7.IN VITRO CELL LINES STUDIES

7.1 INTRODUCTION

In vitro cell line studies are essential as they offer the methods for primary evaluation of direct effects of drugs and formulations on cells and tissues so as to form a basis for *in vivo* animal studies and clinical studies. These studies are important which can give ideas on clinical applicability in pathological conditions and understand molecular mechanisms as well as can screen the test samples for their efficacy and toxicity (1-3).

Mainly, culturing of cells is a technique in which a tissue or outgrowth of a primary explant is isolated, either mechanically or enzymatically and dispersed in culture medium to prepare a cell suspension. This cells suspension may form a solid substrate or an adherent monolayer or a cell suspension in culture medium. The cells undergo proliferation in cultures forming a monolayer or suspension, which constitute a passage. After a number of generations cells can transform into continuous cells having capability of high growth and uniformity of population. Adherent monolayer cell culture is widely used for *in vitro* experiments and the cells of this culture are generally anchorage dependent as for proliferation they require a substrate for attachment of cells. On the other side suspension cultures are rarely explored in experiments and are anchorage independent and can proliferate without attachment.

Primary cell cultures are sometimes not used for experimental studies due to their poor stability as they undergo constant adaptive alterations and it is challenging to select a period of when the total cell population is homogenous or stable. After confluence some cells may transform and become insensitive to contact inhibition and overgrow, therefore it is necessary to keep the cell density low to maintain the original phenotype. After first subculture or a passage, the culture is called cell line. In each subsequent subculture a population of cell having capacity to rapidly grow will predominate while slow growing cells dilute out. In most cases culture becomes stable after three passages (4,5).

The propagation and growth of cell line requires a culture media with distinct chemical composition to confirm consistent quality and reproducibility. Mostly all the cells grow efficiently well at pH 7.4 and in 5% CO_2 environment as CO_2 gas phase

after dissolution into culture medium can establish an equilibrium with HCO_3^{--} ions present in the medium to maintain the pH. Besides HCO_3^- other ingredients such as pyruvate, high concentration of amino acids are used as buffering agent in culture media. The cells also need oxygen;thus, the depth of static culture should be kept within the range of 2-5 mm so as to maintain the rate of oxygen diffusion to the cells. The requirements of temperature rely on body temperature of animal from which cells were obtained and thus kept at 37°C.

7.2 MATERIALS AND INSTRUMENTS

Table 7-1 List of Materials

Sr No	Chemicals/Materials	Source/Manufacturer
1.	A549 cell line	NCCS, Pune, India.
2.	Thiazolyl Blue Tetrazolium Bromide (MTT)	HiMedia, Mumbai, India
3.	Dulbecco's Modified Eagle Medium (DMEM) (high glucose)	HiMedia, Mumbai, India
4.	Fetal Bovine Serum (FBS)	HiMedia, Mumbai, India
5.	Penicillin/streptomycin antibiotic	HiMedia, Mumbai, India
6.	Trypsin EDTA	HiMedia, Mumbai, India
7.	RNAse	HiMedia, Mumbai, India
8.	Trypan blue	HiMedia, Mumbai, India
9.	DAPI	HiMedia, Mumbai, India
10.	Dulbecco's Modified Eagle Medium	ATCC, USA
11.	Fluorescein isothiocyanate (FITC)	HiMedia, Mumbai, 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

SrNo	Instruments	Company
6.	Confocal laser scanning microscope	CarlZeiss LSM 710, Germany
7.	Jouan IGO150 CELL life CO2	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

7.3 METHODS

General Methods and Preparations

Preparation of complete media

To prepare complete media, Dulbecco's Modified Eagle's Medium (DMEM) (incomplete medium) was first filtered through 0.2 µ membrane filter. Then, 1% v/v Antibiotic solution (Penicillin/streptomycin) and 10% v/v FBS (fetal bovine serum) were added in a filtered media. The procedure was carried out in vertical laminar air flow cabinet (Weiber Vertical Laminar Air Flow, India).

Preparation of PBS (Phosphate Buffer Saline) pH 7.4

8 gm of sodium chloride, 200 mg of potassium chloride, 1.44 gm 240 mg di-sodium hydrogen phosphate, potassium dihydrogen phosphate was added in 1 litre of distilled water and pH was checked. The buffer was autoclaved lastly.

Preparation of FACS buffer

0.5% w/v Bovine serum albumin and 0.5% v/v FBS were added in sterilized PBS pH 7.4 to prepare FACS buffer.

Sub-culturing of cells

The A549 cells and H1299 cells were grown and maintained in DMEM (Himedia). The medium of all the cell lines was supplemented with 10% fetal bovine serum (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. The cells were maintained as monolayer culture in T-75 cell culture flasks, and sub-cultured twice every week by taking 10^4 cells in T-75 flasks (6,7).

Following procedure was followed for the sub-culturing;

- The culture flask was taken to the sterile area in laminar air flow unit and culture media was removed.
- The cells were washed one time with sterile phosphate buffer saline pH 7.4 (PBS) to eliminate the traces of serum present in the media which may hinder the action of trypsin.
- 2 mL of Trypsin-EDTA solution was added to flask and shaken gently to allow the detachment of the cells from each other and from surface. Then Trypsin-EDTA was removed to get residual film of cells and cells were kept in incubator for 2-3 minutes for rounding up.
- Cells were observed under the inverted microscope until cell layer was detached (usually within 5 minutes).
- Complete medium (2 ml) was added to disperse the cells, dispersion was made with pipetting as continuous cell line requires vigorous pipetting for complete disaggregation.
- Cells count was performed on haemocytometer.
- Then appropriate seeding concentration was added to the flask and 10 mL of complete medium was added to it. Passage number was marked on the T-75 culture flask.
- The flask was closed and cells were incubated at 37°C, 5% CO_2.

7.3.1 Cytotoxicity studies by MTT assay

Cytotoxicity studies are carried out for the delivery systems developed for delivery of nucleic acids. These studies establish their cytotoxic potential upon administration in the body and hence depend on the route of administration. Additionally, the toxicity studies need to be done on the cells that are the targets for the delivery system. These in vitro cytotoxicity studies can be carried out using MTT/MTS/XTT/HDL assays which work based on the working enzymatic machinery of living cells.

In vitro toxicity testing is the scientific analysis of the effects of toxic chemical substances on cultured bacteria or mammalian cells. *In vitro* testing methods are used either to check potentially toxic effects of chemicals or to confirm the lack of toxic properties of chemicals, which might be useful in the development of new therapeutic agents or their delivery systems, agricultural chemicals, or food additives (8-10)

Different methods used for cell counting and cytotoxicity assays along with the dyes or probes used, the detection method, sensitivity, and features (5). Of all methods the most popular ones are the MTT and XTT dye (tetrazole dyes) based assay methods due to their ease, sensitivity and scalability.

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 coloured formazan compound that gives a direct estimate of number of viable cells when measured spectrophotometrically (Fig 8.1).

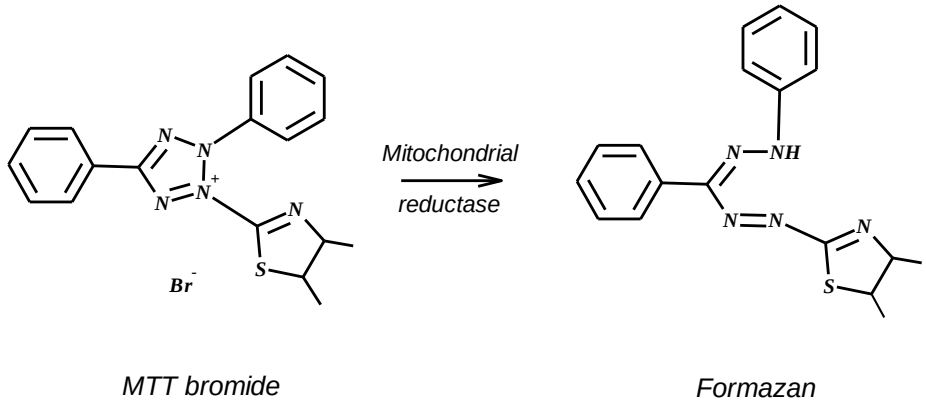

Figure 7-1 MTT Dye Reduction by Mitochondrial Reductase Enzyme of Viable Cells

Formazan formed so 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.

Method

The MTT assay was performed to assess cell cytotoxic potential of siRNA-HNCs complexes in lung cancer cell line A549 by measuring IC50 values. The nanocarriers wascomplexed with siRNA at various N/P ratio (i.e. 2.5, 5, 7.5 and 10) and cells were

treated with 200µL of the prepared formulation. Cells treated with 200 µL of fresh incomplete medium (DMEM) were used as negative control (100% viability will be assumed from the absorbance of wells containing these cells) while those treated with carrier 0.5% Triton X100 were kept as a positive control.

a) Media Preparation

Complete media was prepared by mixing Dulbecco's modified Eagle's medium (DMEM) filtered through sterile 0.2 µ membrane filter with antibiotic solution (1% v/v) and 10% v/v fetal bovine serum (FBS). Media was stored in a sterile screw capped bottle. The bottle was then sealed with parafilm and wrapped with aluminum foil. The whole process carried out in vertical laminar air flow cabinet (Weiber Vertical Laminar Air Flow, India).

b) Sub-culturing of cell line

A549 cell line was obtained in NCCS, Pune. The cells were maintained as monolayer culture in T-75 cell culture flasks, and sub-cultured twice every week by taking 10^4 cells in T-75 flasks. Cell lines obtained from NCCS, Pune were sub-cultured at 37°C in a humidified atmosphere at 95% air and 5% CO_2(Jouan IGO150 CELLlife CO2 Incubator, Thermo Fisher Scientific, India) incomplete media. Fresh complete medium was replaced every 3 days.

Following procedure was followed for the sub-culturing.

- Culture medium was removed from the Tissue culture T-75flask containing cells.
- 2 mL of Trypsin-EDTA solution was added to flask and shaken gently to allow the detachment of the cells from each other. Then Trypsin-EDTA was removed to get residual film of cells and cells were kept in incubator for 2-3 minutes for rounding up.
- Cells were observed under the inverted microscope until cell layer was detached (usually within 5 minutes).
- 10 mL of complete growth medium was added to flask and cells were aspirated gently by pipette. Passage number was marked on the T-75 culture flask.
- Subcultures were incubated at 37°C with 5% CO2 level.

c) Cell Counting Using Haemocytometer

Preparing Haemocytometer:

- Haemocytometer was cleaned properly using 70% ethanol.

- The shoulders of the haemocytometer were moistened and the coverslip was affixed firmly using gentle pressure and small circular motions.

Preparing Cell Suspension:

- The cell suspension to be counted was mixed properly by gentle agitation of the flask containing the cells.
- Before the cells started settling down about 1 mL of cell suspension was sampled using a serological pipette and placed in microcentrifuge tube.
- Using a 100 µl pipette, cells in this sample were mixed again (gently to avoid cell lysis). And then 100 µL was taken out and placed into a new microcentrifuge tube which was then treated with 100 µl trypan blue and mixed with it gently.

Counting:

- Using the micro pipette, some cell suspension containing trypan blue was drawn out and carefully filled in the haemocytometer by gently resting the end of the tip at the edge of the chamber taking care to avoid overfilling of chamber.
- The grid lines of the haemocytometer were focused using the 10X objective of the microscope. One set of 16 corner squares as indicated by the circle in the Fig 8.2.
- 16 squares were focused at a time.

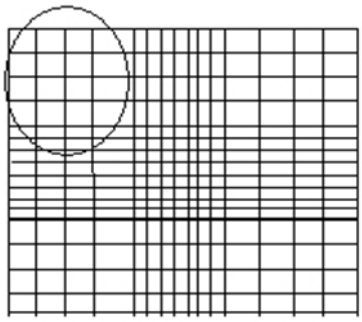

Figure 7-2Haemocytometer diagram indicating the 16 corners squares which should be used for counting

- Using a hand tally counter, the number of cells in this area of 16 squares was counted. When counting, only live cells that look unstained by trypan blue were counted. Cells that are within the square and any positioned on the right hand or bottom boundary line were counted.
- Counting of cells was continued for all other remaining set of 16 corner squares.
- The haemocytometer is designed so that the number of cells in one set of 16 corner squares is equivalent to the number of cells x 10^4 / mL.

- Calculation of the average no. of cells in 4 sets of 16 corners is as follows:The total count from 4 sets of 16 corners = (Average no. of cells/mL) x 10^4 x 2, where 10^4 is conversion factor (Conversion of 0.1 mm^3 to mL) and 2 is dilution factor.

d) Preparation of formulation for treatment

Stock solutions of formulations were sterilized by filtering through 0.2 µ membrane filter. All the dilutions and filtration were carried out in Laminar Air Flow Hood and all the materials and equipment used were sterilized appropriately before use. MTT studies were carried out with the formulation mentioned previously.

e) MTT Assay Protocol

Plating out cells:

- Sub-confluent monolayer culture in one T-25 flask was trypsinized and 5 mL of complete growth medium was added. The medium was removed to remove trypsin and again 5 mL of complete growth medium containing serum was replenished. Cells were aspirated gently with pipette. Tips of pipette were discarded after single use.
- Cells were counted and diluted to 25 $\times 10^3$ cells/mL
- Then cell suspension was transferred to 96-well plates (except for the column 1 which is to be used as a control) with a multichannel pipette to produce cell concentration of 5 × 10^3 cells per well. Then 200 µL of complete medium was added in each well with a multichannel pipette. Lid was placed over the plate. 3 plates were prepared similarly for each study period of 24 hr, 48 hr and 72 hr.
- Cells were incubated in an incubator at 37°C and 5% CO_2 exposure for 24 hr before exposure to the formulation.

Formulation treatment:

- Medium from the wells was removed using multichannel pipette and discarded.
- Cells were treated with formulation diluted in sterile filtered incomplete media (DMEM) as mentioned previously. Cells treated with 200 µL of fresh incomplete medium (DMEM) were used as negative control (100% viability will be assumed from the absorbance of wells containing these cells) while those treated with carrier 0.5% Triton X100 were kept as a positive control. Treatment with each formulation was performed in triplicate.

- Lid was placed on the plates and the plate was returned to incubator (37°C and 5% CO_2 exposure). The cells were incubated for 6 hr exposure period.

Growth period:

- At the end of the exposure period, medium was removed from all the wells and wells were fed with 200 µL of fresh complete medium.
- The plates were replenished with fresh complete medium after 24 hr for 48 hr viability study. For 72 hr viability study, it was done at 24 and 48 hr as per requirement of cell culture condition.

Estimation of surviving cell numbers:

- Plates were fed with 200 µL of fresh complete medium after washing with Phosphate Buffer Saline pH 7.4 at the end of the growth period (24 hr, 48 hr and 72 hr) and 100 µL of MTT solution (1 mg/mL MTT solution was filtered through 0.2 µ membrane filter and stored at 2-8°C in dark.) was added to all of the wells in plate.
- Each plate was wrapped in aluminum foil, and incubated for 4 hr at in the incubator at 37°C at 5% CO_2.
- Medium along with MTT were removed from the wells. Cell lysis and solubilization of formazan crystals was done by adding 200µL of DMSO to all of the wells.
- Absorbance was recorded at 570 nm with a reference filter of 655 nm on microplate reader (ELISA microplate reader, BioRad, USA) immediately, because the product is unstable.

Analysis of MTT assay:

Determination of the cell viability was done by using the following equation.

$$\% \text{ Viability} = \frac{\text{Mean Absorbance of Sample}}{\text{Mean Absorbance of Control}} \times 100$$

where absorbance of sample and control cells represent the amount of formazan determined for cells treated with the different formulations and for control cells (nontreated), respectively.Viability plots were plotted by plotting % viable cells (y-axis) against the concentration of cationic lipids (x-axis) and IC50 values were calculated using Graphpad prism software.

7.3.2 Cellular uptake studies

In order to perform cell uptake studies, FITC labelled siRNA (FITC-siRNA) containing formulations were used. The lyophilized stock supplied was reconstituted with NFW in amber coloured tubes and used for experiments. For quantitative measurements flow-cytometry was used while qualitative evaluation was conducted using confocal microscopy.

7.3.2.1 Qualitative uptake studies using confocal microscopy

The laser scanning confocal microscope (LSCM) is an essential component of modern-day biomedical research applications. In a conventional microscopy the entire specimen is illuminated from a mercury of xenon source. However, in confocal microscopy the illumination is achieved by scanning one or more laser beams across the specimen to create an optical section of specimen in a non-invasive manner. It uses confocal pinholes that allow light coming only from the plane of focus to reach the photomultiplier tube detector and excludes the 'out of focus' light coming to the detector. This enables imaging of the living specimens and generation of 3-dimensional data in the form of Z-stacks. It uses laser as light source, a sensitive photomultiplier tube detector and a computer to control the scanning mirrors and build images.

The optical path used in confocal microscopy is based on conventional reflected light wide-filed epi-fluorescence microscope with a point light source and a pinhole in front of detector which are confocal with each other. The specimens are labeled with one or more fluorescent probes. The confocal microscopy also offers the advantage greater resolution due to use of highly sensitive photomultiplier tube detectors. The series of time-lapse run can be converted into a 3-D image from the obtained data with time as the z-axis. This can be useful for observing physiologic changes during development. Further a 4-dimension data set can be produced consisting of three spatial dimensions X, Y, Z and time as fourth dimension. In cellular biology confocal microscopy has been used for visualizing intracellular organelles, cellular uptake, intracellular localization of drugs and drug delivery systems using fluorescent probes.

Protocol: A549 Cells were seeded at a density of 10^4 cells/well in a 24 well plate containing 0.17 mm thick flame sterilized cover glass and were allowed to grow for

24 h in DMEM at 37°C temperature and 5% CO_2. After 24 h cells the media was removed and cells were washed with sterile PBS. Then the cells were exposed to formulations containing FITC labeled negative control siRNA (FITC-siRNA) at siRNA concentration of 100 nM. Transfection step was performed by manufacturer's instruction (Santacruze biotechnology, USA) using transfection reagent, transfection medium and dilution buffer. After 6 h of exposure cells were washed with PBS for twice to ensure removal of residual formulation. Then the cells were fixed with 4% paraformaldehyde solution (1 mL/well) and incubated at room temperature for 3-5 min. The paraformaldehyde was immediately removed after exposure time and cells were washed with PBS three times accompanied by intermittent shaking for each wash to remove the traces of paraformaldehyde. Then the nuclei of the cells was stained with 4',6-diamidino-2-phenylindole (DAPI) at 1 ug/mL concentration with enough volume to cover the cells and kept for 15 min at room temperature for dye permeation under protection by aluminum foil. Then cells were washed once with PBS. The coverslips were mounted on glass slide using PBS:glycerin solution (50:50 and confocal microscopy was performed using confocal laser scanning microscope. The negative control for the experiment was Naked FITC-NC-siRNA and positive control was FITC labelled-NC-siRNA complexed Lipofectamine 2000 (L2K). Cell line treatment parameters are as per following:

Table 7-3 Cell line treatment parameters for confocal microscopy and flow cytometry of HNCs based formulations

Formulations	Cells	Treatment	Conditions
Naked siRNA	A549 lung adenocarcinoma cells	FITC labelled siRNA (100 nM)	Incubation time = 6 hr Incubation temperature = 37°C
siRNA-HNCs complexes		FITC labelled siRNA (100 nM)	
Lipofactamine-2000		FITC-NC-siRNA (100 nM)	

7.3.2.2 Quantitative uptake studies using Flow cytometry

Flow-cytometry is a powerful technique for characterizing cells in clinical diagnosis and biomedical research for quantifying aspects about their size, internal complexity

and surface markers. In a flow cytometer the suspension of cells is hydro-dynamically focused in a single cell wide stream of fluid containing a fast-moving sheath fluid around the slow-moving cell suspension emerging through a 70 µm nozzle. This is achieved by with air or gas pressure and the differential pressure between the streams controls the samples introduction rate. This laminar steam stream of particles is subsequently interrogated by one or more laser beams placed perpendicular to it and only illuminate single cell at a time. At this point the laser is scattered at the same wavelength at different directions. The light scattered in forward direction (FSC) is proportional to the size of the cells. While the light scattered in perpendicular direction (SSC) correlates with intracellular granularity or complexity. Thus, scattering itself gives information about the size and composition of the cells.

The second technique of detection relies on use of fluorescent probes attached to cells, which fluorescence after interaction with laser at interrogation point and emit light at longer wavelengths. Here, the non-fluorescent cells will be counted as negative while the fluorescent cells will be called as positive cells. Further, the intensity of emission gives information about the number of fluorescent probes. Downstream the interrogation points, the particle stream is broken into discrete droplets which can be selectively charged and deflected using an electric field into a collector and the remainder are disposed. The results of fluorescence and scatter are displayed as histogram.

Before starting the experiment appropriate controls are needed to enable interpretation of the results in the context of the purpose of experiment. At least three controls are essential in any experiment which are: set up control (instrument), specificity control (gating) and biological comparison control. 'Setup controls' are required to ensure that instrument is properly set up with respect to photomultiplier voltage gains and compensation; 'Specificity or gating controls' are used to set location of gates or graphical regions to classify the cells as required for the purpose of the experiment. A 'biological comparison control', consisting of unstained/unstimulated cells in a biologically relevant conditions, is required to set up positive/negative boundaries (11, 12)

Protocol: For FACS analysis, A549 cells were seeded in 24 well plate at cell density of 5 X 10^5 cells/well. The cells were allowed to grow for 24 h in EMEM. After 24 h

the cells were treated with formulations containing FITC-NC-siRNA at a concentration of 100 nM and kept for 6 h in incubator maintained at 37°C with 5% CO_2 in a humidified condition. During this period the cells were supposed to internalize the formulations depending on the transfection efficiency. After incubation the cells were washed three times with cold PBS pH 7.4 to remove the residual formulations and harvested using trypsin to obtain a cell suspension in PBS pH 7.4. Before analysis the cell suspension was passed through 70 μm cell strainer to disperse cell aggregates, if any and analyzed for % cell uptake using fluorescence activated cell sorter (FACS BD, USA). The positive control was FITC-NC- siRNA complexed Lipofectamine 2000 (L2K).

7.3.2.3 Uptake efficiency in folate positive and folate negative cells

Cellular uptake of folate targeted HNCs was assessed in folate over expressing H1299 lung carcinoma cells and very low or without folate expressing A549 lung adenocarcinoma cells. 1×10^5 cells/well were seeded in six well plates. The cells were transfected with FITC labelled siRNA (100 nM) anchored HNCs in serum free medium. The cells were harvested and analysed by fluorescence intensity at various time (1, 6, 12, 24 hr) points after transfection. The cellular uptake was reported quantitatively using an Envision multilabel plate reader by measuring fluorescence unit.

7.3.3 Chemosensitization studies

Although a number of chemotherapeutic treatments have been shown to be effective at inhibiting or eliminating cancer cell growth in preclinical studies, clinical applications are often limited due to the toxic side effects associated with anticancer drugs. Patients are often unable to tolerate the level of a drug needed to effectively eliminate malignant cells while levels that can be tolerated are insufficient therapeutically. As a result, chemo-resistance and subsequent tumor recurrence are often the outcome of such therapies. An example of this all too common event is the use of taxanes (paclitaxel and its semi-synthetic analogue, docetaxel) in the treatment of a variety of cancers including ovarian, breast, prostate, and non-small cell lung cancers (13,14). While surgery along with taxanes- and platinum-based chemotherapy for advanced ovarian cancer has allowed up to 80% of women to achieve a clinical response (15), cancers in most patients initially diagnosed with late stage disease

eventually recur. Development of methods to circumvent resistance may ultimately improve the impact of adjuvant therapy, resulting in prolonged disease-free intervals and survival. Novel targeted therapies that interfere with specific molecular signalling pathways affecting cancer cell survival are being developed as potential treatment options to render cancer cells more sensitive to cytotoxic chemotherapy. Targeted therapies that increase cancer cell sensitivity to chemotherapies offer the benefits of lowering unwanted side effects and increasing the likelihood of destroying resistant cells while avoiding healthy cells where there is little or no expression of the targeted entity.

Protocol:

In vitro cytotoxicity of anticancer drug cisplatin at sequential concentrations was assessed with siRNA-HNCs complexes in A549 cells. IC50 values were calculated using Graphpad prism software by performing MTT assay as described in procedure 7.3.1. Chemosensitization was confirmed by performing and comparing MTT assays of two formulations i.e. cisplatin caprylate loaded HNCs and cisplatin caprylate loaded siRNA-HNCs complexes. Aliquots of 10^6 cells were seeded in 96 well plate with suitable dilution. Lipofectamine 2000 was used as a positive control. The final concentration of ABC1 siRNA was 100 nM. Transfection step was performed by manufacturer's instruction (Santacruze biotechnology, USA) using transfection reagent, transfection medium and dilution buffer. After 24 hr seeding, Formulation treatments were given as per following; each treatment for 24 hr, 48 hr and 72 hr; cisplatin loaded HNCs, cisplatin loaded siRNA-HNCs complexes and cisplatin solution, then the culture medium was replaced with fresh DMEM supplemented with 10% FBS and antibiotics. and 20 µl of a 5 mg/ml MTT was added to detect IC 50 values.

7.3.4 Wound scratch assay method

Wound healing assay was performed as per the earlier reports (1,2). The *in vitro* wound healing assay stands out as a simple, easy, inexpensive and affordable method to evaluate cell migration and proliferation.A549 cells were grown in 6-well plates and allowed to reach 80% confluency. Wounds were created carefully using pipette tip to remove cell monolayer as a strip. The average size of the wounds was around 300µm and wound width within 5% variation was taken into account for the

study. The wounds were washed with sterile PBS twice to ensure removal of partly adhered cells on the plates due to wound. The wells were treated with serial dilution of R2 HNCs formulation in incomplete media at 20 µM, 10µM, 5 µM and 1 µM drug concentrations. For comparison purpose, another well was treated with Cisplatin solution at 10µM concentration tested against equimolar amount of R2 HNCs to determine the comparative inhibition of cell migration. Incubation was done at 37°C in CO_2 incubator (5%) for 48 hr. One well was not treated with any formulation and acted as control. After incubation period of 48 hr, the treatment was removed and the cells were washed with PBS thrice. Cells were fixed using 70% ethanol, images were taken and the wound width were measured using Nikon Eclipse TS100 inverted microscope (NIS elements imaging software). Width of the untreated group at 0 hr was considered 100% and relatively % recovery of wound was compared.

7.3.5 Apoptosis detection and cell cycle analysis

A549 cells were transfected using FITC labelled siRNA-HNCs batch R2 (100 nM siRNA concentration) as described in the cell viability assay above. The cells were collected, processed and were subjected to FACS analysis to locate cell death progression through four quadrants of dot plot histogram. Control Cells were also incubated having no treatment (medium only). Cells were trypsinized after 6 hr, 12 hr and 24 hr and collected in complete media and cells were collected by centrifugation at 2000 rpm at room temperature for 5 min and resuspended in PBS then 70 % Ethanol was used to make cells permeable for PI and cell suspension is left at 4°C overnight. Cells were washed with PBS and 10 µl of RNAse were added. 5 µl of PI (1 mg/ml stock solution) were added to this cell suspension and incubated at 37°C for 1 hr then prior to analysis through FACS.

7.3.6 Gene knockdown efficiency

RT-PCR is a powerful tool for the detection and quantification of mRNA. It is popular because of high sensitivity, good reproducibility, and wide dynamic quantification range [5]. It allows researchers to amplify specific pieces of DNA more than a billion-fold [6]. In PCR a thermostable polymerase synthesizes a complementary sequence of bases to any single strand of DNA containing a double stranded starting point. The starting points can be chosen by user corresponding to gene of interest and they are known as primers. During PCR the temperature cycling

is used to control the activity of thermostable polymerase and binding of primers. At the beginning the temperature is kept at 95^{o}C where all double stranded DNA will melt. Then temperature is reduced to ~60^{o}C, depending on primer, to allow the primer to bind the target gene. The polymerase subsequently binds the double stranded DNA and starts copying. This temperature when repeated several times leads to exponential increase in number of copies of target DNA sequence. The amplified gene can be observed at the end of process by running on agarose gel and staining it wherein the brighter bands will indicate higher copies of DNA. However, in conventional PC the gel based analysis cannot give time dependent quantity curve.

In Real Time PCR this process is monitored in real time using fluorescent probes of double stranded DNA and detecting them with a camera. The RT-PCR offers several advantages such as: a direct look into the reaction, the precise calculation of efficiency of reaction, eliminating the need to run gels and performing a truly quantitative analysis of gene expression rather than semi-quantitative as in normal PCR [7]. The RT-PCR uses asymmetric cyanine dyes e.g. SYBR green I and BEBO, which do not interfere with polymerase chain reaction. The primer-dimer formation can be easily identified from melt curve analysis. The primer design is also a crucial aspect of RT-PCR. It depends on choice of amplicon as well. The amplicon is generally kept to < 300 base pairs SYBR green based detection while 50-150 base pairs for probe-based detection. The primers are generally 15 – 20 base pair and contain 20-80% CG units. Care should be taken to avoid formation of dimers in SYBR green based detection or should be verified from melt curve.

The quantification using PCR can be standard/absolute or relative. For absolute quantification a standard curve of Ct vs log (conc. Of standard gene) is required to be generated. While in relative quantification a mathematical equationare used to calculate expression level relative to non-treated sample. Further it is normalized to a housekeeping gene which is expressed at constant level and has same amplification efficiency. The Ct values are used for quantification purpose which are fractional PCR cycle number at which the reporter fluorescence level is greater than minimal detection level. This ensures increased accuracy and reproducibility, since; the difference in fluorescence of end product and blank cannot be used for quantification as fluorescence cease to increase proportionately at the end of reaction

as the dye to base binding ratio decreases over the course of reaction. The Ct values are arbitrarily chosen by the software based on the standard deviation of the baseline and it is generally kept 10 times the SD of baseline signal from cycle 3 to 15.

Transfection protocol for siRNA

- **In a six well tissue culture plate, seed 2 x 10^5cells per well in 2 ml anti- biotic-free normal growth medium supplemented with FBS.**

  NOTE: This protocol is recommended for a well from a 6 well tissue culture plate. Adjust cell and reagent amounts proportionately for wells or dishes of different sizes.

  80 % confluent flask contains 20 lacs cells approx. so make suitable dilution to add cells 3.5 lacs cells per well (per 3 ml) i.e. 1 ml trypsin + 17 ml complete media so 3 ml (3.5 lacs cells) per well

- **Incubate the cells at 37° C in a CO_2incubator until the cells are 60-80% confluent. This will usually take 18-24 hours.**

  NOTE: Healthy and sub-confluent cells are required for successful transfection experiments. It is recommended to ensure cell viability one day prior to transfection.

- **Prepare the following solutions:**

  Solution A: (siRNA + transfection medium) For each transfection, dilute 2-8 µl of siRNA duplex (i.e.0.25-1 µg or 20-80 pmols siRNA) into 100 µl siRNA Transfection Medium: sc-36868.

  Solution B: (transfection reagent + transfection medium) For each transfection, dilute 2-8 µl of siRNA Transfection Reagent: sc-29528 into 100 µl siRNA Transfection Medium: sc-36868. Peak activity should be at about 6 µl siRNA Transfection Reagent.

  NOTE: Do not add serum and antibiotics to the siRNA Transfection Medium: sc-36868.

  NOTE: Optimal siRNA amount used for transfection may vary for each target protein and should be determined experimentally.

  NOTE: If a lower siRNA concentration is desired, dilute siRNA appropriately with siRNA Dilution Buffer: sc-29527.

  NOTE: Although highly efficient in a variety of cell lines, siRNA Transfection Reagent: sc-29528 may not be suitable for use with all cell lines.

- **Add the siRNA duplex solution (Solution A) directly to the dilute Transfection Reagent (Solution B) using a pipette**. Mix gently by pipetting the solution up and down and **incubate the mixture 15-45 minutes at room temperature**.

- **Wash the cells once with 2 ml of siRNA Transfection Medium:** sc-36868 Aspirate the medium and proceed immediately to the next step.

  (wash with 1 ml because of limited quantity of transfection medium)

- **For each transfection, add 0.8 ml siRNA Transfection Medium to each tube containing the siRNA Transfection Reagent mixture (Solution A + Solution B). Mix gently** and overlay the mixture onto the washed cells.

- **Incubate the cells 5-7 hours at 37°C in a CO_2incubator.**

  NOTE: Longer transfection times may be desirable depending on the cell line. However prolonged serum starvation may result in unwanted cell detachment or death.

  NOTE: Fluorescein Conjugated Control siRNA should only be incubated for a total 5-7 hours at 37°C in a CO_2incubator. At the end of incubation, they are ready to be assayed by fluorescent microscopy.

- **Add 1 ml of normal growth medium containing 2 times the normal serum and antibiotics concentration (2x normal growth medium) without removing the transfection mixture**. If toxicity is a problem, remove the transfection mixture and replace with 1x normal growth medium.

- **Incubate the cells for an additional 18-24 hours.**

- **Aspirate the medium and replace with fresh 1x normal growth medium.**

- **Assay the cells using the appropriate protocol 24-72 hours after the addition of fresh medium in the step above.**

  NOTE: For RT-PCR analysis isolate RNA using the method described by P. Chomczynski and N. Sacchi (1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162: 156-159.) or a commercially available RNA isolation kit.

The reaction protocol and specification followed are described below:

Selection of primers

Primer design tool of NCBI (National Center for Biotechnology Information) was referred for primer selection (Table 4). Forward Primer and reverse primer for cDNA amplification of ABCC3 gene were custom synthesized from Eurofins scientific, India.

Total RNA isolation

The gene expression analysis also depends on integrity of isolated RNA, therefore isolation of intact total RNA is primary requirement for gene quantification. The absolute quantification that normalize specific mRNA expression against total RNA (g/g of total RNA). The long mRNA are prone to degradation by RNase during tissue sampling, RNA purification and RNA storage. In addition to the cellular RNase there are several RNases that are present in environment. The RNA samples may get contaminated by DNA and even minor quantities can get amplified in PCR. Therefore, a properly optimized laboratory procedure was used for RNA extraction:

- One mL of TRIzol reagent (1 mL/10cm^2) was added to each well and allowed to incubate for 5 min at room temperature.
- The sample was transferred to 2ml of autoclaved eppendorf (DEPC treated, RNase free). Then 200µl of chloroform was added and mixed vigorously for 15 sec and incubated at room temperature for 2-3 min.
- The machine was pre-maintained and samples were centrifuged at 12000g for 15 min at 2-8^{0}C.
- Then 50% of the aqueous phase, above the fairly visible interphase, was transferred to fresh tubes. The aqueous phase contains both RNA and DNA, however, RNA, being of smaller fragments, resides in the top of the aqueous phase.
- To the aqueous phase 0.5 mL of isopropyl alcohol was added and incubated at for 10 min at room temperature. Further, sample was incubated at -20^{o}C to precipitate the RNA.
- The sample was centrifuged at 12000g for 10min at 2-8^{0}C to obtain the RNA pellet.

- The supernatant was removed and 75% ethanol was added to wash the pellet by mixing with vortex again centrifuged at 7500g for 5min at 2-8^{0}C
- The supernatant was removed and pellet was allowed to semi air dry.
- The washed pellet was dissolved in 50µl DEPC treated water by incubation at 55-60^{0}C for 10min.
- The RNA was checked on 1.2% agarose by loading 2 ul of sample with loading dye.
- The RNA concentration was checked by OD (1 O.D = 33 µg/ml) using nanodrop and the purity was assessed from A260/A280 ratio which was between 1.8-2.1

Table 7-4 Details of primers

Primer	Sequence (5'->3')	Template strand	Molecular weight	Length	Tm	GC %
	ABCC3 siRNA					
Forward primer	AGGGAGTGTTACAGGGTCCA	Plus	6222.07	20	59.35	55.00
Reverse primer	GGTACCAAGGCCACAGTTCT	Minus	6101.98	20	59.35	55.00
	GAPDH primers					
Forward primer	ATCCCATCACCATCTTCCAGG	Plus	6347.83	21	59.8	52.4
Reverse primer	CAAATGAGCCCCAGCCTTCT	Minus	6537.94	21	60.9	55.0

RNA to cDNA conversion

RNAs are highly unstable and sensitive, and are prone to degrade by the RNases. On the other hand DNA is fairly stable, therefore; RNA is converted to cDNA to store the information in RNA in a stable form. The RNA to DNA conversion is brought about by RNA-dependent DNA polymerase, known as reverse transcriptase. Using RNA as template it can produce cDNA. It also needs a primer with a free 3′-hydroxyl group. In eukaryotic mRNAs, a poly-A tail is present at their 3′-ends, therefore; a poly-T

oligonucleotide can be used as a primer. During reaction the primer gets annealed to the 3′-end of the mRNA. 3′-end of the primer is extended by the reverse transcriptase producing a RNA-DNA hybrid molecule. Finally using RNase H or alkaline hydrolysis, the RNA strand of this RNA-DNA hybrid molecule is digested. The following step wise protocol was used for cDNA synthesis:

To convert RNA to cDNA the high capacity RNA-to-cDNA Conversion Kit was utilized.

Kit components were removed from their storage conditions and allowed to thaw on ice.

1.5 microgram of RNA /20 µL of reaction was used for conversion.

The Reaction set up used is given in Table 7.5.

RNA to cDNA conversion parameters

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

To the each well of 48 well plate, 20 µL of RT (reverse transcription) reaction mix was added for real time PCR reaction.

Plate was sealed with sealer and centrifuged to spin down the contents and to remove air bubbles. Plate was placed in the sample holder of PCR system and following cycle was run:

Steps of PCR cycle

Parameters	**Step 1**	**Step 2**	**Step 3**
Temperature(^{o}C)	45	95	4
Time (min)	30	10	Storage

Real Time PCR

Once the cDNA was obtained from mRNA. The quantification of gene expression was performed on RT-PCR using SYBR green based detection and gene knock-down was accessed with respect to the control. The reaction was set as per below composition.

Table 7-5mRNA quantification – reaction parameters

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

To the each well of 48 well plate 15 µL of RT reaction mix was added for real time PCR reaction. Plate was sealed with sealer film and centrifuged to settle down the contents and to remove any air bubble. Plate was placed in the sample holder of RT-PCR machine and following cycle was run:

Table 8.8RT-PCR cycle steps

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

7.4 RESULTS AND DISCUSSION

7.4.1 Cytotoxicity studies by MTT assay

In vitro cell line studies for cytotoxicity of siRNA-HNCs were performed to assess effect of cationic lipid and siRNA complexation on cell cytotoxicity. It was observed that formulations were non-cytotoxic to cells. More than 90 % cells were viable in all cases except in case of siRNA-HNCs formulation having N/P ratio of 10 at 100 nMsiRNA concentration (Figure 7-3). Cell viabilities were 67.91 % and 58.82 % for 48 hr and 72 hr respectively. Greater cytotoxicity was observed due to more amount of cationic lipid (DOTAP). High cationic charge is responsible for cell cytotoxicity due to charge based stronger interaction between positive charged cationic lipids and negatively charged cell membrane components.

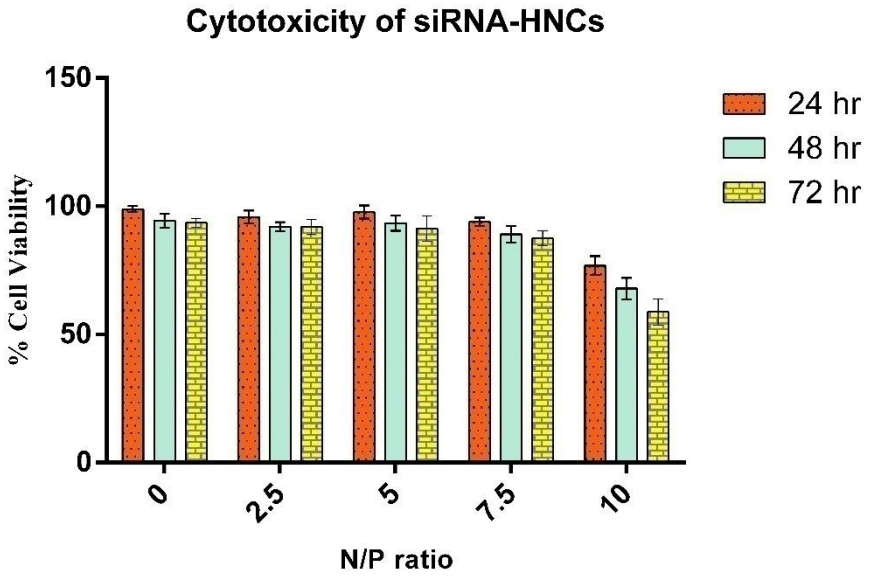

Figure 7-3Cytotoxicity siRNA-HNCs

7.4.2 Cellular uptake studies of HNCs

7.4.2.1 Qualitative uptake studies using confocal microscopy

Confocal microscopy images of the cell uptake studies are shown in Figure 7-4. It depicts the cellular uptake of developed Docetaxel loaded FITC labelled siRNA-HNCs. complexes in comparison to Lipofactamine-2000 as reference control and FITC labelled naked siRNA. The lesser green fluorescence was observed in case of the naked siRNA while HNCs formulation showed significant siRNA presence in cytosol as indicated by intense green fluorescence from the cytosol inside the cells. The least green fluorescence of naked siRNA may be due to the nuclease activity which might degrade siRNA. Blue fluorescence is due to nucleus staining by DAPI and merged confocal image clearly demonstrated uptake of formulation into cytosol thus it can be concluded that HNCs could successfully carry and deliver siRNA into the cytosol i.e. transfection efficiency is improved with HNCs.

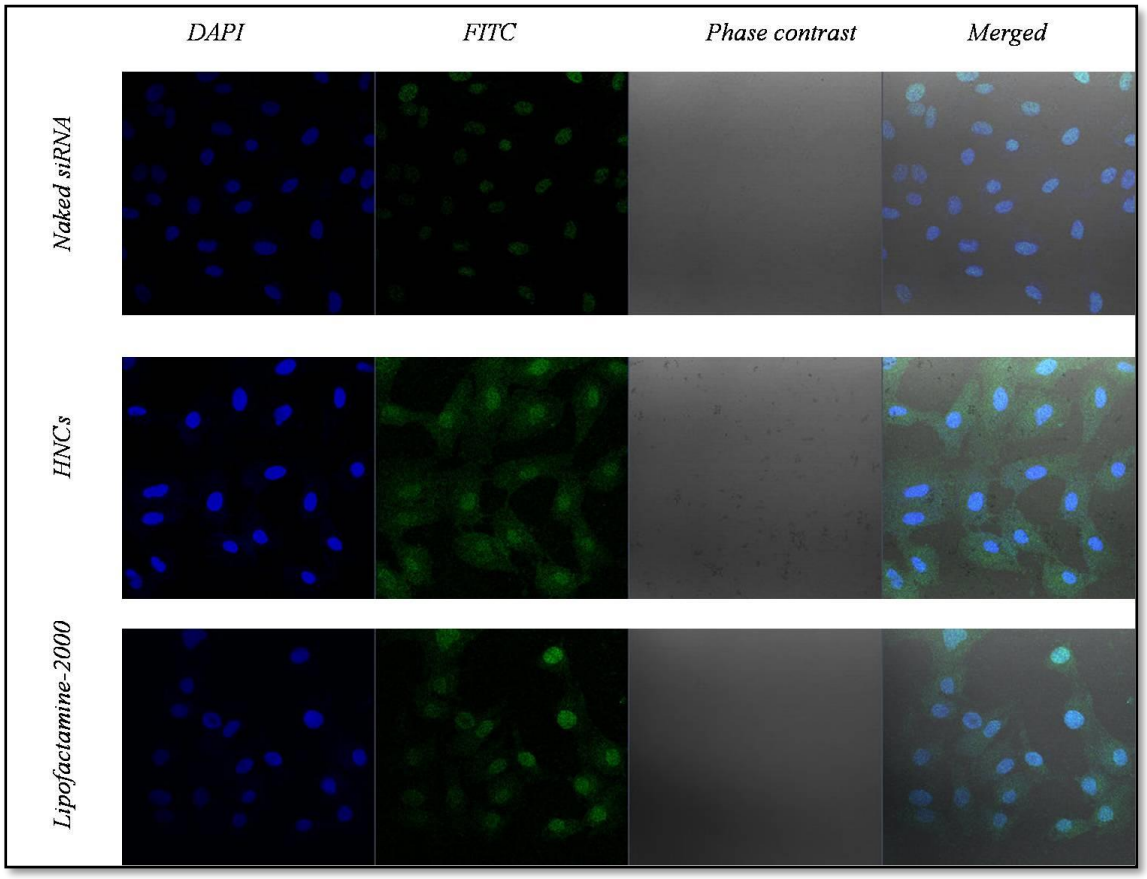

Figure 7-4Confocal images for qualitative cellular uptake study

7.4.2.2 Quantitative uptake studies using Flow cytometry

FACS histograms of cellular uptake of developed HNCs are shown in Figure 7-5. The quantitative representation of the cellular uptake of HNCs after transfection with FITC labelled siRNA. It was observed that the naked siRNA showed very less fluorescence indicating very marginal transfection efficiency. Examining the histograms gives the idea about the HNCs uptake after transfection. There is a rightward shift of the histograms. Fluorescence intensity of HNCs is very much near to transfection standard lipofactamine-2000 complexed siRNA (Figure 7-6). Successful transfection of the using HNCs depict that HNCs are easily uptaken by the cells through endocytosis due to their cationic characteristic.

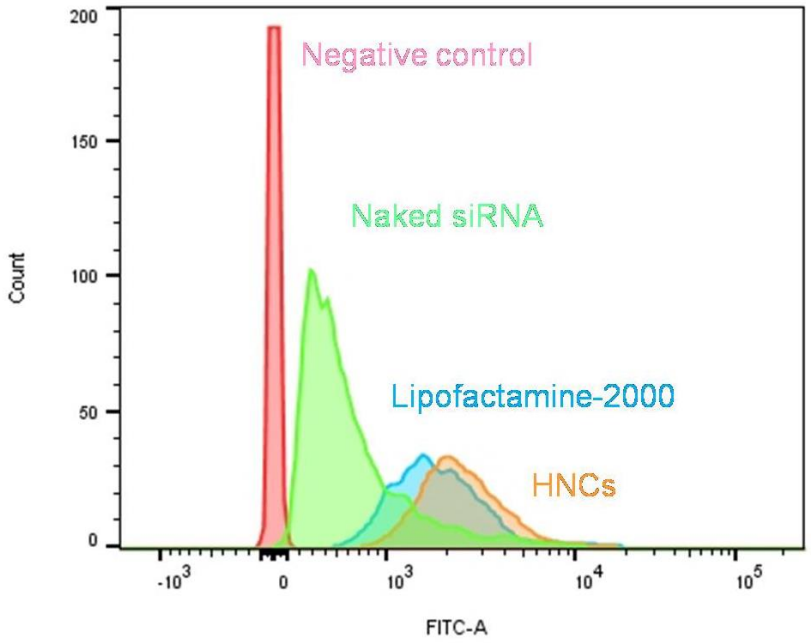

Figure 7-5 FACS histogram

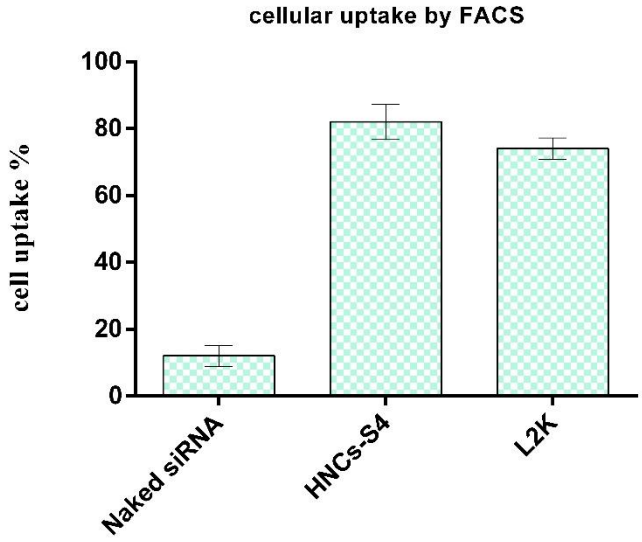

Figure 7-6quantitative cellular uptake by FACS

7.4.2.3 Uptake efficiency in folate positive and folate negative cells

To examine the cell uptake efficiency of folate ligand (DSPE-PEG 2000-folate) anchored HNCs, H1299 and A549 cells were transfected with HNCs containing FITC labelled siRNA. The uptake of siRNA-HNCs containing DSPE-PEG 2000-folate by the cells was determined over time and expressed as fluorescence unit. A time-dependent increase in fluorescence was observed in FITC-labelled siRNA-HNCs in

H1299 cells compared to A549 cells (Figure 7-7). It revealed significant more uptake and delivery of DSPE PEG-2000-folate anchored HNCs in folate overexpressing H1299 cells than low or no folate expressing A549 cells. Cellular uptake by endocytosis is the major pathway associated with the delivery of HNCs. Particles under 150 nm size are effectively endocytosed. Nanoparticles with a diameter less than 200 nm were able to enter the cell for presentation to the lysosomes via clathrin-mediated endocytosis. Here in these vesicles, nuclease and other degradative enzymes are absent and the delivery systems may be directly transported to the golgi or endoplasmic reticulum and thereby escaping the lysosomal degradation. Additionally, hybrid vesicles having DOPE in their formulation as fusogenic lipid promotes an inverted hexagonal phase which would destabilize endosome by disrupting lysosome membrane, thereby facilitating siRNA release into the cytosol.

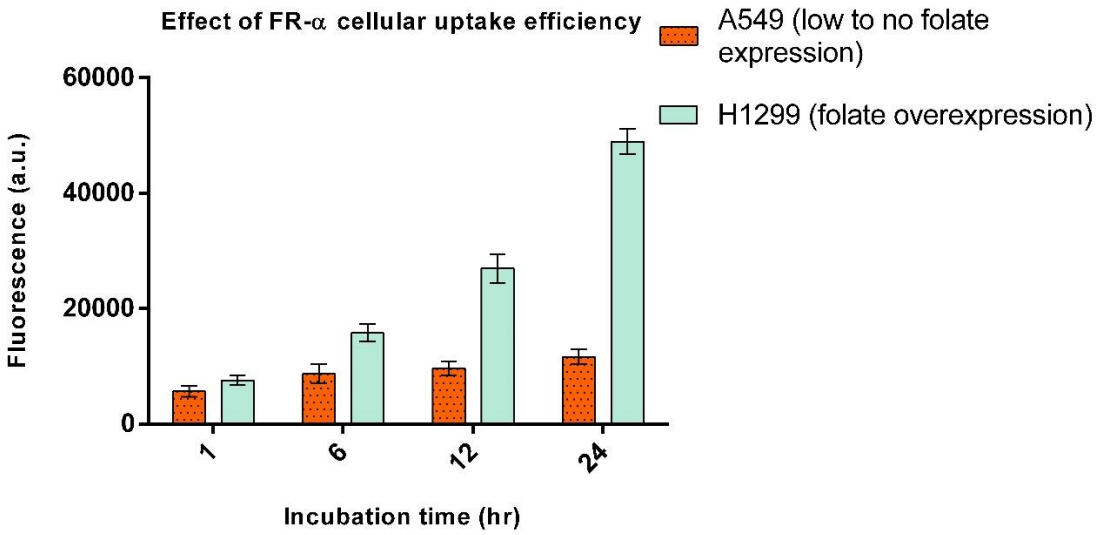

Figure 7-7Effect of folate on cellular uptake efficiency

7.4.3 Chemo-sensitization study

The results of chemo-sensitization studies are depicted in Figure 7-8, Figure 7-9 and Figure 7-10along with IC50 values in Table 7-6. Chemo sensitization effect (performed to evaluate cytotoxic potential of drug) was proved by the fold change in IC50 values when siRNA was complexed with cisplatin caprylate loaded HNCs. All Cisplatin loaded siRNA complexed HNCs showed significantly less IC50 value as compared to Cisplatin loaded HNCs. The Fold change in IC50 values of siRNA complexed HNCs are 1.52, 1.92 and 3.25 for 24 hr, 48 hr and 72 hr respectively.

Highest chemo-sensitization was observed at 72 hr. Furthermore, the lesser IC50 values imply the lesser requirement of anti-neoplastic drug to kill 50 % of cancer cell populations due to greater cytotoxic effect. Cytotoxicity to cancer cells occur due to passive targeting of tumor tissues/cells by enhanced permeability and retention effect (EPR effect) and active targeting through folate receptor uptake followed by clathrin mediated endocytosis and thereby facilitating endosomal uptake.

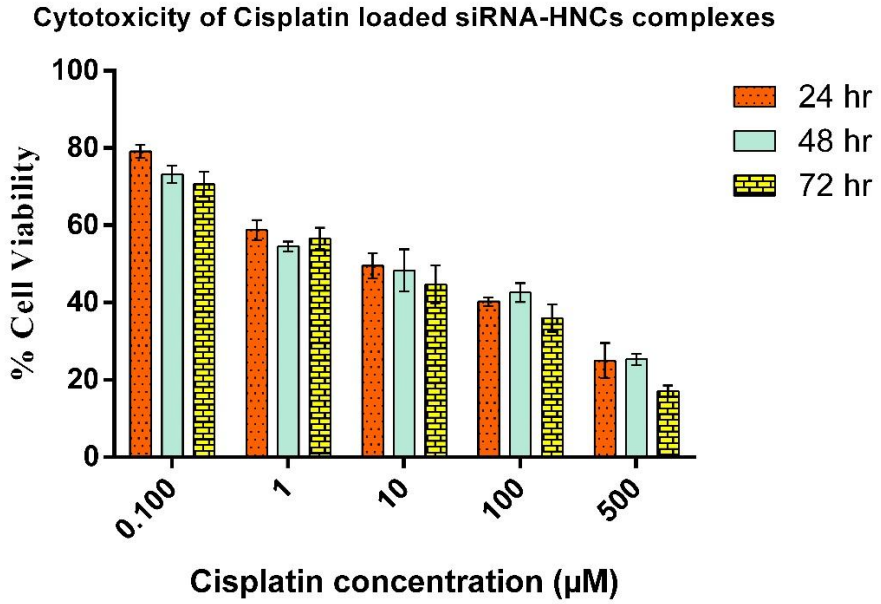

Figure 7-8 MTT assay for cisplatin loaded siRNA-HNCs

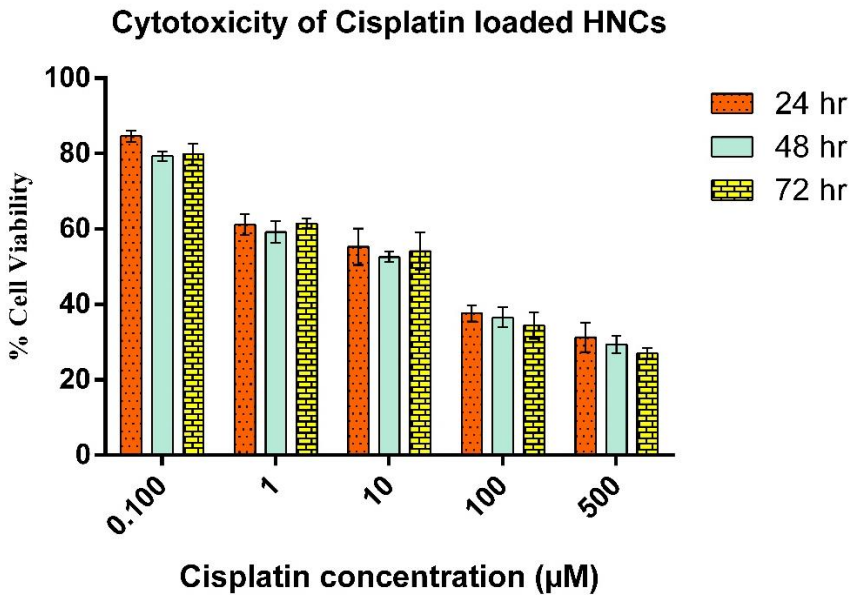

Figure 7-9 MTT assay for cisplatin loaded HNCs

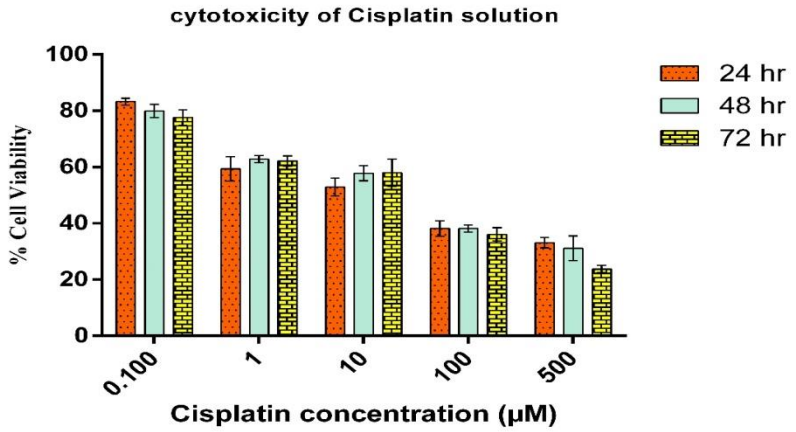

Figure 7-10 MTT assay for cisplatin solution

The cytotoxicity displayed by Cisplatin solution and cisplatin caprylate loaded HNCs at various concentration and time were quite similar and fold change in IC_{50} of later with respect to former were only 1.04, 1.18 and 1.03 at 24 h, 48 h and 72 h respectively. This could indirectly imply that Cisplatin solution and cisplatin caprylate loaded HNCs presented equivalent amount of drug to the cells. Further, a probable reason for non-improvement in the cytotoxicity would be due to the slow release of drug from the formulation due to sustained release characteristics and is consistent with the results reported in studies earlier[37, 38]. The most significant impact on cell viability profile was observed with dual loaded HNCs formulation, wherein a 3.35-fold improvement in IC_{50} value was observed at 72 h. This reduction in value may be due to the impact of siRNA on the drug efflux inhibition. At 24 h and 48 h, the fold reduction in values were 1.57 and 2.27 respectively. A549 cell lines are known to express ABC efflux pumps that readily efflux the various chemotherapeutics including cisplatin from the cells[39]. Thus, this strategy of co-delivering siRNA would have led to higher cellular level of cisplatin for cell cidal effects due to synergistic effect of co-loading. It is anticipated that the cytotoxicity to the cells observed herein could favourably replicated in in-vivo study in animal tumor xenograft models due to characteristics this formulation possesses for selective extravasation in tumor cells that would occur by enhanced permeability and retention effect (EPR effect) and passive uptake by cancer cells without adversely affecting the normal cells[40].

Table 7-6 IC50 Values of Cisplatin caprylate loaded HNCs, Cisplatin caprylate loaded siRNA-HNCs and Cisplatin solution

Formulation	IC50 (µM)		
	24 hr	48 hr	72 hr
Cisplatin caprylate loaded HNCs	14.41	11.20	10.79
Cisplatin caprylate loaded siRNA-HNCs	9.48	5.81	3.32
Cisplatin solution	14.92	13.18	11.11

7.4.4 Wound scratch assay

For assessment of migration of cell, wound scratch assay was performed. For untreated well (control), the recovery of wound occurred to greatest extent covering the entire surface of wound made at initial time point (Reference). In other treatment group for R2 HNCs as evidenced from the Figure 7.11(A), a concentration dependent inhibition of wound recovery was observed. There was a higher decrease till 10µM concentration whereas comparative %recovery was observed at 20µM concentration against 10µM. Figure 7.11(B) shows the % wound recovery for the different concentration of treatment with R2 HNCs. The recovery observed was concentration dependent ($p<0.01$); lowest for the well with highest concentration of HNCs i.e. for 20 µM it was 42.3±4.6 % while the recovery was highest for well treated with 1 µM concentration 86.1 ± 3.0%. Also, at equimolar concentration of treatment given to the cells by R2 HNCs, the % recovery of the wound was 1.5 times lower than cisplatin solution indicating the superior performance of formulation in inhibiting cell proliferation (Figure 7.11(C)).

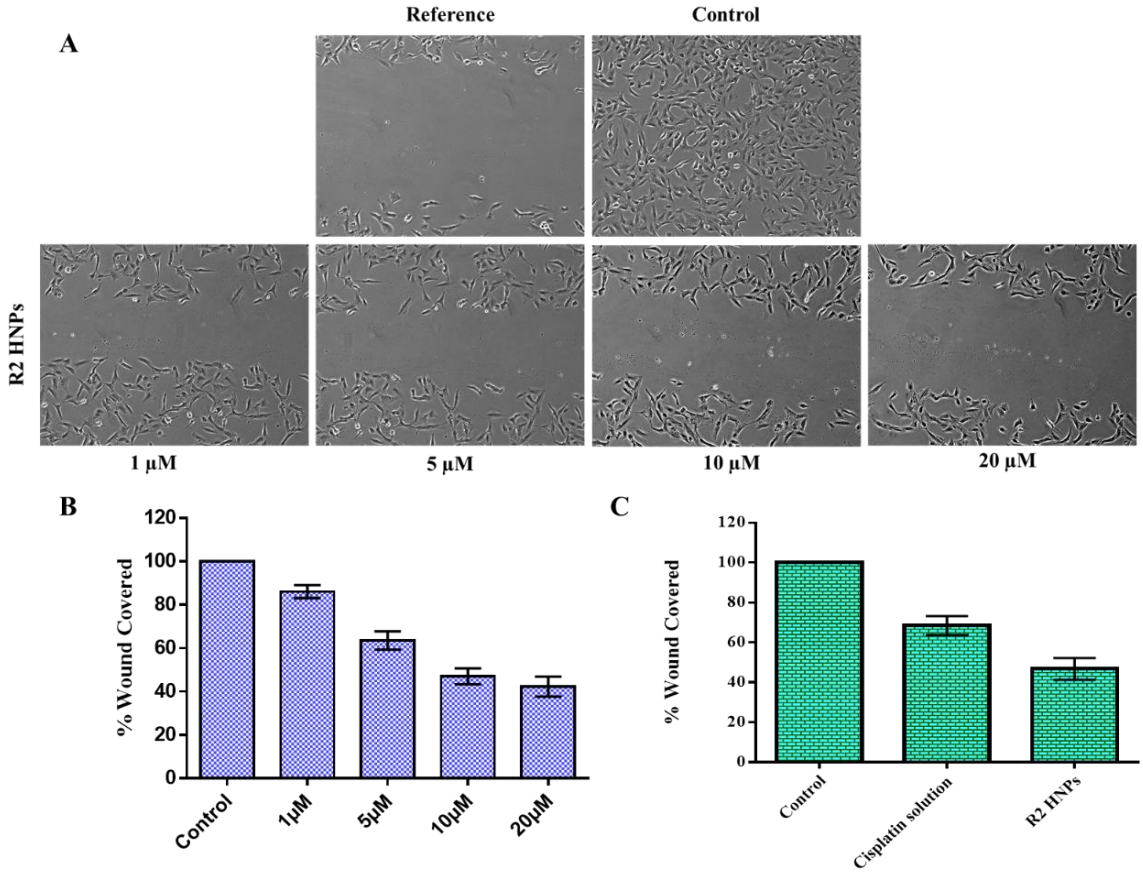

Figure 7-11 Wound scratch assay results

(A) Wound healing images in presence of various concentration of HNCs. (B) Percent wound covered after 48 hr in comparison to untreated wound at 0 hr for R2 HNCs at various concentrations. (C) Percent wound covered for R2 HNCs against drug solution at 48 hrs.

7.4.5 Apoptosis detection and cell cycle analysis

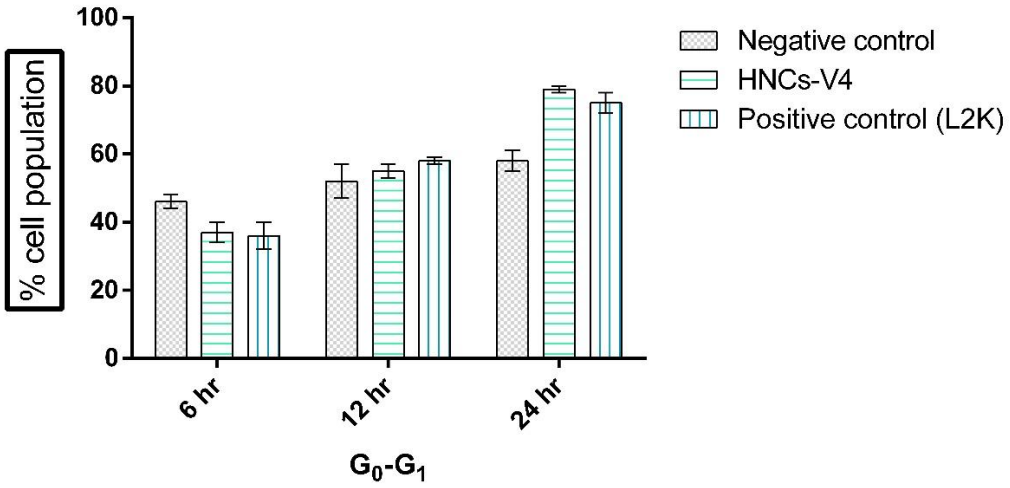

Figure 7-12Dot plot histogram apoptosis detection FACS 1

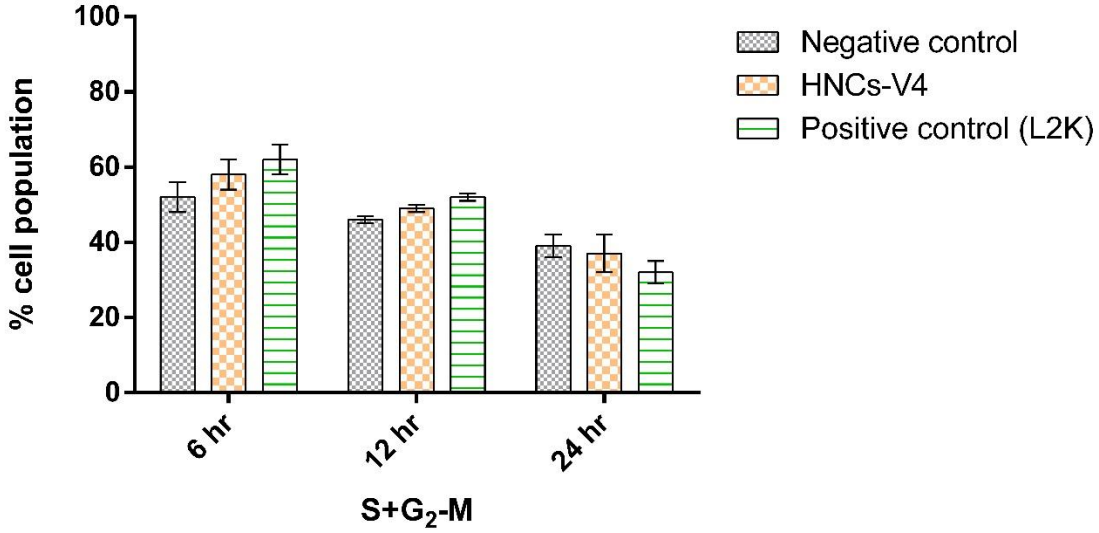

Figure 7-13 cell cycle analysis S+G2-M

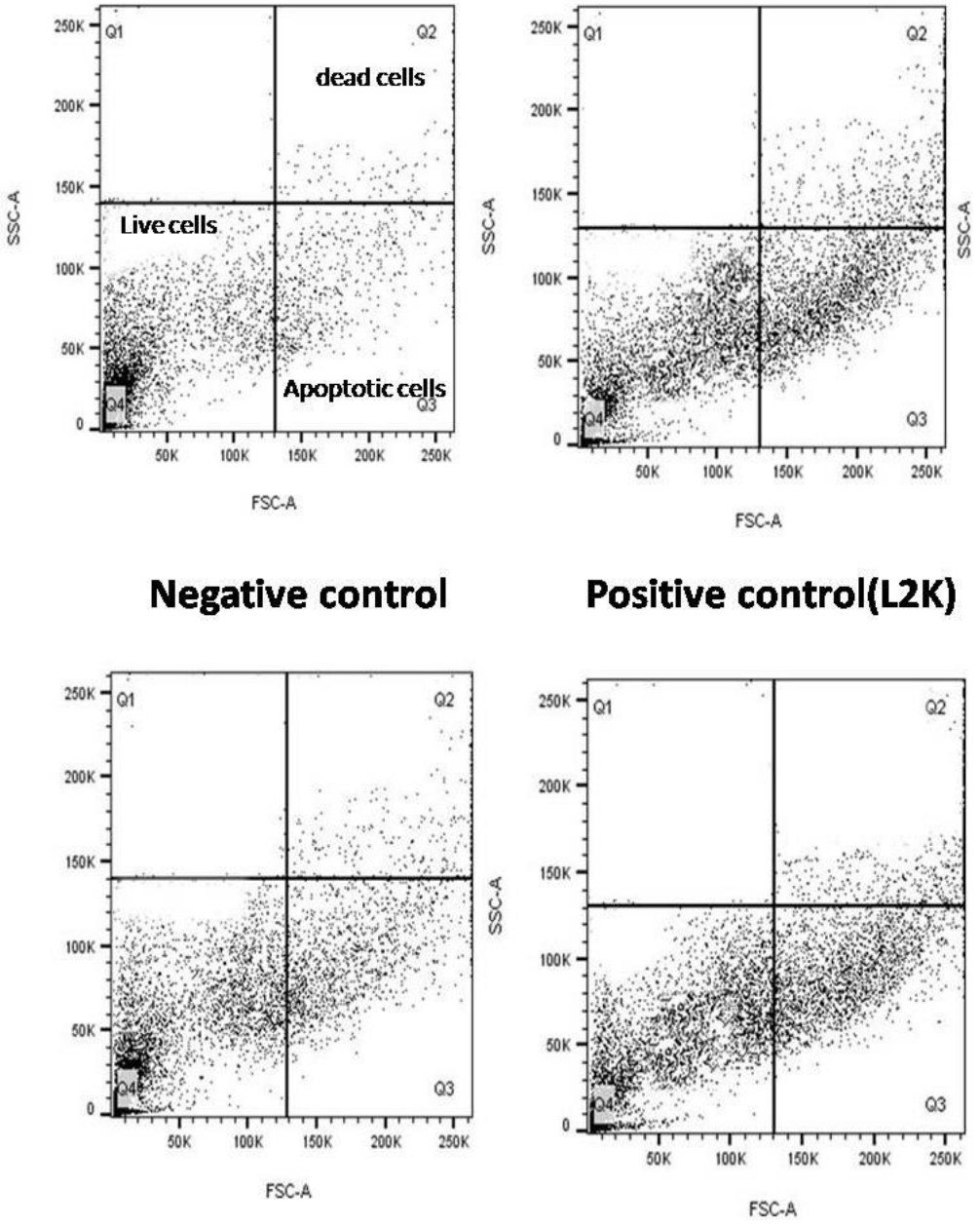

Figure 7-14 Cell cycle analysis G0-G1 in negative control, positive control, R2-HNCs (L) and R2 siRNA-HNCs (R)

Cell cycle analysis of cisplatin caprylate treated A549 cells was performed using FACS to know mechanism of action of cisplatin & cell cycle progression. The cell population in a specific cell division stage is proportional to the amount of DNA present in the cell. Cell cycle analysis graph was generated and analyzed using FACS and the results were represented as cell populations at different stages of cell division. Figure 7-12 and Figure 7-13showed that there is gradual increment in cell population in all treatments over the period of 6, 12 and 24 h which indicates the transfer to

S+G2 phase of cell division after G0-G1 phase. In G0-G1 phase, it was observed that cells treated with batch R2 behaved in the similar fashion as control. However, almost cell populations had been able to move on to S+G2+M phase of cell cycle and all phases showed similar amount of cell populations therefore it was established that cisplatin is cell cycle non-specific and it exerted its action on all phases of cell populations. Figure 7-14 depicts the dot plot for the formulations compared to controls groups.

7.4.6 Gene knock-down by RT PCR

Gene knock down study revealed transfection efficiency of ABCC3 siRNA anchored HNCs targeting ABCC3 gene. NC-siRNA-HNCs-S4 showed very less knock down compared to control. Transfection at 100 nM and 200 nM strongly down regulates (Figure7-15) the ABCC3 concentration as compared to 50 nM which was sub growth inhibitory concentration. From the gene expression study, it can be seen that 50 pM is sub growth inhibitory concentration. There was not any significant difference at 100 nM and 200 nM concentration for gene expression using L2K and HNCs.

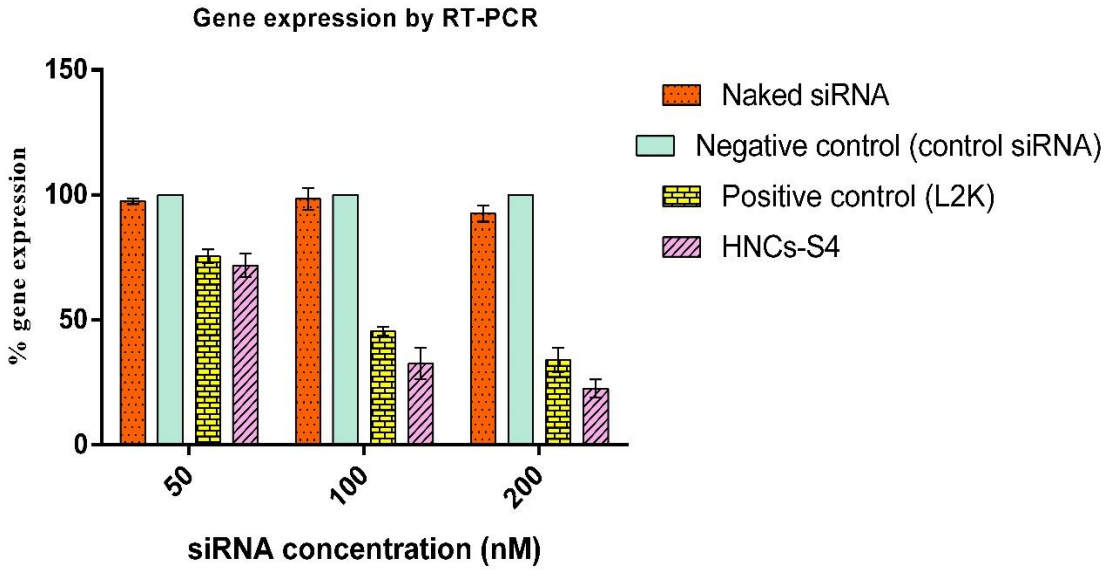

Figure7-15 Gene expression study

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