7 IN-VITRO CELL LINE STUDIES

7.1 INTRODUCTION

Cell lines have been seen as an exceptional element for the determination of the various cellular pathways, cell-cell interaction, and characterization of a specific gene involved in cancer development. Nevertheless, cell lines provide comprehensive fundamentals of carriers' system before its actual application to animal models (1). Characterization of cell lines provides better insight into a polygenetic etiology of the cancer cells along with the biological reflexes involved in the reinforcement of a disease. Cell characterization has played a significant role in the development of novel carriers, understanding of different uptake mechanisms along with alterations involved in current therapy resistivity, and development of targeted carriers with available chemotherapeutic agents. In fact, the appropriate in-vitro model can help in determination of the cellular pathway, genetic as well epigenetic pathway, to study the downregulation of proliferation, apoptotic pathways, progression of the disease, to define the endocytic route for uptake, quantification of cellular uptake and characterization of the novel cancer therapeutics (2). The results of the cell line study have been extrapolated to the in-vivo studies on animals and humans. The developed polymeric lipid hybrid nanocarriers (PLHNCs) have been characterized further for the relative in-vitro and in-vivo characterization. To assess the biological effects of the developed PLHNCs, the in-vitro characterization was performed on modified A549 cell lines.

7.2 MATERIALS AND EQUIPEMENTS

7.2.1 Materials

Table 7-1 List of Materials

Sr No	Chemicals/Materials	Source/Manufacturer
1.	A549 cell line	NCCS, Pune, India.
2.	Docetaxel resistant cell line	Developed in laboratory
3.	Thiazolyl Blue Tetrazolium Bromide (MTT)	HiMedia, Mumbai, India
4.	Dulbecco's Modified Eagle Medium (DMEM) (high glucose)	HiMedia, Mumbai, India
5.	Fetal Bovine Serum (FBS)	HiMedia, Mumbai, India
6.	Penicillin/streptomycin antibiotic	HiMedia, Mumbai, India
7.	Trypsin EDTA	HiMedia, Mumbai, India
8.	RNAse	HiMedia, Mumbai, India

Sr No	Chemicals/Materials	Source/Manufacturer
9.	Trypan blue	HiMedia, Mumbai, India
10.	DAPI	HiMedia, Mumbai, India
11.	Dulbecco's Modified Eagle Medium	ATCC, USA
12.	Fluorescein isothiocyanate (FITC)	HiMedia, Mumbai, India
13.	SYBR Green	HiMedia, Mumbai, India
14.	Rhodamine 123	SD Fine chem, Mumbai, India.
15.	Verapamil	Sun pharma, Vadodara, India.
16.	CASP-3-C kit,	Sigma-Aldrich,USA

All other chemicals used were of analytical reagent grade and were used without any further purification.

Table 7-2 List of Instruments

SrNo	Instruments	Company
1.	BOD Shaker Incubator	Orbitek, Scigenics
2.	Centrifuge (CPR-30)	Remi Elektrotechnik Ltd., India.
3.	UV Visible Spectrophotometer (1800)	Schimadzu, India
4.	Laminar air flow (HEPA filter)	Weiber vertical laminar air flow
5.	BD FACS AriaIII	BD Biosciences, USA
6.	Confocal laser scanning microscope	CarlZeiss LSM 710, Germany
7.	Jouan IGO150 CELL life CO2 Incubator	Thermo Fisher Scientific, India
8.	Inverted microscope	Nikon Eclipse TS 100
9.	Deep Freeze ((-70 ^{0}C)	E.I.E Instrument Ltd., Ahmedabad
10.	ELISA micro plate Reader	Bio-Rad, Model 680 XR, Mumbai,
11.	Multichannel micropipette	Himedia, Mumbai, India
12.	96 well plates and culture flasks	Tarsons, India
13.	Olympus inverted microscope	Olympus lifescience solution,USA

7.3 GENERAL METHODS AND PREPARATIONS

7.3.1 Preparation of complete media

Dulbecco's Modified Eagle's Medium (DMEM) (called as incomplete medium) was first passed through a 0.2 µ membrane filter for complete media preparation. Then, in a diluted form, 1% v/v Antibiotic solution (Penicillin/streptomycin) and 10% v/v FBS (fetal bovine

serum) were introduced. The whole experiment was carried out in the vertical laminar air flow cabinet.

7.3.2 Preparation of PBS (Phosphate Buffer Saline) pH 7.4

Sodium chloride (8 gm),potassium chloride (200 mg), di-sodium hydrogen phosphate (1.44 gm), potassium dihydrogen phosphate (240 mg) was added in 1 liter of distilled water and pH was checked. Finally, the buffer was autoclaved.

7.3.3 Preparation of FACS buffer

Bovine serum albumin (0.5% w/v) and fetal bovine serum (0.5% v/v) were added in sterilized phosphate buffer pH 7.4 to prepare FACS buffer. The buffer also contains 2 mM EDTA as well as 2 mM NaN3 as preservatives (3).

7.3.4 Sub-culturing of cells

The A549 cells were grown and maintained in DMEM along with 10% FBS and 1% penicillin/streptomycin antibiotics. The cell cultures were incubated in a humidified atmosphere of 5% CO_2 (Jouan IGO150 CELL life CO_2 Incubator, Thermo Fisher Scientific, India) at 37°C temperature. In T-25 cell culture flasks, the cells were persisted as monolayer culture, and sub-cultured twice every week by capturing 10^4 cells in T-25 flasks (4).

The sub-culturing was preceded by the following protocol;

- In the laminar air flow unit, the culture flask was transported to the sterile region and culture media was extracted.
- To remove the residues of serum present in the media that may impede the activity of trypsin, the cells were washed once with sterile phosphate buffer saline pH 7.4 (PBS).
- 2 mL of Trypsin-EDTA solution was introduced to the flask and gently shook, to allow the cells to differentiate from each other and from the surface. Then Trypsin-EDTA was removed to acquire residual cell film and cells were maintained for 2-3 minutes for rounding up in the incubator.
- Under the inverted microscope, cells were examined before the cell layer was removed (usually within 5 minutes).
- To disperse the cells, complete medium (2 ml) was introduced, dispersion was rendered with pipetting as for complete disaggregation cell line requires vigorous pipetting.
- On a haemocytometer, the cell count was carried out.
- Then the flask was added with sufficient seeding concentration and 10 mL of full medium was added to it. On the T-25 culture flask, the passage number was labelled.

- The flask was closed and cells were incubated at 37 °C, 5 % CO_2.

7.4 IN-VITRO CELL LINE STUDIES

7.4.1 Development of Docetaxel resistant A-549 cell lines

The A549 cells were cultured in a DMEM growth medium containing 10% FBS along with 1% penicillin-streptomycin solution. Cells were incubated at 37 °C in a humidified atmosphere having 5% CO_2. Docetaxel resistant A549 cell line was developed through increasing contact of A549 cells with Docetaxel over the subsequent passage of the cell lines. Dose escalation process with docetaxel using concentration more than IC_{50} was performed to generate stable strains of Docetaxel resistant A549 (DR-A549) cells (5). IC_{50} of the cells was noted for the initial passage of the cells and cells were kept in contact with Docetaxel containing complete media for 2-3 days depending upon the cell growth and replaced with fresh Docetaxel containing media. Initially, cells were contacted with IC_{50} concentration of the Docetaxel. After 72 h of exposure, only the resistant clone of the A549 cells were able to survive. Replacement of the Docetaxel containing media with completely fresh media and allowing the same for incubation until the 70-80 % confluency resulted into an achievement of Docetaxel resistant strains. The resistant cells were again exposed to the higher Docetaxel concentration in multiple of IC_{50} until the stable clone was achieved to the 10 times concentration of Docetaxel, when compared to the IC_{50} initial passage (6). The detailed protocol of the development has been mentioned below.

a. Plate A549 cells in 25 cm^2 flasks containing 10 mL of media with 5 x 10^3 cells per flask. To have enough cells at the end of the protocol, 20 flasks were used as per recommendation.
b. After 24 h, when cells were at about 70-80% confluent, Docetaxel was added at the IC_{50} concentration (0.059 µm forA549 cells as performed in laboratory).
c. After 72 h, drug containing media was aspirated and fresh, Docetaxel free media was added. Media was changed every 3-4 days. Within approximately 1-2 weeks, resistant clones appeared evident under the microscope.
d. Media was removed and cells were carefully washed with 15 ml PBS and incubated with 4 mL 0.05% Trypsin-EDTA for 3-5 min at 37 °C to detach cells from the flask surface.
e. Trypsinized cell were resuspended from the flask using 8 mL of fresh media. Cells were pooled from all treated flasks and centrifuged to get cell pellets. (300 x g, 3 min, RT).

f. Supernatant was removed and the obtained cell pellet was resuspended, in 20 mL of fresh media and cells were fixed in T25 flasks.

g. After 24 h, when cells were at about 70-80% confluency, 0.059 µm Docetaxel was added (initial IC_{50} concentration) again.

h. On day 3 steps were repeated from c to f.

i. After 24 h, when cells were at about 70-80% confluency, cells were treated with 2X IC_{50} Docetaxel concentration (0.1 µm for the cells described in this protocol).

j. Steps were repeated from c to j, following a dose-escalation of Docetaxel concentrations (0.025, 0.05, 0.1, 0.2, 0.4 and 1.0 µm), to generate a stable Docetaxel resistant population of cells growing in flasks under the final highest concentration.

7.4.2 In-vitro cytotoxicity

MTT assay is a colorimetric assay for determining the viable cell count depending on the mitochondrial dehydrogenase activity measurement (7). The MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole dye) is taken up and reduced inside the living cells which have mitochondrial dehydrogenase (reductase) enzyme activity intact. This reduction produces a purple-colored formazan compound that gives a direct estimate of number of viable cells when measured spectrophotometrically (As shown in Figure 7-1).

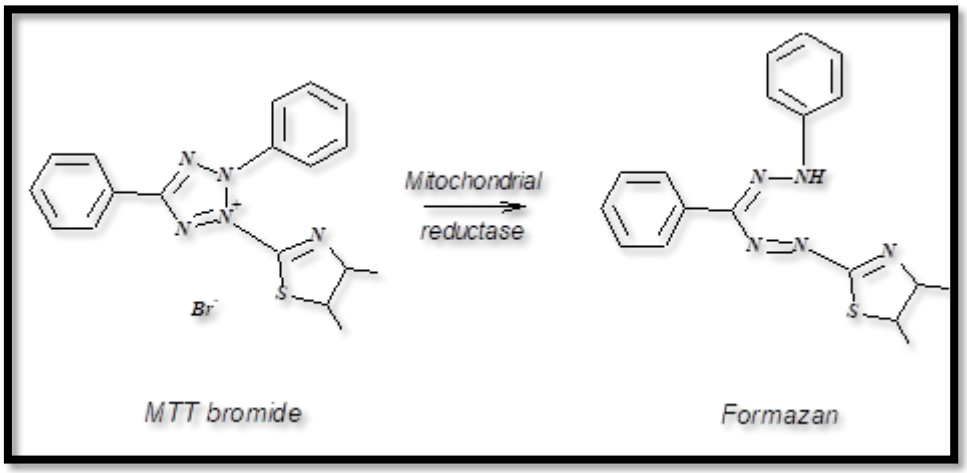

Figure 7-1 MTT Dye Reduction by Mitochondrial Reductase Enzyme of Viable Cells

Formazan formed is water insoluble, so solubilization of formazan precipitates is done by using chemicals like dimethyl sulfoxide (DMSO), an acidified ethanol solution or a surfactant (Sodium lauryl sulphate) solution in diluted hydrochloric acid. Formazan derivative absorbs UV radiation which can be used for estimation of quantity of formazan formed. This

assay can be of use to determine the viable cell count of any culture. This in turn can be useful in determining the cytotoxicity of any chemical of medical interest or any delivery system developed for delivery of drug or therapeutic genes.

In-vitro cytotoxicity of the PLHNCs was estimated with the help of MTT assay (7). Drug resistant (DR-A549) cells were seeded into 96 well-plates with a calculated cell density of 5000 cells per well. After the growing phase of 24 h, cells were exposed to various ranges of formulation (Docetaxel equivalent to 0.025, 0.05, 0.1, 0.2, 0.4 and1.0 µM) in complete media. The cells were incubated for 24 h, 48 h, and 72 h. After incubation, 100 µL of MTT solution (1 mg/mL) was added to each well and incubated for 4 h. Once the MTT incubation was conducted, the culture medium was removed and 200 µL of DMSO was added to each well. Live cells were able to reduce the MTT dye (yellow colored) to formazan crystals (purple colored) with mitochondrial enzymes. The absorbance of the plate was recorded at 570 nm with a reference band at 655 nm on the ELISA microplate reader, BioRad, USA. Micro well with 0.5 % Triton X100 was taken as positive control in 8 wells for each experiment, whereas for the negative control, wells of an untreated cell were considered. Cell viability was calculated and plotted on the basis of the concentration of Docetaxel on the X-axis against % viability on the Y-axis.

7.4.3 Cell migration assay

Migration of the cancer cells was assessed using the scratch assay (8). Briefly, the confluent monolayer of the cells was grown in 6 well-plates at a concentration of 5000 cells/well. The cells were allowed to grow for a period of 24 h. Consequently, scratches were made on the centre of wells using a 200 µL sterile pipette tip. Markings of the scratch were highlighted from the bottom with the help of a marker. The later treatment was delivered to all the wells with the D-PLHNCs, sh-D-PLHNCs, Docetaxel solution at dose of IC_{50} as found in MTT assay and incubated for 24 h. Images of the scratch were captured after incubation period with Olympus inverted microscope. Captured images were quantified using ImageJ software to assess the inhibitory effect on scratch width concerning formulation and % scratch closure was then computed.

7.4.4 Cellular uptake studies

Cell uptake studies were performed using FITC labeled sh-RNA plasmid PLHNCs. The lyophilized stock of shRNA plasmid was reconstituted with nuclease-free water and it was used for further investigations. In order to measure the qualitative cellular uptake, confocal

microscopy was performed; while to analyze the quantitative uptake, flowcytometric technique was employed.

7.4.4.1 Qualitative cellular uptake by confocal microscopy

DR-A549 cells were seeded into 6 well-plates with an initial cell density of 10,000 cells/well on the covered surface of a sterilized glass coverslip. After the span of 24 h, the cells were transfected with different formulation and naked shRNA plasmid concentration of 100 nM. Subsequently, cells were incubated for 6 h and then, washed with cold phosphate buffer saline (PBS). Finally, washing was followed by live imaging of the cells, which was subjected to a confocal laser scanning microscope for the ultimate visualization (9).

7.4.4.2 Quantitative uptake by flow cytometry

DR-A549 cells were seeded with an initial density of 5000 cells/well and proliferated for 24 h. After proliferation, cells were treated with FITC labeled negative control shRNA plasmid at a concentration of 100 nM. Treated cells were incubated with coumarin-6 D-sh-PLHNCs and incubated for 6 h at 37 °C with a condition of 5 % CO_2 in an incubator. Then, the cells were harvested and further collected in FACS buffer containing 0.5 % bovine serum albumin (BSA) & 0.5 mL FBS. At last, cells were sorted using fluorescence intensity using a fluorescence-activated cell sorter. Naked FITC loaded NC-shRNA plasmid was considered as a negative control, while Lipofectamine (L2K)-shRNA plasmid complex was considered as a positive control. Raw data was analyzed and processed using FlowJo software (10).

7.4.5 P-gp inhibition assay

DR-A549 cells were cultured in 24 well-plates with an initial cell density of 20,000 cells in each well. Cells were allowed to attach overnight and then, treated with different concentrations (20,40,60,100 ng) of shRNA plasmid containing PLHNCs. Subsequently, they were incubated at 37 °C for 24 h. Wells were treated with rhodamine123 alone as well as with sh-PLHNCs/Verapamil (p-gp inhibitor) and further incubated for a span of 60 min and washed twice with PBS. To observe the qualitative uptake of rhodamine123, fluorescence microscopy was used (11).

7.4.6 Endocytic routes of sh-PLHNCs and FA-sh-PLHNCs

A number of pathways exist that helps the cellular internalization of nanocarriers i.e., caveolae-, clathrin-, as well as receptor-mediated endocytosis, phagocytosis, and/or micropinocytosis (12). The phagocytosis pathway is specifically regulated by special cells i.e., monocytes and macrophages. While clathrin-mediated, caveolae-mediated, and

micropinocytosis are important routes that help the receptor-mediated internalization based on surface properties and particle size of the carrier. For efficient intracellular delivery, effective uptake of the PLHNCs is very crucial. Hence, clear understanding of different endocytic routes of sh-PLHNCs and FA-sh-PLHNCs is necessary. Herein, FITC labeled shRNA plasmid was incorporated to visualize the internalization. Usually, endocytosis is divided into four routes i) clathrin-mediated endocytosis ii) caveolae-mediated endocytosis iii) clathrin/caveolae-mediated endocytosis iv) micropinocytosis (13).

To understand the clear pathway of endocytosis, DR-A549 cells were incubated in 96 well-plates with various endocytosis inhibitors for a period of 30 min at 4 °C. Trypan blue exclusion assay was performed to determine their optimal concentration for endocytosis inhibition (14). After that, cells were washed with PBS thrice to remove the traces of extra inhibitors, incase if any. Subsequently, the treatment of sh-PLHNCs and FA-sh-PLHNCs (Containing 40 ng of shRNA pDNA) was offered to cells and incubated for 2 h at 37 °C. After 2 h, images were examined under the confocal microscope and a fluorescence microplate reader (Varioskan Flash, Thermo-Fisher Scientific, USA) for the qualitative as well as quantitative determination of the endocytic pathway, respectively. 15 µM of Chlorpromazine hydrochloride and 30 µM of nystatin were used for the determination of clathrin-mediated and caveolae-mediated endocytoses, respectively. Additionally, 500 µM of Amiloride hydrochloride was employed as an inhibitor of micropinocytosis.

7.4.7 Apoptosis detection and cell cycle analysis

7.4.7.1 Caspase-3 Induction assay

Apoptosis plays a major role in the assessment of chemotherapeutic agents, since it is a major pathway for cell death. The role of caspase-3 in apoptosis is well determined. Hence, we measured the activity of caspase executioner in response to Docetaxel formulation. The colorimetric assay is based on Ac-DEVDpNa (acetyl Asp-Glu-Val-Asp p-nitroanilide) hydrolysis by the caspases-3 enzyme. The resulting hydrolysis would release p-nitroaniline which could show absorbance at 405 nm (15). The absorbance of treated cells compared to untreated control cells showed the fold increase in the activity of caspase-3. Briefly, DR-A549 cells were seeded at an initial density of 10,000 cells/well in a 96 well-plate. Once the 80 % confluency was achieved, cell were treated with various formulations (Docetaxel solution, Docetaxel PLHNCs. Docetaxel-shRNA-PLHNCs and FA conjugated at dose of IC_{50} found in MTT assay) and incubated for 6 h and 12 h with individual plates. After the treatment, cells were washed thrice with PBS and caspase assay buffer containing 50 mM HEPES, 1 mM

EDTA, 100 mM NaCl, 10% glycerol, 10 mM DTT, and 0.1% CHAPS with final pH of 7.4. After that, cell lysis was carried out with cell lysis buffer (1X) and cells were pelletized with centrifugation at 2000 rpm for 10 min. Lysate of cells were then transferred to another well plate and incubated with the caspase-3 colorimetric substrate (CASP-3-C, Sigma-Aldrich) at 37 °C for at least 2 h. After incubation, absorbance was measured using an ELISA plate reader at 405 nm.

7.4.7.2 Cell apoptosis detection

The detection of cell apoptosis was accomplished using a flow cytometer. DR-A549 cells were seeded at an initial cell density of 2000 cells per well in 6-well plates. Cells were incubated for 24 h. Cells were treated with Docetaxel solution, Docetaxel PLHNCs, Docetaxel-shRNA-PLHNCs and FA conjugated D-sh-PLHNCs (at dose of IC_{50} found in MTT assay) and incubated at 37 °C for 24 h. Untreated cells were selected as control. Once the incubation period was over, cells were trypsinized using 0.0025% trypsin solution and harvested in 200 mL of binding buffer (0.2 µm sterile filtered 0.1M HEPES (pH 7.4), 1.4M NaCl, and 25 mM CaCl2 solution) in the form of a cell suspension. Immediately, propidium iodide (8 mL) and annexin V-FITC (5 mL) were added to the cell suspension and gently vortexed for uniform mixing, and kept aside for 2.5 hrs for permeation of dyes into the cells (16). The proportion of the apoptotic cells and stained cells were measured using confocal laser scanning microscope.

7.4.7.3 Cell cycle analysis

DR-A549 cells were seeded at an initial cell density of 2000 cells per well in a 6-well plate. The cells were allowed to attach for 24 h and then, treated with Docetaxel solution, Docetaxel PLHNCs. Docetaxel-shRNA-PLHNCs and FA conjugated D-sh-PLHNCs at dose of IC_{50} as found in MTT assay. Cells were allowed to incubate at 37 °C for 24 h. Then cells were trypsinized using trypsin and centrifuged at 1500 rpm for 4 min in sterilized cell centrifuge tubes. Pellets of the cells were washed twice with PBS and cells were fixed using 75 % ethanol at 4 °C. After that, the cells were centrifuged again and washed with PBS twice and resuspended in PBS containing propidium iodide at a concentration of 5 mg/mL and ribonuclease A (deoxyribonuclease-free) at a concentration of 50 mg/mL (16). The resulting cell suspension was incubated in dark place for 25 min and patterns of the cell cycle were analysed using FACS.

7.4.8 Quantitative RT-PCR

RT-PCR is an useful technique for mRNA detection and quantification due to its high sensitivity, good reproducibility and broad dynamic quantification range, it is extensively used

(17). It enables researchers to amplify more than a billion times precise pieces of DNA (18). In PCR, a thermostable polymerase synthesizes any single strand of DNA containing a double stranded starting point with a complementary sequence of bases. Users that correspond to the gene of interest will pick the starting points and they are known as primers. Temperature cycling is used during PCR to monitor the operation of thermostable polymerase and primer binding. The temperature is held at 95^{o}C at the beginning, where all double stranded DNA can melt. In order to allow the primer to bind the target gene, the temperature is then reduced to ~60^{o}C, depending on the primer. Subsequently, the polymerase binds the double stranded DNA and begins copying. As replicated many times, this temperature contributes to an exponential increase in the number of target DNA sequence copies. The amplified gene can be detected by running on agarose gel at the end of the process and staining it where higher copies of DNA are shown by the brighter bands. However, the gel-based analysis in traditional PC does not provide a time-dependent quantity curve.

This approach is tracked and recorded in Real Time PCR using fluorescent probes of double-stranded DNA and detected by a camera. The RT-PCR provides many advantages, such as: a direct look at the reaction, accurate reaction efficiency measurement, removal of the need to run gels, and true quantitative rather than semi-quantitative analysis of gene expression as in typical PCR (19). The RT-PCR uses asymmetric cyanine colors such as SYBR green I and BEBO, that would not interact with the reaction of the polymerase chain. From melt curve observation, the formation of the primer-dimer can be simply explained. The configuration of the primer is also a key feature of the RT-PCR. It also relies on amplicon selection. The amplicon is normally retained for SYBR green-based detection to < 300 base pairs, whereas for probe-based detection, 50-150 base pairs. Generally, the primers are 15 to 20 base pairs and contain 20-80 percent CG units. In order to avoid the formation of dimers in SYBR green-based detection, care should be taken or the melt curve should be checked.

Standard/absolute or relative the quantification of cDNA can be done using PCR. A standard curve of Ct vs log (conc. of the standard gene) must be generated for absolute quantification. A statistical equation is used in proportional quantification to determine the degree of expression relative to the non-treated set. In addition, a housekeeping gene that is expressed at a constant level and has the same quality of amplification is normalized. For quantification purposes, the Ct values are used as a fractional PCR cycle number at which the level of reporter fluorescence is greater than the minimum detection level. This guarantees improved accuracy and reproducibility, as the difference between end product and blank fluorescence cannot be used for quantification because at the end of the reaction, the

fluorescence ceases to rise proportionately as the dye-to-base binding ratio falls over the duration of the reaction. Based on the standard deviation of the baseline, the Ct values are randomly selected by the program and are normally holding 10 times the SD of the baseline signal from period 3 to 15.

7.4.8.1 Transfection protocol for shRNA pDNA

a. **Seed 2,00,000 cells (cell population were calculated using hemocytometer) per well with 2 ml of anti-biotic-free normal growth medium (supplemented with FBS) in a six well tissue culture plate.**

   (*__NOTE__: For a well from a six well tissue culture plate, this procedure is recommended. For wells of varying sizes, adjust cell and reagent volumes proportionately. 80% of the confluent flask contains approx. 20 lac cells. 3.5 lacs cells per well (per 3 ml) i.e. 1 ml trypsin + 17 ml complete media such that 3 ml (3.5 lacs cells) per well are applied to the required dilution.)

b. **Incubate the cells in a CO_2 incubator at 37 ° C before the cells converge by 60-80 %. Usually, this takes 18-24 hours.**

   (***NOTE:** For successful transfection experiments, healthy and sub-confluent cells were desired. One day prior to transfection, Cell viability was ensured.)

c. **Prepare the following solutions:**

   - **Solution A:** For each transfection, dilute 10 µl of resuspended shRNA Plasmid DNA (i.e. 1 µg shRNA Plasmid DNA) into 90 µl shRNA Plasmid Transfection Medium: sc-108062.
   - **Solution B**: For each transfection, dilute 1 - 6 µl of shRNA Plasmid Transfection Reagent: sc-108061 with enough shRNA Plasmid Transfection Medium: sc-108062 to bring final volume to 100 µl.

     (**NOTE:** Do not add antibiotics to the shRNA Plasmid Transfection Medium: sc-108062.)

     (**shRNA Plasmid sc-108062**: Reduced-serum medium suitable for addition to shRNA 20 ml Transfection suspension and shRNA Transfection Reagent immediately Medium prior to cell transfection; modification of Eagle's Minimal Essential Medium, buffered with HEPES and sodium bicarbonate, and supplemented with hypoxanthine, thymidine, sodium pyruvate, L-glutamine, trace elements, growth factors and phenol red.)

(**NOTE:** Optimal results may be achieved by using siliconized microcentrifuge tubes.)

d. **shRNA Plasmid DNA solution (Solution A) was added directly to the dilute shRNA Plasmid Transfection Reagent (Solution B) using a pipette. Mixing was done gently by pipetting the solution up and down and incubated the mixture for 15-45 minutes at room temperature**

e. **Cells were washed twice with 2 ml of shRNA Transfection Medium: sc-108062. Medium was aspirated and subjected to the next step.**

(**NOTE:** Do not use PBS as the residual phosphate may compete with DNA and bind the shRNA Plasmid Transfection Reagent, thereby reducing the transfection efficiency.)

f. **For each transfection, add 0.8 ml shRNA Plasmid Transfection Medium to well.**

g. **200 µl shRNA Plasmid DNA/shRNA Plasmid Transfection Reagent Complex (Solution A + Solution B) was added dropwise to well, covering the entire layer.**

h. **Mixing was done by swirling the plate to ensure that the entire cell layer was immersed in solution.**

i. **Cells were incubated for 5-7 hours at 37° C in a CO2 incubator. (Longer transfection times may be desirable depending on the cell line.)**

j. **Following incubation, 1 ml of normal growth medium containing 2 times the normal serum and antibiotics concentration was added. (2x normal growth medium).**

k. **Cells were incubated for an additional 18-24 hours under conditions normally used to culture the cells.**

(NOTE: For RT-PCR analysis isolate RNA using the method described by P. Chomczynski and N. Sacchi (20) or a commercially available RNA isolation kit.)

7.4.8.2 Reaction procedure and specifications

7.4.8.2.1 Selection of primers

The NCBI (National Center for Biotechnology Information) primer design tool has been referred for primer collection **Table 7-3**. Custom synthesized forward primer and reverse primer for ABCB1 gene cDNA amplification was purchased from Eurofins research, India.

Table 7-3 Details of primers

Primer	Sequence (5'->3')	Template strand	Molecular weight (D)	Length (base pair)	Tm (° C)	GC%
	ABCB1 shRNA pDNA					
Forward primer	5′-ACCAGGCTCGCCAATGA-3′	Plus	5164.4	17	57.25	59.00
Reverse primer	5′-CGGGTGGGATAGTTGAA-3′	Minus	5330.5	17	57.25	53.00
	GAPDH primers					
Forward primer	ATCCCATCACCATCTTCCAGG	Plus	6347.83	21	59.8	52.4
Reverse primer	CAAATGAGCCCCAGCCTTCT	Minus	6537.94	21	60.9	55.0

7.4.8.2.2 Total RNA isolation

The study of gene expression also relies on the consistency of isolated RNA, so the primary criterion for gene quantification is isolation of intact complete RNA. An absolute quantification which normalizes the expression of specific mRNA against total RNA (g/g of total RNA). During the process of tissue sampling, RNA purification and RNA preservation, the long strain of mRNA is vulnerable to degradation by RNase. There are other RNases that are present in the environment, aside from the cellular RNase. DNA can contaminate the RNA samples and even small amounts in PCR may be amplified. Therefore, for RNA extraction, a correctly optimized laboratory technique was used: 1 mL of TRIzol reagent (1 mL/10cm^2) was added to each well and allowed to incubate for 5 min at room temperature.

- A sample was shifted to 2 ml Eppendorf (DEPC treated, RNase free). 200µl of chloroform was then added and mixed vigorously for 15 seconds and incubated for 2-3 minutes at room temperature.
- The system was pre-maintained and specimens were centrifuged at 12000g at 2-8^{0}C for 15 minutes.
- Then 50 % of the aqueous phase was transferred to fresh tubing, above the reasonably visible interphase. The aqueous phase comprises both RNA and DNA, but RNA remains at the tip of the aqueous phase due to smaller fragments.
- 0.5 mL of isopropyl alcohol was added to the aqueous phase and incubated for 10 minutes at room temperature. In addition, the sample was incubated at -20^{o}C for RNA precipitation.

- To extract the RNA pellet, the sample was centrifuged at 12000g for 10min at 2-8^{0}C.
- The supernatant was separated and 10 % ethanol was applied to wash the pellet by mixing again with vortex and centrifuged at 7500g at 2-8^{0}C for 5min.
- The supernatant was separated and semi-air drying of the pellet was facilitated.
- The washed pellet was dissolved by incubation at 55-60^{0}C for 10 minutes in 50µl DEPC treated water.
- 2 ul of sample was analysed for RNA on 1.2 % agarose gel with loading dye.
- The RNA concentration was tested using nanodrop by OD (1 O.D = 33 µg/ml) and the purity was estimated from the A260/A280 ratio, which was between 1.8-2.1.

7.4.8.2.3 RNA to cDNA conversion

The RNAs are incredibly unstable and vulnerable, and the RNases are susceptible to degradation. Therefore, RNA is translated to cDNA to store RNA information in a stable manner as DNA is stable compared to RNA. RNA-dependent DNA polymerase, also known as reverse transcriptase, is the catalyst for RNA-to-DNA conversion. It can generate cDNA by using RNA as a template. A primer with a free 3′-hydroxyl group is also desired. A poly-A tail is found in eukaryotic mRNAs at their 3' ends, so a poly-T oligonucleotide can be used as a primer. The primer is recycled to the 3'-end of the mRNA during conversion. The 3' end of the primer is expanded by an RNA-DNA hybrid molecule producing the reverse transcriptase. Lastly, the RNA strand of this RNA-DNA hybrid molecule is digested using RNase H or alkaline hydrolysis. For cDNA synthesis, the following step wise protocol was employed:

- The high-capacity RNA-to-cDNA Conversion Kit (ReadyScript™ cDNA Synthesis Mix) was used to convert RNA to cDNA.
- Kit components were removed and allowed to thaw on ice from their storage conditions.
- For conversion, 1.5 microgram RNA /20 µL reaction was used.
- RNA to cDNA conversion parameters are given in Table 7-4

Table 7-4 RNA to cDNA conversion parameters

Component	Volume/Reaction
Sample	9
2✕ RT (reverse transcription) Buffer	10
20✕RT (reverse transcription) Enzyme	1

- For real time PCR reaction, 20 µL of RT (reverse transcription) reaction mix was applied to each well of 48 well plates.

- The plate was sealed and centrifuged with a sealer to spin the contents down and eliminate air bubbles.
- The plate was kept in the PCR device sample holder and the cycle was carried out as per Table 7-5:

Table 7-5 Steps of PCR cycle

Parameters	Step 1	Step 2	Step 3
Temperature (°C)	45	95	4
Time (min)	30	10	Storage

7.1.1.1.1 Real Time PCR

Once the cDNA was received from mRNA. Gene expression quantification was accomplished with SYBR green-based detection on RT-PCR and gene knock-down was accessed with respect to the monitor. The reaction was configured as per the composition shown in Table 7-6.

Table 7-6 Reaction parameters for mRNA quantification

Component	Volume/ Reaction (µL)
Forward primer	0.7
Reverse primer	0.7
cDNA	1.5
Master Mix	7.5
Nuclease-free H_2O	q.s. to 15

For real time PCR reaction, 15 µL of reverse transcription reaction mix was applied to each well of 48 well plates. The plate was coated and centrifuged with sealer film to settle the contents and eliminate any air bubbles. The plate was kept in the RT-PCR system sample holder and the cycle was executed as mentioned in Table 7-7:

Table 7-7 RT-PCR cycle steps

Parameters	Step 1	Step 2	No. of cycles
Temperature (°C)	95	60	45
Time (seconds)	15	60	

7.4.8.2.4 Brief procedure

Quantification of specific mRNA knockdown with transfection of specific ABCB1 shRNA plasmid was attained by real-time polymerase chain reaction technique (RT-PCR).

Initially, DR-A549 cells were incorporated in 24 well-plates with a cell density of 5000 cells per well for 24 h. The optimized formulation of D-sh-PLHNCs were added in different concentrations of ABCB1 shRNA plasmid at a concentration of 20 nM, 40 nM, 60 nM, and 100 nM. The untreated cells were considered as the positive control to quantify gene expression. Lipofectamine 2000 treated cells were used to determine 100 % transfection of shRNA plasmid. After 48 h, TRIzol reagent was used for the isolation of the RNA from the cells. After that, RNA was treated with DNase enzyme to substrate the residues of genomic DNA. cDNA was synthesized from RNA (5 µg) using reaction mix 5x (ReadyScript™ cDNA Synthesis Mix), reverse transcriptase enzyme, and water (nuclease-free) and incubated 20 min at 46 °C. The further reaction was incubated for 1 min at 95°C with a final volume of 20 µL. qRT-PCR (Quantitative RT-PCR) was performed and the level of expression of each RNA sample was compared with the control. The sample was analyzed by Step One real-time PCR (Applied Biosciences, USA) using 10 µL SYBER green master mix (Applied Biosciences, USA) and 1 µL cDNA with nuclease-free water to make a final reaction volume of 20 µL. In qRT-PCR, denaturation was performed at 95 °C for 30 s following 40 cycles of 30 s at 95°C, followed by 5 s at 65 °C with a final annealing temperature at 60 °C. PCR reaction product was measured at real-time with increasing level of fluorescence due to SYBR Green I binding.

7.5 RESULTS AND DISCUSSION

7.5.1 Functional characterization of developed Docetaxel resistant A549 cell line.

MTT assay was performed to characterize the acquired Docetaxel resistance by the A549 cells. From Figure 7-2, it can be said that a higher number of cells remained viable in DR-A549 compared to parent cells after 24 hrs. As per the IC_{50} values, the DR-A549 had IC_{50} of 1.066 µM when treated with Docetaxel solution in different concentration while parent A549 cells possessed IC_{50} of 0.0504 µM. Hence, the drug resistance of the DR-A549 was almost 100 times higher than the parent cell line after calculating the ratio. The increased IC_{50} values of DR-A549 suggested that parent cell lines have acquired the Docetaxel resistance. Hence, developed cell lines can be used for further studies.

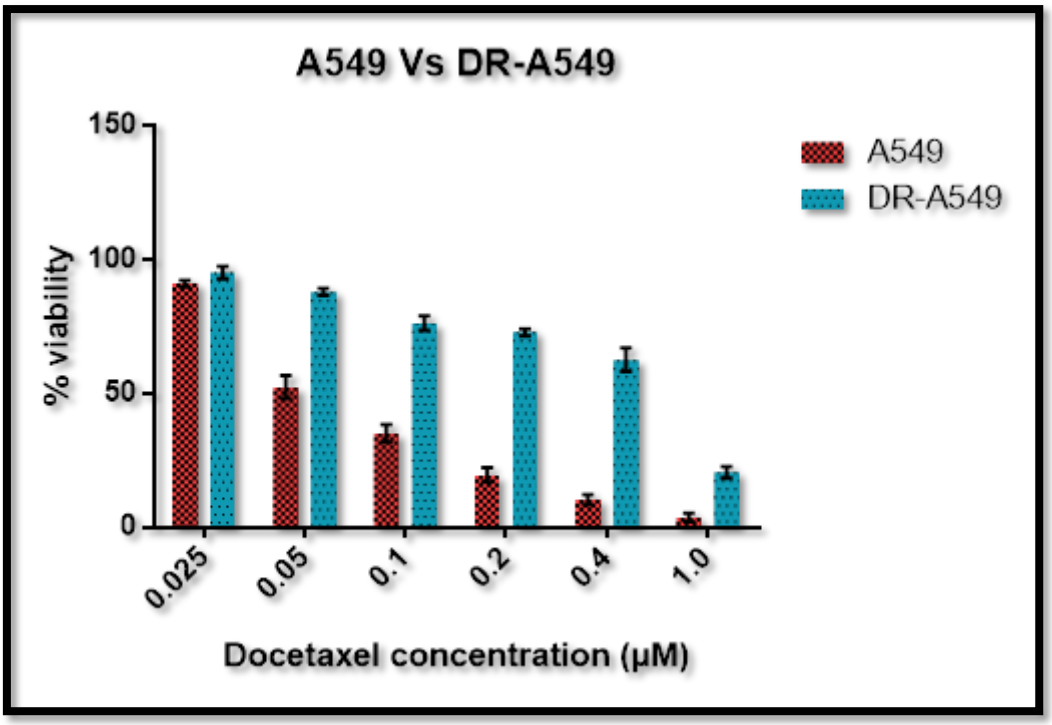

Figure 7-2 Change in % viability of the parent A549 cells and DR-A549 cells on treatment with Docetaxel solution.

7.5.2 Cytotoxicity assay of various PLHNCs formulations

The results of the cytotoxic studies are shown in Figure 7-3 and IC_{50} of the DR-A549 with various formulations is depicted in Table 7-8, whereas the cytotoxicity of the placebo PLHNCs and PLHNC-DPI can be seen in Figure 7-3A, which shows more than 90 % viability, implying that carriers and excipients for the dry powder for the inhalation are non-toxic in nature. Similarly, cytotoxicity of the different formulations of PLHNCs were demonstrated in IC_{50} at 24, 48, and 72 h. After 24 h, there was a non-significant improvement in IC_{50} of D-PLHNCs and sh-D-PLHNCs due to sustained release of Docetaxel from the PLHNCs. The fold change in the IC_{50} of the Docetaxel solution and D-PLHNCs were found to be 2.00, 1.96, 1.84 at the period of 24, 48, and 72 h, respectively. The most significant effect on cell cytotoxicity was observed in sh-D-PLHNCs (Redispersed formulation) and Docetaxel solution, where fold change in IC_{50} was 2.68, 2.84, and 2.64 at 24, 48, and 72 h, respectively. The increased cytotoxicity of sh-D-PLHNCs showed the efficiency of ABCB1 shRNA in the inhibition of the P-gp efflux pump, which accounts for the increased Docetaxel concentration in the cells. Thus, the combinatorial approach for simultaneous delivery of the ABCB1 shRNA plasmid along with Docetaxel led to a higher cellular accumulation of Docetaxel. It can be anticipated that the higher cell-cidal effect of the combinatorial approach (sh-D-PLHNCs) possibly mimicked

with in-vivo models due to selective extravasation of PLHNCs in tumor vasculature driven by EPR effect (enhanced permeability and retention effect) and passive uptake by tumor cells with a narrow distribution in normal tissues.

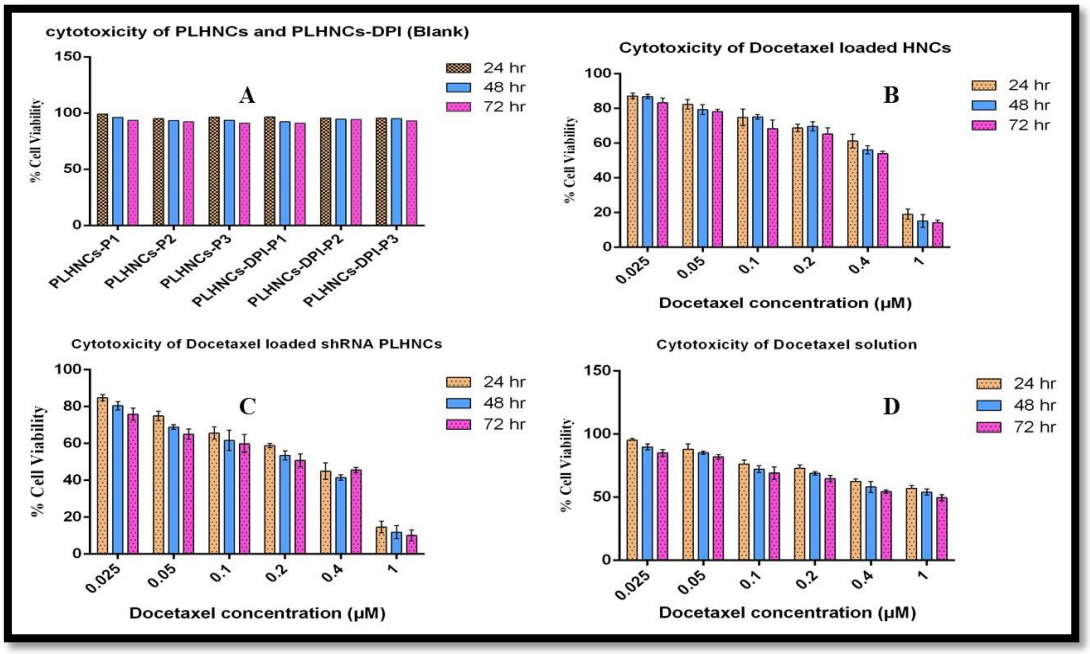

Figure 7-3 Cytotoxicity studies on DR-A549 cell line.

A) Cytotoxicity of the blank formulation. B) Cytotoxicity of the Docetaxel loaded PLHNC formulation. C) Cytotoxicity of the shRNA loaded Docetaxel PLHNCs. D) Cytotoxicity of the standard Docetaxel solution.

Table 7-8 IC_{50} (µM) of Various formulation on DR-A549 cell lines

Formulation Treatment	IC_{50} (µM) on DR-A549 cell line		
	24H	48H	72H
Docetaxel solution	1.066	0.969	0.837
D-PLHNCs	0.532	0.493	0.454
sh-D-PLHNCs	0.408	0.343	0.316

7.5.3 Characterization of cell migration (Scratch assay)

Cells of the DR-A549 were cultured and they were subjected to the described formulations for the period of 0, 24 and 48 h. From Figure 7-4, it can be observed that there was a significant reduction in the scratch area after 24 h in control cells and cells had fully interacted after a span of 48 h (i.e., no scratch was visible). A significant decrease was noticed in the D-PLHNCs and sh-D-PLHNCs formulations as the scratch area remained almost the

same even after 48 h. Again, standard Docetaxel solution showed some cell-cell interaction and migration after 24 h and 48 h which showed that cells had effluxed the Docetaxel, making the Docetaxel solution treatment ineffective over time. The results were quantified (using ImageJ software) and % migration was found as 0.63 % and 1.26 % in shRNA and Docetaxel loaded PLHNCs at 24 and 48 h, respectively. Control cell had fully grown throughout 48 h and showed 100 % migration. Docetaxel solution and D-PLHNCs showed 23.58 % and 93.68% of cell migration at 48 h, which implies that the formulation had effectively inhibited the cell migration of DR-A549 cells compared to Docetaxel solution. Hence, from Figure 7-4 and Table 7-9, it can be concluded that both D-PLHNCs and sh-D-PLHNCs have enhanced the anti-migratory effect of the Docetaxel, which could prove to be a great tool for the shrinkage of the tumors and regression of tumor metastasis.

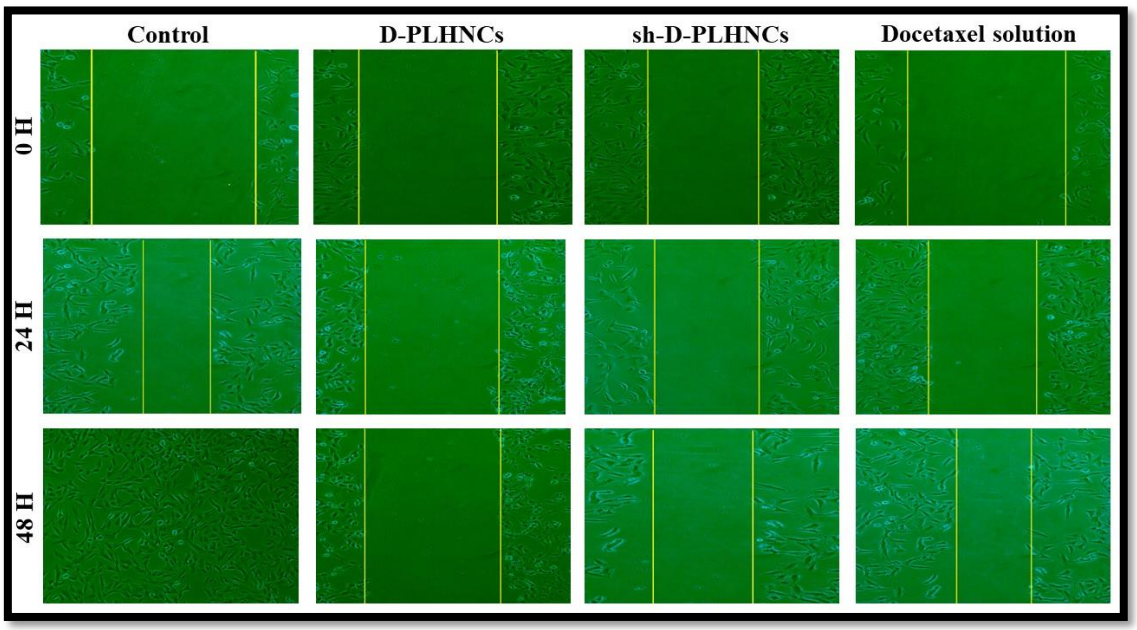

Figure 7-4 Cell migration assay

Table 7-9 Quantitative estimation of cell migration assay

Treatment	Time (hr)	Initial width (µm)	Final width (µm)	% Cell migration
Control	24	209	108	48.33
	48	209	0	100.00
D-PLHNCs	24	186	178	4.30
	48	186	168	9.68
sh-D-PLHNCs	24	159	158	0.63
	48	159	157	1.26
Docetaxel solution	24	212	184	13.21
	48	212	162	23.58

7.5.4 Qualitative uptake studies using confocal microscopy

Confocal images shown in Figure 7-5 suggests the effect of targeting on the cellular internalization and the role of formulation development in targeting tumor cells. From Figure 7-5, it can be observed that the cellular uptake of folate targeted formulation drastically increases over time compared to non-targeted PLHNCs. An increase in fluorescence with time has suggested the internalization process of PLHNCs. At any time-point, non-targeted PLHNCs have shown less fluorescence compared to the targeted ones, while in the same way naked shRNA plasmid showed less fluorescence at all time-points compared to the targeted and non-targeted PLHNCs. Images of naked shRNA plasmid uptake showed that pDNA remained outside the cell surface and produced maximum fluorescence around cells, which might be due to the negative charge of the naked shRNA plasmid that affects its intracellular penetration. PLHNCs formulation, both targeted and non-targeted, could penetrate cellular bilayer very easily and with high efficiency. Folate targeted PLHNCs had more uptake (i.e., more fluorescence inside the cell) due to an enhanced engulfing of folate receptors on the cell surface. Even fusogenic properties of the cationic lipids facilitated cellular uptake via micropinocytosis transfer and resulted in more fluorescence in both targeted and non-targeted PLHNCs compared to naked shRNA plasmid.

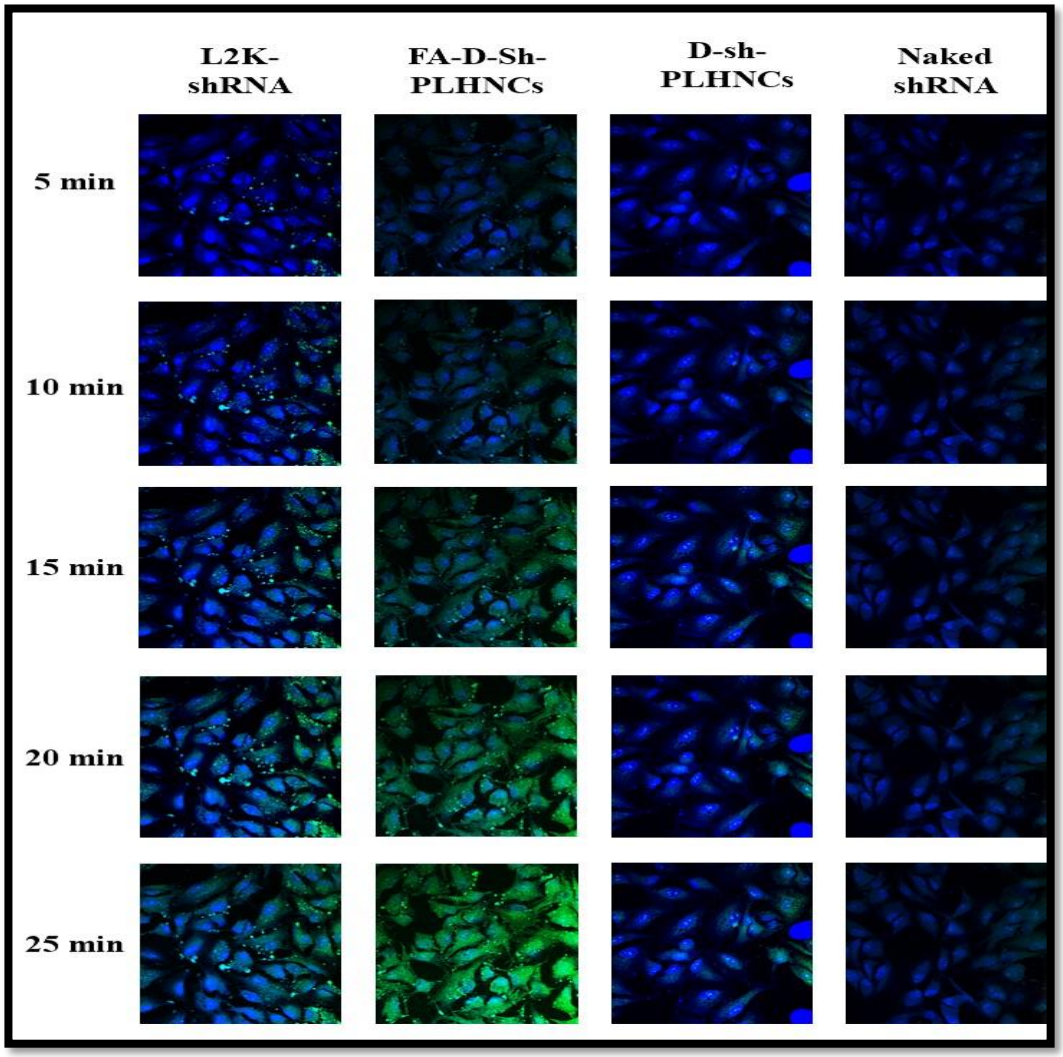

Figure 7-5 Live confocal microscopy of cellular uptake at different time intervals for naked shRNA pDNA, sh-PLHNCs, FA-sh-PLHNCs, and Lipofectamine 2000-shRNA pDNA complex.

7.5.5 Quantitative uptake studies using FACS

Fluorescence of the images was quantified using ImageJ software and results are depicted in Figure 7-6, which suggests the lowest fluorescence intensity of naked shRNA plasmid at 25 min, where highest fluorescence intensity was achieved intracellularly with lipofectamine L2K and FA-sh-PLHNCs. Cumulative quantification of the cellular uptake was studied by flow cytometry. A very low FITC fluorescence in naked shRNA plasmid indicates marginal transfection efficiency. A rightward shift of histogram (Figure 7-7) for lipofectamine and FA-sh-PLHNCs showed the highest intensity of formulation near to positive control giving proof of current concept. A remarkable hike in fluorescence intensity was noticed in targeted and non-targeted PLHNCs formulation compared to the Docetaxel solution. Even cellular uptake was increased for sh-PLHNCs with an increase in incubation time from 30 min to 60 min.

Successful transfection of sh-PLHNCs into DR-A549 cells might be due to the fusogenic nature of cationic lipid with the cell lipid bilayer.

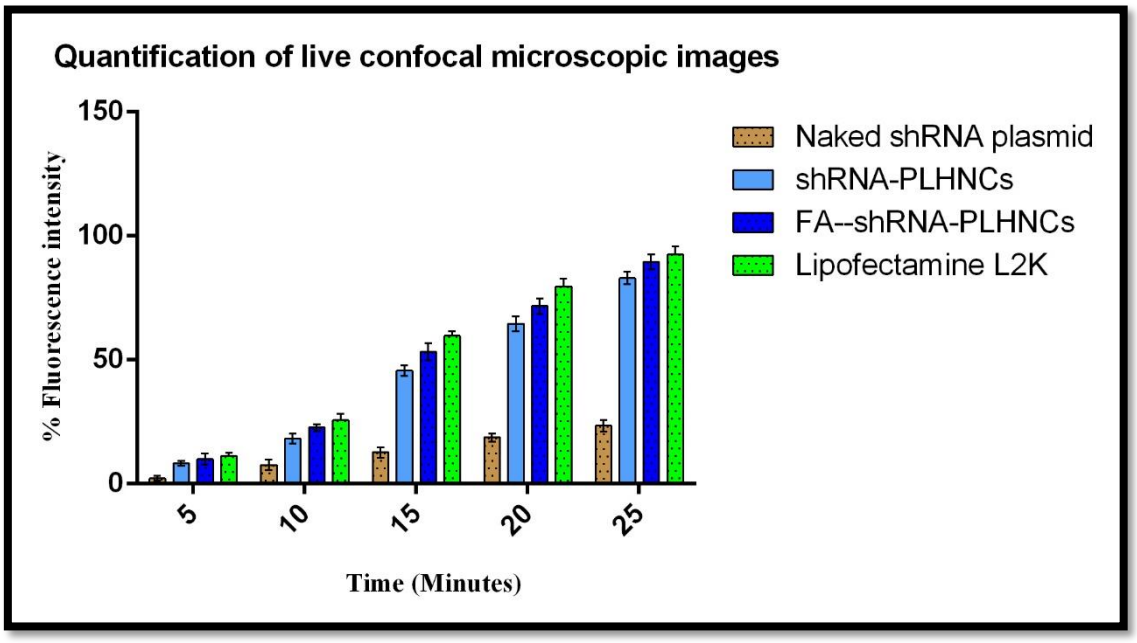

Figure 7-6 Quantification of live confocal images at the period of 5- min, 10-min, 15-min, 20-min, and 25 min interval with various treatment using ImageJ software

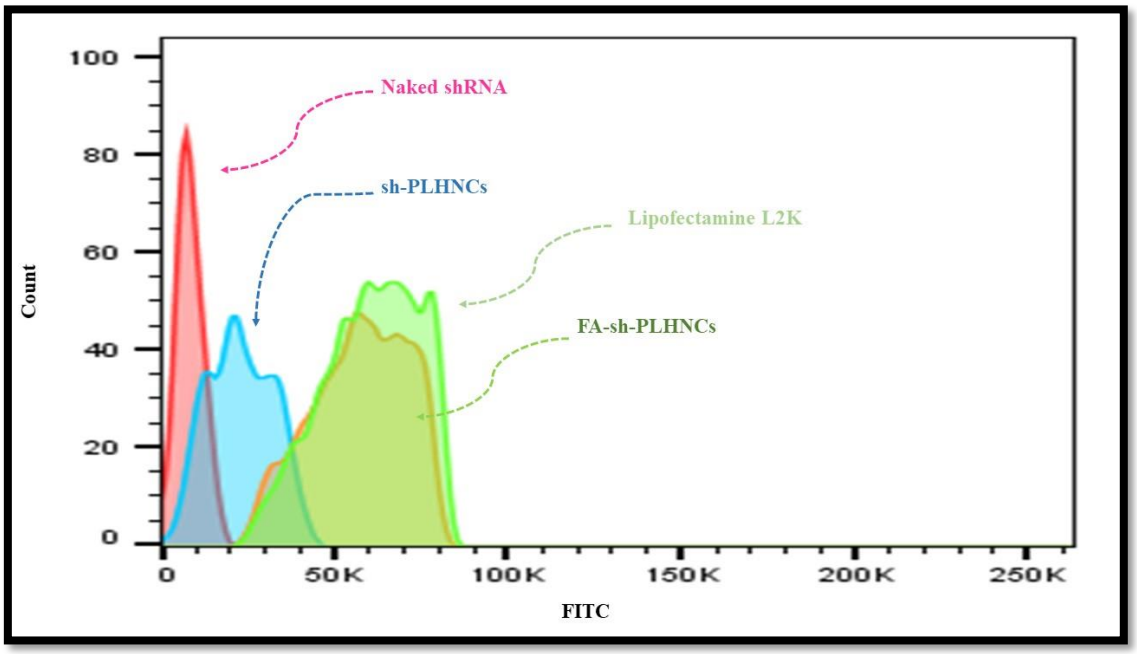

Figure 7-7 Quantitative uptake using FACS histogram

7.5.6 Effect of ABCB1 shRNA plasmid on P-gp activity

To determine the activity of shRNA plasmid on P-gp efflux pump, rhodamine exclusion assay was performed on DR-A549 cells. The confocal images are shown in Figure 7-8. Combination of verapamil with Rh123 was considered as a positive control, while Rh123 alone was selected as a negative control. Lipofectamine-2000 with ABCB1shRNA was taken as a reference control in the exclusion assay. Different levels of exclusion of the Rh123 were noted with different treatment suggesting an alteration in the P-gp activity. The highest cell accumulation of the Rh123 was observed in the verapamil treated positive control, as verapamil is well known P-gp inhibitor (Confirmed by highest fluorescence in the cells). sh-PLHNCs showed reduced Rh123 exclusion compared to naked shRNA plasmid. It was clearly visible that Rh123 efflux after treatment of 40 ng ABCB1shRNA-PLHNCs was decreased compared to 20 ng ABCB1shRNA-PLHNC treatment. However, further increase in the shRNA levels to 60 and 100 ng could not show any significant reduction in Rh123 efflux.

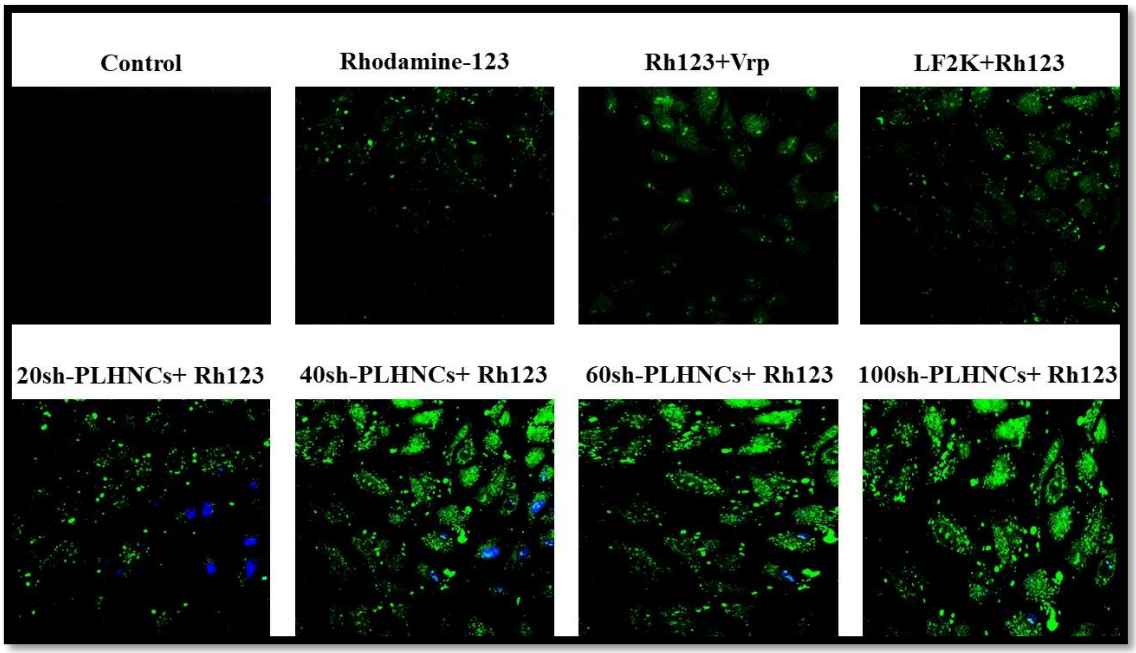

Figure 7-8 Estimation of P-gp inhibition using Rh123.

Confocal images of DR-A549 cells after incubation with Rhodamine123 dye and other formulations. Rh123 = Rhodamine123, Vrp = verapamil, LF2K+Rh123 = Lipofectamine-2000 and Rhodamine123 complexes. 20,40,60,100 ng sh-PLHNCs+Rh123= 20,40,60 and 100 ng of ABCB1shRNA plasmid Encapsulated in PLHNCs treatment along with Rhodamine123.

7.5.7 Determination of endocytic route

The result of the study revealed the unsigned agreement between confocal images and fluorescence intensity measured by a microplate reader. From Figure 7-9, it is inferred that DR-A549 cells without any inhibitors produces the highest fluorescence in both targeted as well as non-targeted PLHNCs. Formulation without Docetaxel has been chosen for endocytosis studies as Docetaxel formulation may account for cell apoptosis which can overlay the fluorescence intensity. In contrast, cell with specific inhibitors generates less fluorescence, as it inhibits the uptake of PLHNCs to the cells. For all endocytic pathways, ATP plays a significant role in cellular internalization and at 4 °C, cells undergo hibernation. Hence, cells show the least cellular internalization (21). For non-targeted PLHNCs, fluorescence intensity was declined in nystatin treated cells, whereas other inhibitors could not show any changes in fluorescence intensity. The significant reduction in fluorescence indicates that non-targeted PLHNCs could be transported through caveolae-mediated endocytosis. The cholesterol and glycosphingolipid present in the caveolae showed considerable hydrophobicity and makes a fusion with the cationic lipid of the PLHNCs. Contrary, non-significant reductions were noted with chlorpromazine hydrochloride and amiloride hydrochloride inhibitors, which suggested that targeted PLHNCs could adopt more than one pathway. The presence of a hydrophobic coat over a polymeric coat was responsible for the micropinocytosis which can be seen by reduced fluorescence of amiloride hydrochloride treated cells with FA-sh-PLHNCs formulation. Micropinocytosis has to offer a distinct advantage over other pathways for gene delivery, as it allows endosomal escape and prevents lysosomal degradation of RNA (22). However, no common physicochemical parameter explains the associated mechanism for the involved endocytic pathway/s. Taken together, the results of qualitative and quantitative endocytosis uptake suggested that nontargeted PLHNCs could utilize the specific caveolae-mediated pathway for endocytosis, while folic acid targeted PLHNCs could adopt additional pathways specifically micropinocytosis with a different mechanism. Hence, FA-sh-PLHNCs could facilitate better intracellular delivery with the adoption of different endocytic pathways.

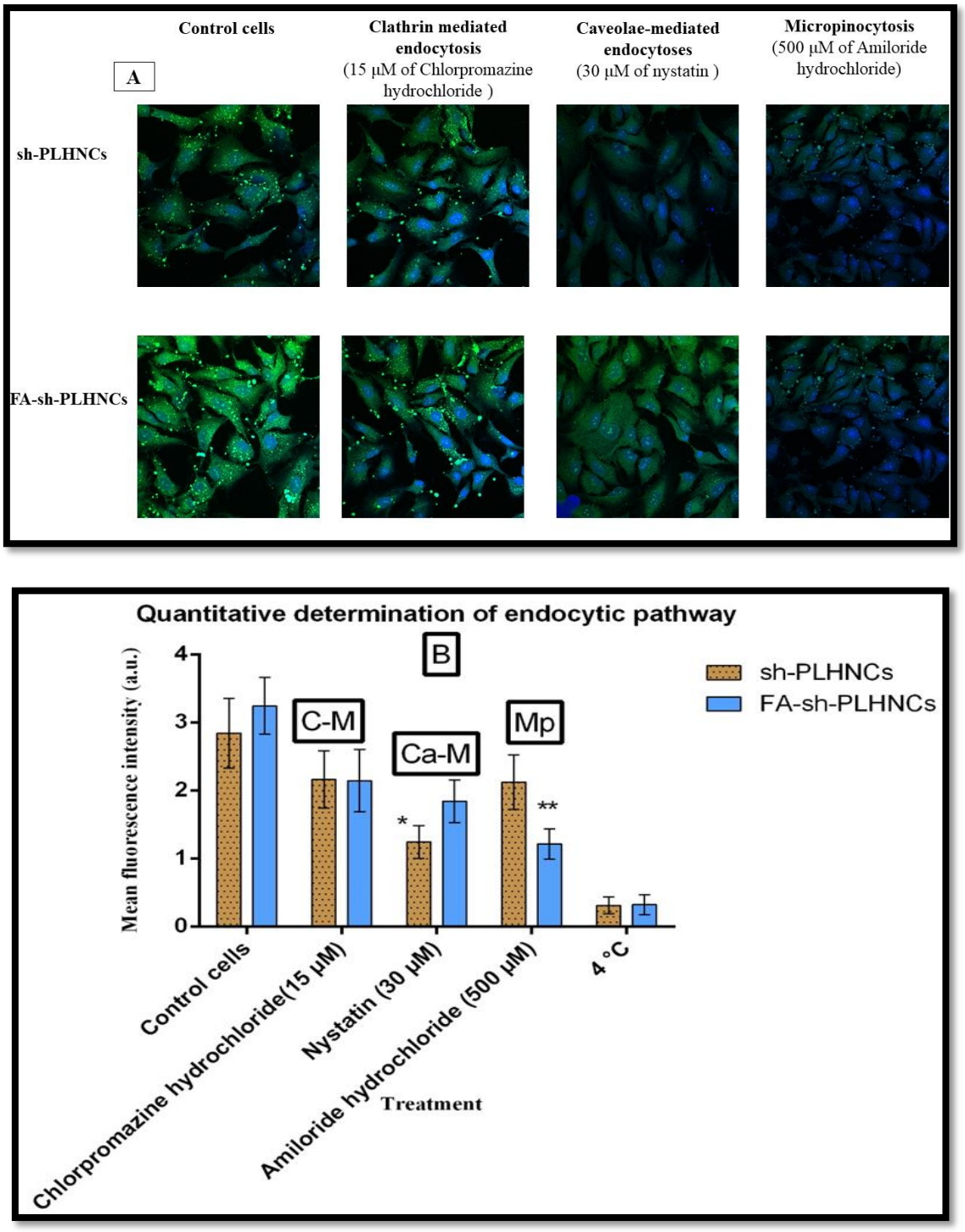

Figure 7-9 A) Qualitative measurement of endocytic uptake using Confocal images B) Quantitative measurement of fluorescence intensity using a fluorescent microplate reader

Quantitative endocytic uptake was measured via clathrin-mediated endocytosis (C-M), caveolae-mediated endocytosis (Ca-M), and micropinocytosis (Mp) with the treatment of different inhibitors. * and ** shows a significant change in intensity compare to control cells.

7.5.8 Cell apoptosis determination and cell cycle analysis

7.5.8.1 Effect of combinatorial approach caspase-3 enzyme

Caspase-3 acts as a mediator in nuclear apoptosis which includes chromatin condensation, DNA fragmentation along with cell blebbing (23). From Figure 7-10, it can be concluded that control cells with zero treatment showed almost negligible cell apoptosis, while cells with Docetaxel solution possessed some caspase-3 activity but no significant rise was noted. A significant rise in caspase activity was noted in D-sh-PLHNCs, which could show almost three-fold higher apoptotic activity compared to Docetaxel solution in DR-A549 cells. Folate targeted formulation could not show any extraordinary results on apoptotic induction. These results indicate the capability of the D-sh-PLHNCs in apoptotic induction and confirmed its anticancer activity on DR-A549 cells which was apoptosis enabled.

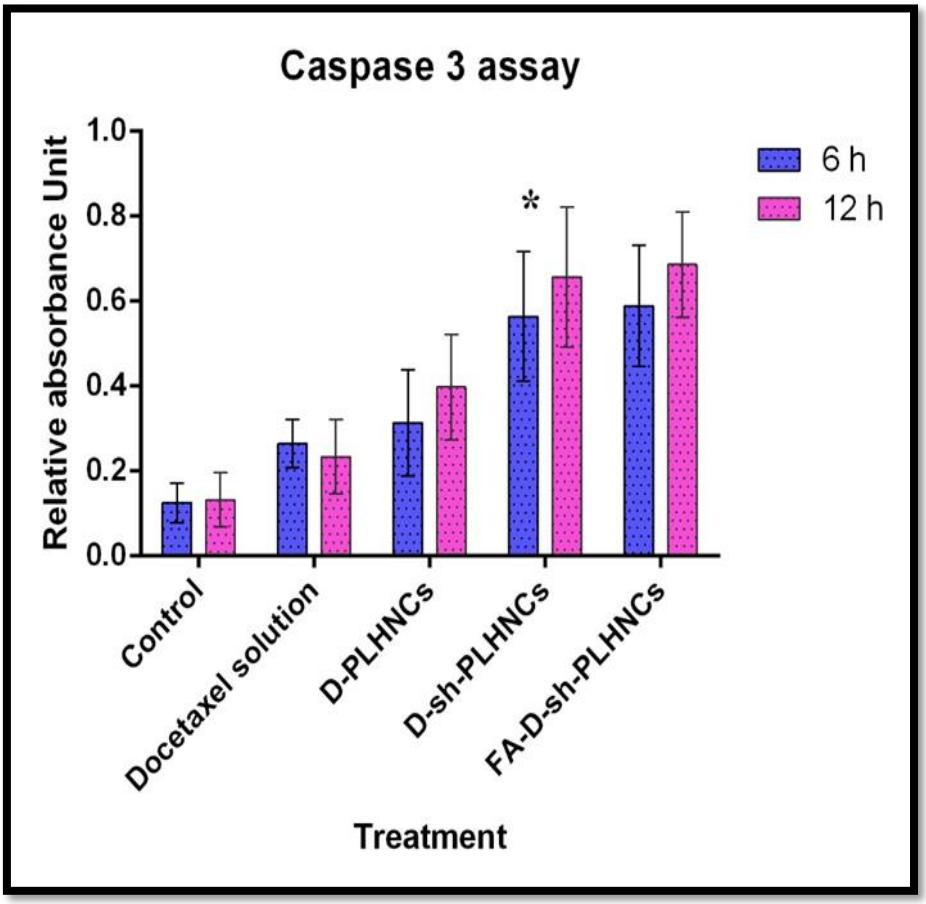

Figure 7-10 Detection of caspase-3 level in DR-A549 cell line using CASP-3-C kit, treated with different docetaxel and hybrid formulation and incubated for 6 and 12 h. *- shows significance increase in caspsase-3 concentration.

7.5.8.2 Effect of combinatorial approach on cell apoptosis

FITC- Annexin V conjugation along with Propidium Iodide (PI) was used to differentiate live cells, early apoptotic cells, and late apoptotic cells. The intact membranes of viable cells do not permit PI interaction, while dead cells permit the PI interaction. Hence, FITC-Annexin V/ PI staining would help to differentiate early apoptosis from late apoptosis in cell populations (24). As seen in the Figure 7-12, after the incubation with DR-A549 cells for 24 h, Docetaxel-shRNA loaded hybrid nanocarriers treated cells shows a shift towards the upper right quadrant in the cell chamber that indicates initiation of early as well late apoptosis. Control cells show a majority of cells in the lower left quadrant of the cell chamber which suggested a high number of live cells with negligible DNA fragmentation. An extreme upward right shift of the folate targeted formulation suggested a predominant number of cells in the early as well as late stages of apoptosis, which proves the high DNA fragmentation and condensation of chromatin due to Docetaxel inside the cells. Further, the time-dependent study (Figure 7-11) confirmed that the apoptosis phase of the cells increased gradually with the incubation time for both targeted as well as non-targeted PLHNCs. A peak in the late apoptotic cells has been observed with a longer incubation time (up to 80 h) in both the systems.

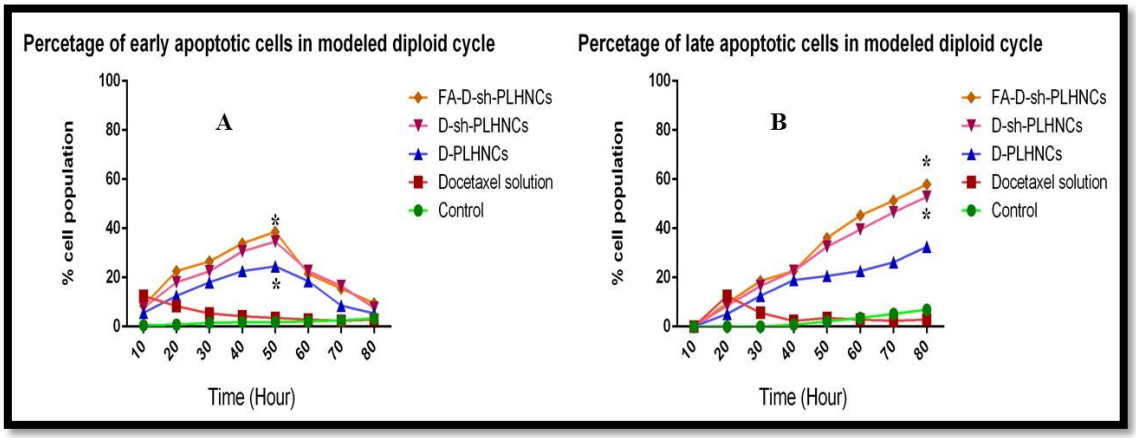

Figure 7-11 Apoptotic analysis of DR-A549 cells in a time-dependent manner with different treatment.

A. Graph % cell population in early apoptotic phase B. Graph % cell population in late apoptotic phase *- shows significant results

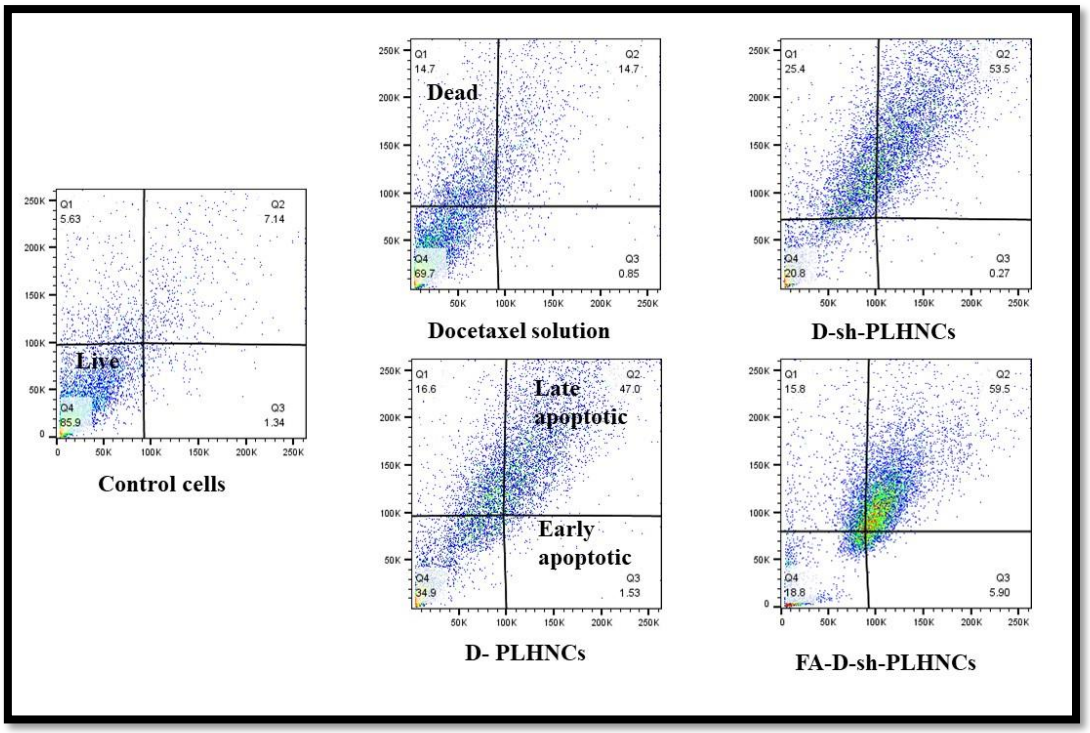

Figure 7-12 Apoptosis cell analysis of DR-A549 cell line after different Docetaxel formulation upon 24h incubation.

7.5.8.3 Determination of cell cycle arrest induced by combinatorial PLHNCs

Docetaxel is known to arrest the mitotic phase of the cell cycle by binding and stabilization of microtubules. A typical G2/M phase arrest of Docetaxel is induced by mitosis impairment and permanent damage in chromosomes (25). The previously determined IC_{50} dose of 0.532 μM dose was given with all the formulation as well as Docetaxel solution treatment. The results in the Figure 7-13 depicts that control cells were largely present in the G1 phase (Normal DNA content, 2N) while only a few populations of the cell were present in the G2/M phase (Double DNA content, 4N). Docetaxel solution treated cells could show almost negligible population of cells in G2/M, as IC_{50} of Docetaxel solution was much higher than the given dose. The gradual increment in cell population in S (DNA synthesis) +G2 stages indicated the transfer of cells after cell division from subG0 to G2/M phase with the treatment of non-targeted and targeted PLHNCs as shown in the Figure 7-14. PLHNCs could show a sustained release profile for Docetaxel. Hence, apoptosis occurred at lower doses and after some time, it might account for high cell deaths. FA-conjugated PLHNCs revealed remarkable population in the subG0-G1 phase, which indicated the apoptotic or hypodiploid cells, while an increase in G2/M phase population indicated the synergistic cytotoxic action of Docetaxel. However, in the case of targeted hybrid PLHNCs, highest G2/M phase arrest was achieved as

there was more amount of drug available at the site of action as compared to non-targeted PLHNCs.

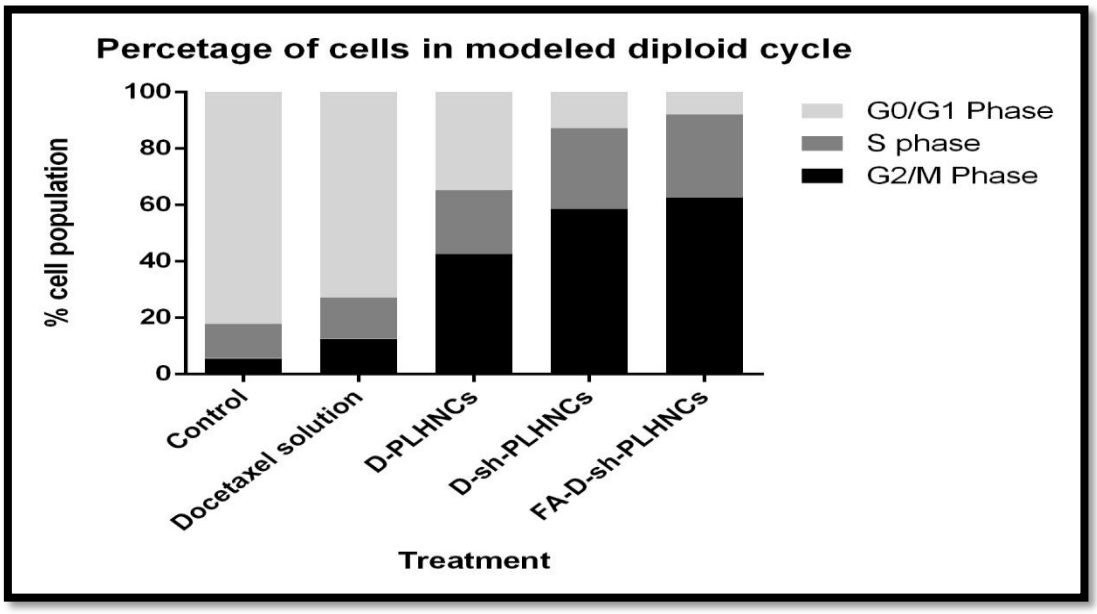

Figure 7-13 Distribution of cell cycle in DR-A549 cells after 24 h incubation.

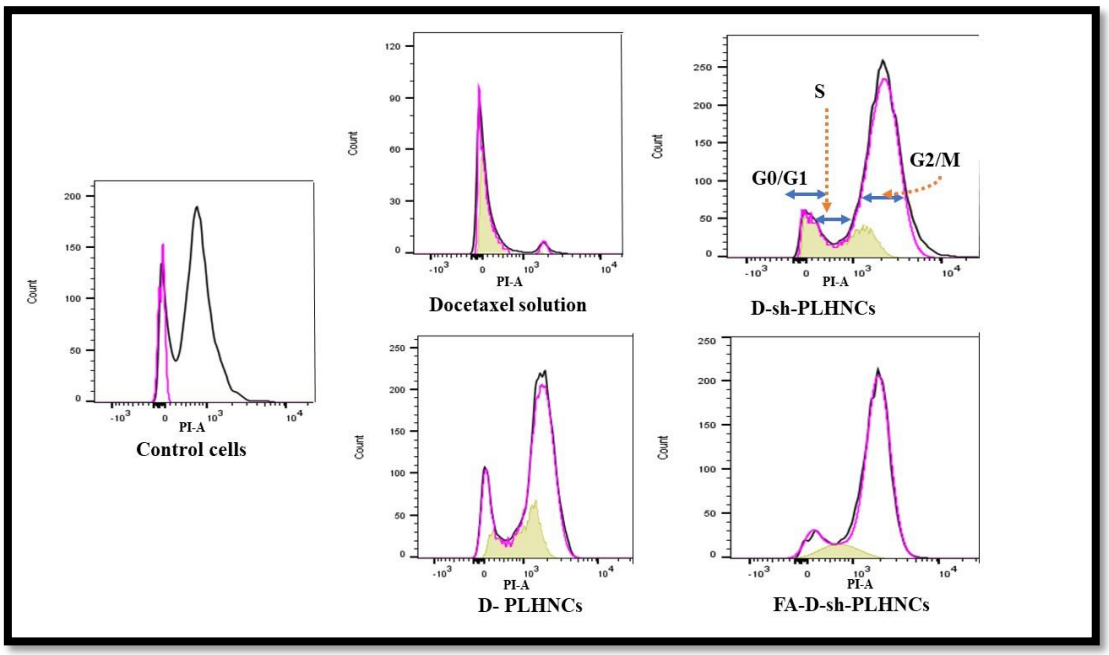

Figure 7-14 Cell cycle histogram after 24 h incubation with different Docetaxel formulation.

7.5.9 Inhibition of MDR1 mRNA levels by ABCB1 shRNA plasmid

After the reverse transcription of the cell RNA into cDNA, expression of the MDR1 mRNA was quantified using RT-PCR. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was utilized as a housekeeping gene which can be used as negative control plasmid and

did not possess any silencing activity on MDR1 mRNA (26). Results are shown in Figure 7-15, where it was discerned that increasing dose of shRNA pDNA from 20 ng to 40 ng, level of mRNA in cells was reduced almost ~20 % and as compared to control cells, it was significantly reduced almost ~ 40%. Surprisingly, no significance in expression was noticed when doses ranged from 40 ng to 100 ng. Hence, it can be considered that inhibition of MDR1 mRNA expression was found effective in the range of 40-60 ng and further increment in dose could not show any significant changes in mRNA expression level.

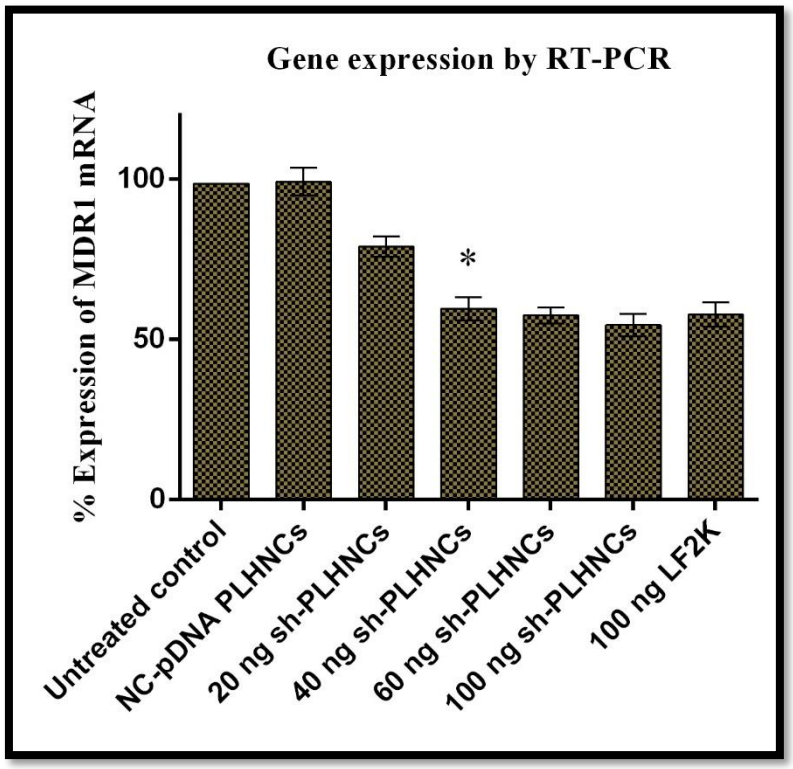

Figure 7-15 Impact on MDR1 mRNA upon treatment with various concentrations of ABCB1 shRNA in DR-A549 cells.

(The bar graph depicts the % expression of MDR1 as compared to untreated control cells. The results are shown in mean where n=3. NC-pDNA-PLHNCs possess 100 ng of negative control pDNA while LF2K100 shows expression by ABCB1 shRNA-Lipofectamine-2000 complex (100 ng). 20, 40, 60 and 100 ng sh-PLHNCs possesses respective ng of ABCB1 shRNA plasmid. *- shows significant reduction in mRNA expression.)

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