics of liposomal and free drug conjugates and there was no significant difference between FT and SL. As shown in Figure 6.5 and 6.6, FR-targeted liposomes showed much greater cellular uptake and liposome internalization

compared to SL and CL liposomes. These results indicated that these liposomes have efficient interactions with the cellular folate receptor. FR is a tumor marker that is consistently over expressed in ovarian carcinomas therefore FT liposomes shows more internalization in HeLa cells compare.

6.5.4 Conclusion

It has been concluded that liposomal suspension shows more internalization in both the HeLa and A549 cells, among this FT liposomes shows greater internalization then CL and SL liposomes.

6.6 Cell Uptake by Live Cell Confocal Imaging

6.6.1 Introduction

Live cell imaging system delivers all the key performance functions required from a confocal laser scanning microscope and the unique dual scanner, allowing stimulation and observation at the same time. It minimizes specimen damage during high-speed imaging of living organisms and accurately captures a full range of related information, with high sensitivity, high speed and high precision. In this scanner incorporates two independent fully synchronised laser scanners in a single compact design for simultaneous laser light stimulation and high-resolution confocal observation. This unique scanning capability ensures that confocal image observation is no longer interrupted during laser light stimulation, e. g. photoactivation, or laser manipulation. Thus the benefit of this technique is that we will not miss rapid fluorescence changes that occur during or immediately following laser stimulation or manipulation (http://www.iolympus.cz/mikroskopy/prospekty/IX71-81_UIS2.pdf, http://www.olympusamerica.com/seg_section/product.asp?product=1037).

6.6.1.1 Applications

The unique structured illumination microscopy (SIM) concept offers distinct advantages for sophisticated applications including FRAP (Fluorescent Recovery After Photobleaching), FLIP (Fluorescent Loss In Photobleaching), photoactivation, photoconversion, uncaging, laser ablation and many more.

6.6.2 Materials and methods

6.6.2.1 Cellular uptake by live cell imaging

A549 cells were cultured in complete growth media in confocal dish containing a glass coverslips and seeded with 1000 cells per dish at 37°C in humidified atmosphere of 5% CO_2 in air for 24 hrs. After cell adhesion, treated with 6 coumarin containing liposomal formulation, the advantages of 6-coumarin include the requirement of low dye due to its high fluorescence activity. The uptake of these liposomes can be easily visualized by confocal microscopy. The entry of 6-coumarin loaded liposomes was similar to that of drug loaded liposomes hence, it is expected that their cellular uptake would be similar to drug loading into liposomes. Images were taken at 10, 15, 20, 25,

30 and 60 min by using LIVE cell imaging station - IX81 ZDC-Olympus which is connected to co_2 incubator.

6.6.3 Results and Discussion

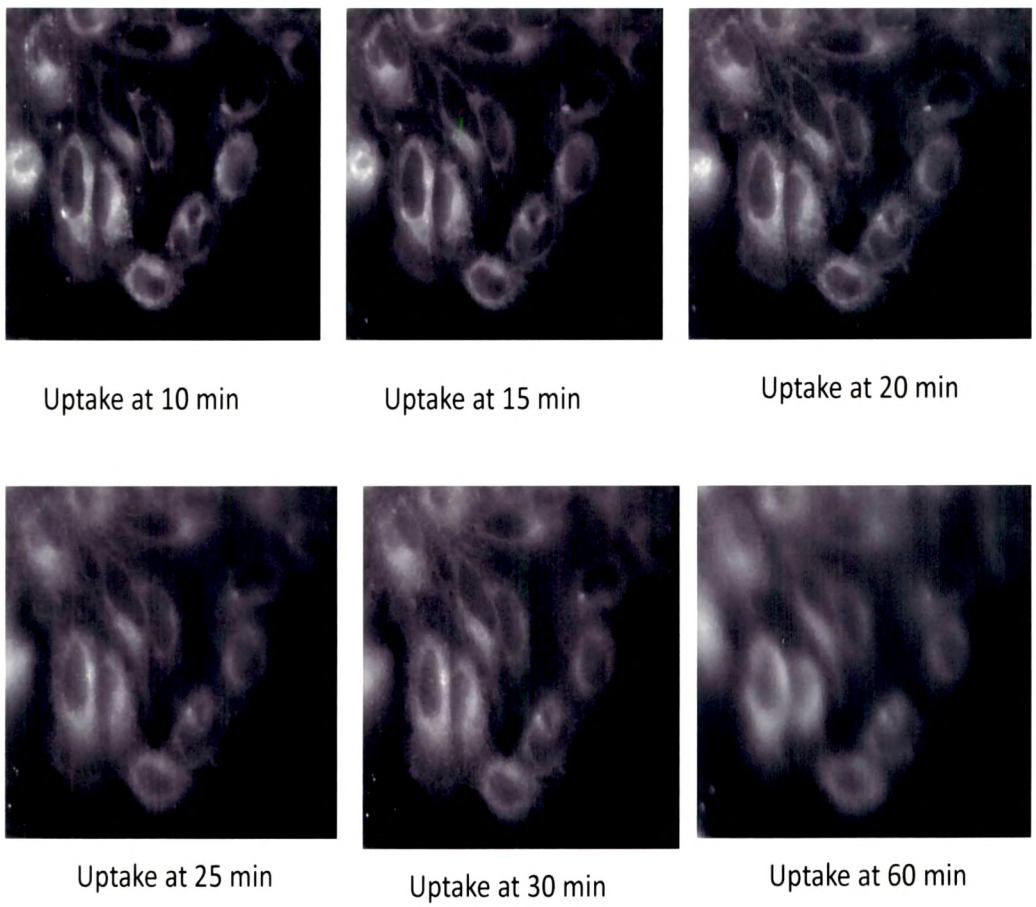


Figure 6.7 Confocal microscopy images of uptake of coumarin-loaded FT liposomes at different time intervals

Cellular drug levels were estimated at different time intervals, after treating A549 cells with 6-coumarin FT liposomes, it has shown that the uptake of liposomal suspension takes place through surface as been confirmed by intensity of fluorescence increases in cells as time shown in Figure 6.7. Initially it shows uptake at 10 min and the uptake was increased and intensity was more after 30 min and almost saturation takes place at 60 min. This shows an increase in uptake directly proportional to time.

6.6.4 Conclusion

It has been concluded that the uptake of A549 cells takes place initially after 10 min and exponential increase in uptake after 20 min as by shown by increasing fluorescence intensity.

6.7 Western Blotting

6.7.1 Introduction

The western blot (alternatively, protein immunoblot) is an analytical technique used to detect specific proteins in a given sample of tissue homogenate or extract. It uses gel electrophoresis to separate native or denatured proteins by the length of the polypeptide (denaturing conditions) or by the 3-D structure of the protein (native/ non-denaturing conditions). The proteins are then transferred to a membrane (typically nitrocellulose or PVDF), where they are probed (detected) using antibodies specific to the target protein (Hempelmann et al., 1987; Towbin et al., 1979; Renart et al., 1979).

6.7.2.1 Tissue preparation

Samples may be taken from whole tissue or from cell culture. In most cases, solid tissues are first broken down mechanically using a blender (for larger sample volumes), using a homogenizer (smaller volumes), or by sonication. Cells may also be broken open by one of the above mechanical methods. However, it should be noted that bacteria, virus or environmental samples can be the source of protein and thus western blotting is not restricted to cellular studies only.

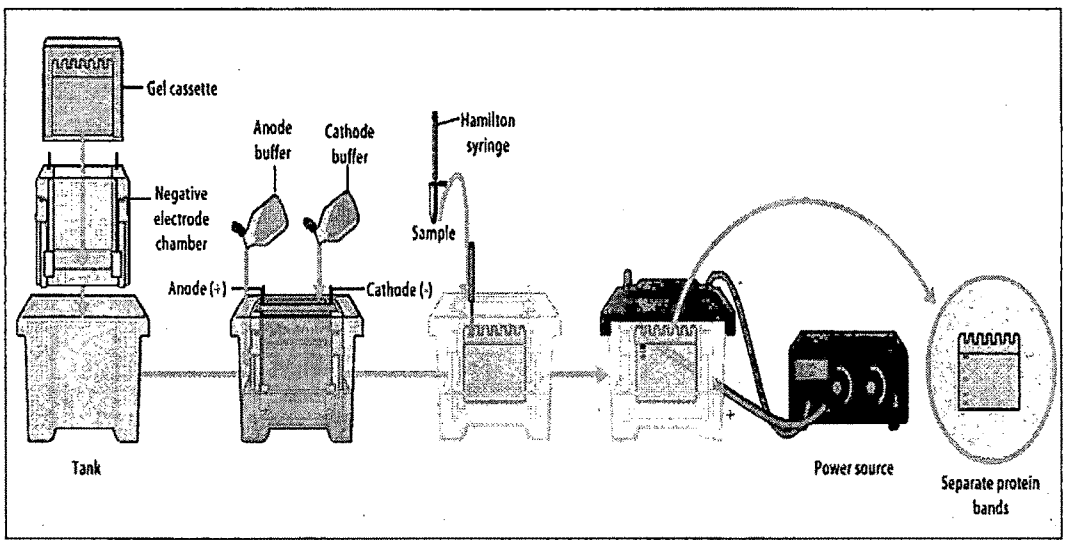
Assorted detergents, salts and buffers may be employed to encourage lysis of cells and to solubilize proteins. Protease and phosphatase inhibitors are often added to prevent the digestion of the sample by its own enzymes. Tissue preparation is often done at cold temperatures to avoid protein denaturation. A combination of biochemical and mechanical techniques including various types of filtration and centrifugation can be used to separate different cell compartments and organelles.

6.7.2.3 Gel electrophoresis

The proteins of the sample are separated using gel electrophoresis. Separation of proteins may be by isoelectric point (pI), molecular weight, electric charge or a combination of these factors. The nature of the separation depends on the treatment of the sample and the nature of the gel. This is a very useful way to determine a protein.

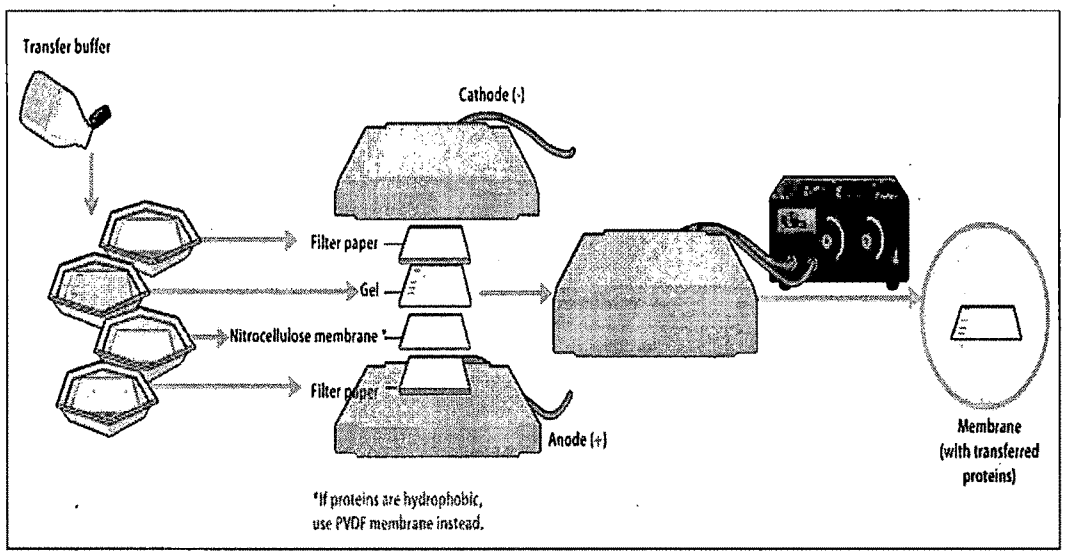
By far the most common type of gel electrophoresis employs polyacrylamide gels and buffers loaded with sodium dodecyl sulfate (SDS). SDS-PAGE (SDS polyacrylamide gel ectrophoresis) maintains polypeptides in a denatured state once they have been treated with strong reducing agents to remove secondary and tertiary structure (e.g. disulfide bonds [S-S] to sulfhydryl groups [SH and SH]) and thus allows separation of proteins by their molecular weight. Sampled proteins become covered in the negatively charged SDS and move to the positively charged electrode through the acrylamide mesh of the gel. Smaller proteins migrate faster through this mesh and the proteins are thus separated according to size (usually measured in kilodaltons, kDa). The concentration of acrylamide determines the resolution of the gel, the greater the acrylamide concentration the better the resolution of lower molecular weight proteins. The lower the acrylamide concentration the better the resolution of higher molecular weight proteins. Proteins travel only in one dimension along the gel for most blots.

Samples are loaded into wells in the gel. One lane is usually reserved for a marker or ladder, a commercially available mixture of proteins having defined molecular weights, typically stained so as to form visible, coloured bands. When voltage is applied along the gel, proteins migrate into it at different speeds. These different rates of advancement (different electrophoretic mobilities) separate into bands within each lane.



6.7.2.4 Transfer

In order to make the proteins accessible to antibody detection, they are moved from within the gel onto a membrane made of nitrocellulose or polyvinylidene difluoride (PVDF). The membrane is placed on top of the gel, and a stack of filter papers placed on top of that. The entire stack is placed in a buffer solution which moves up the paper by capillary action, bringing the proteins with it. Another method for transferring the proteins is called electro blotting and uses an electric current to pull proteins from the gel into the PVDF or nitrocellulose membrane. The proteins move from within the gel onto the membrane while maintaining the organization they had within the gel. As a result of this "blotting" process, the proteins are exposed on a thin surface layer for detection (see below). Both varieties of membrane are chosen for their non-specific protein binding properties (i.e. binds all proteins equally well). Protein binding is based upon hydrophobic interactions, as well as charged interactions between the membrane and protein. Nitrocellulose membranes are cheaper than PVDF, but are far more fragile and do not stand up well to repeated probings.



6.7.2.5 Blocking

Since the membrane has been chosen for its ability to bind protein, and both antibodies and the target are proteins, steps must be taken to prevent interactions between the membrane and the antibody used for detection of the target protein (since

the antibody is a protein itself). Blocking of non-specific binding is achieved by placing the membrane in a dilute solution of protein, typically Bovine serum albumin (BSA) or non-fat dry milk (both are inexpensive), with a minute percentage of detergent such as Tween 20. The protein in the dilute solution attaches to the membrane in all places where the target proteins have not attached. Thus, when the antibody is added, there is no room on the membrane for it to attach other than on the binding sites of the specific target protein. This reduces "noise" in the final product of the western blot, leading to clearer results, and eliminates false positives.

6.7.2.6 Detection

During the detection process the membrane is "probed" for the protein of interest with a modified antibody which is linked to a reporter enzyme, which when exposed to an appropriate substrate drives a colourimetric reaction and produces a colour. For a variety of reasons, this traditionally takes place in a two-step process, although there are now one-step detection methods available for certain applications.

6.7.2.7 Two step

6.7.2.7.1 Primary antibody

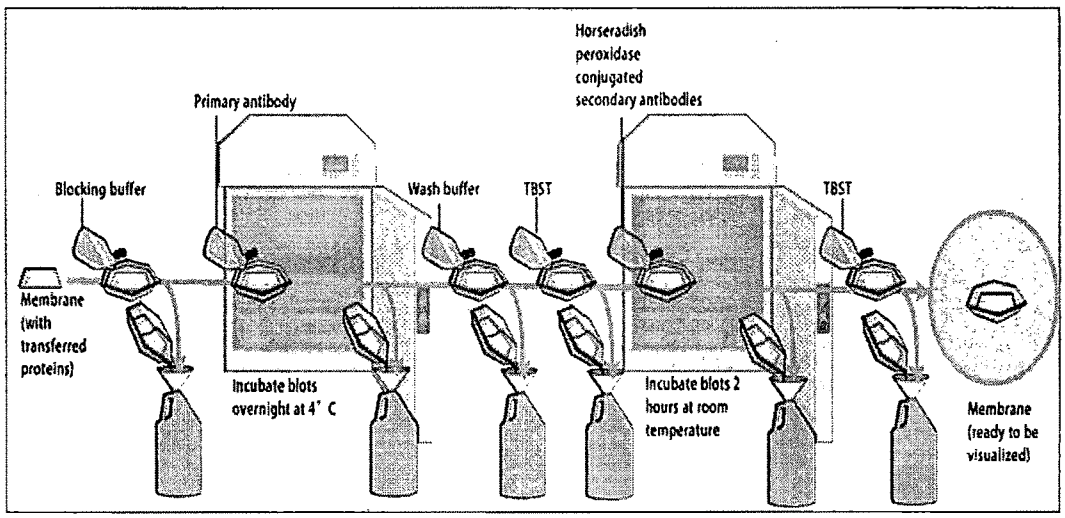
Antibodies are generated when a host species or immune cell culture is exposed to the protein of interest (or a part thereof). Normally, this is part of the immune response, whereas here they are harvested and used as sensitive and specific detection tools that bind the protein directly.

After blocking, a dilute solution of primary antibody (generally between 0.5 and 5 micrograms/mL) is incubated with the membrane under gentle agitation. Typically, the solution is composed of buffered saline solution with a small percentage of detergent, and sometimes with powdered milk or BSA. The antibody solution and the membrane can be sealed and incubated together for anywhere from 30 minutes to overnight. It can also be incubated at different temperatures, with warmer temperatures being associated with more binding, both specific (to the target protein, the "signal") and non-specific ("noise").

6.7.2.7. 2 Secondary antibody

After rinsing the membrane to remove unbound primary antibody, the membrane is exposed to another antibody, directed at a species-specific portion of the primary antibody. This is known as a secondary antibody, and due to its targeting properties, tends to be referred to as "anti-mouse," "anti-goat," etc. Antibodies come from animal sources (or animal sourced hybridoma cultures); an anti-mouse secondary will bind to almost any mouse-sourced primary antibody. This allows some cost savings by allowing an entire lab to share a single source of mass-produced antibody, and provides far more consistent results. The secondary antibody is usually linked to biotin or to a reporter enzyme such as alkaline phosphatase or horseradish peroxidase. This means that several secondary antibodies will bind to one primary antibody and enhance the signal.

Most commonly a horseradish peroxidase linked secondary is used to cleave a chemiluminescent agent, and the reaction product produces luminescence in proportion to the amount of protein. A sensitive sheet of photographic film is placed against the membrane, and exposure to the light from the reaction creates an image of the antibodies bound to the blot. A cheaper but less sensitive approach utilizes a 4-chloronaphthol stain with 1% hydrogen peroxide; reaction of peroxide radicals with 4-chloronaphthol produces a dark brown stain that can be photographed without using specialized photographic film.



6.7.2.8 Analysis

After the unbound probes are washed away, the western blot is ready for detection of the probes that are labeled and bound to the protein of interest. In practical terms, not all westerns reveal protein, only at one band in a membrane. Size approximations are taken by comparing the stained bands to that of the marker or ladder loaded during electrophoresis. The process is repeated for a structural protein, such as actin or tubulin, which should not change between samples. The amount of target protein is indexed to the structural protein to control between groups. This practice ensures correction for the amount of total protein on the membrane in case of errors or incomplete transfers.

6.7.2.9 Chemiluminescent detection

Chemiluminescent detection methods depend on incubation of the western blot with a substrate that will luminesce when exposed to the reporter on the secondary antibody. The light is then detected by photographic film (Renart et al., 1979; exactantigen.com, ; Neil Burnette, 1981; Ambroz, 2006).

6.7.3 Materials and methods

6.7.3.1 Caspase activity by western blotting

The A549 cells of 2× 10^6 cells were seeded in 30 mm dish after 24 hrs the formulations were treated with cells for 2 hrs with PD, CL and FT. After 2 hrs treatment, the media was removed from the dish wash with 1mL of 1X PBS, then add 500 µl of lysis buffer and scrap the cells with syringe/ scraper and spin it with 1500 rpm for 5 min take a supernatant for further studies as discussed earlier like separation of proteins by Gel electrophoresis, transfer of proteins from gel to nitrocellulose membrane, blocking of nonspecific binding is achieved by placing the membrane in non-fatty dry milk with a minute 0.2% of detergent such as tween 20 for 2hrs, the membrane is treated with primary antibody of anti rabbit 1mL of 1:2000 dilution keep it overnight in a shaker at 4°C, remove the membrane and wash with TBST solution for 10 min three times, take the membrane and add a secondary antibody rabbit polyclonal antibody for Caspase-3 in 1:1500 dilution and keep it in shaker for 2hrs, remove the membrane and wash with TBST as earlier then spots are

detected by chemiluminescent detection method by transfering the spots to the X-ray sheet by using 1mL mixture of peroxide (50 µl/ 0.5 mL) and Lumiglo (50 µl/ 0.5 mL).

6.7.4 Results and Discussion

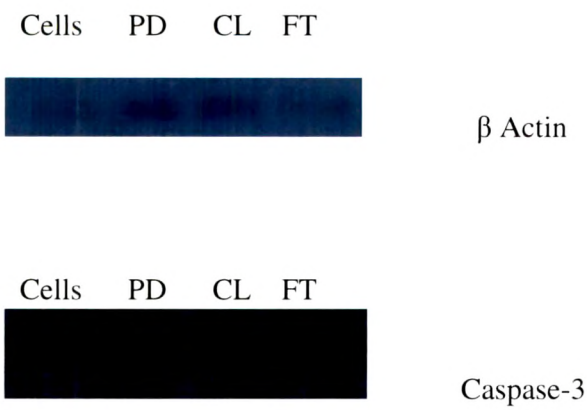


Figure 6.8 Liposomal suspension activity on Caspase in A549 cell line, β Actin was used as standard

Caspase-3 is a frequently activated death protease, caspase-3 is a 32 KDa activated during apoptosis; however, the specific requirements of this (or any other) caspase in apoptosis were until now largely unknown. Recent work, reviewed here, has revealed that caspase-3 is important for cell death in a remarkable tissue, cell type or death stimulus-specific manner and is essential for some of the characteristic changes in cell morphology, and certain biochemical events associated with the execution and completion of apoptosis. Gemcitabine liposomal solution increases caspase-3 activity in A549 cell has been shown by western blotting analysis as comparatively plain drug, β Actin was used as standard.

6.7.5 Conclusion

By the western blotting analysis we can conclude that the apoptosis takes place by increase in the caspase-3 activity and it may be one of the possible mechanisms in apoptosis in A549 cell line.

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