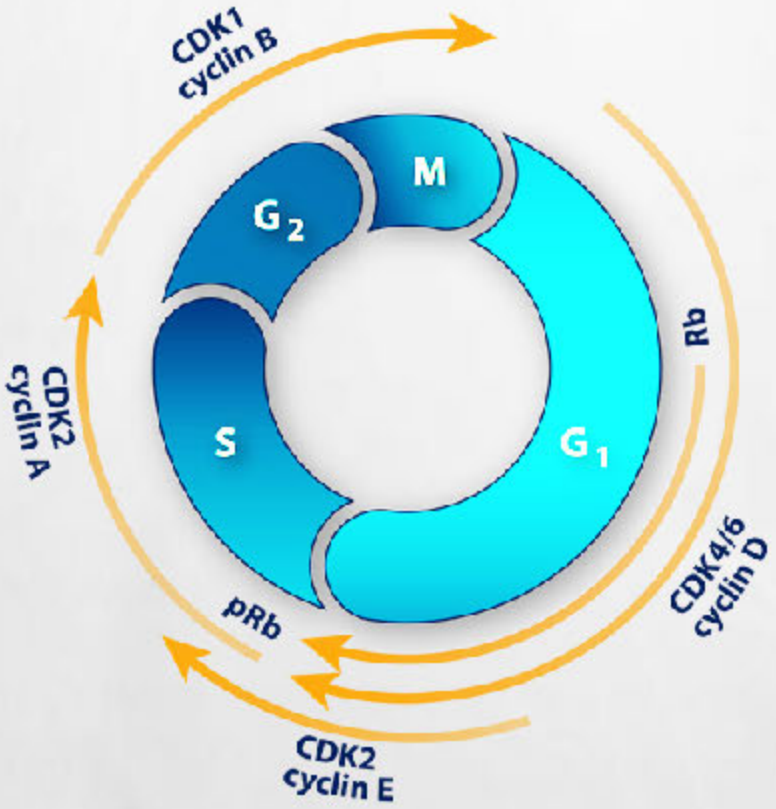
Chapter-7
In vitro Studies



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.

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 a 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. Anchorage dependent adherent monolayer cell cultures can be cultured on surface treated plastics with high energy ionizing radiations or electric ion discharge such as polystyrene. The extracellular matrix (ECM) is secreted by these types of cells which first bind to the previously surface treated charged surface substrate and then via specific receptors cells bind to that matrix to form an adherent monolayer. The space between cells is filled with ECM after cell attachment. The ECM, stays in dynamic balance with cells and its complexity is important contributor to phenotypic expression of cells attached to it. The constituents of ECM comprise of collagen, proteoglycans, fibronectin, laminin, hyaluronan and bound growth factors or cytokines. There are various transmembrane proteins or receptors involved in cell-cell and cell-substrate interactions. Self-interactive proteins such as cell adhesion molecules (CAM) are responsible for the cell-cell interactions in which calcium independent addressins are homologous molecules those interact with each other from two opposing cells and calcium dependent cadherins are the examples. Moreover, integrins are calcium independent CAM those are responsible for cell-substrate or

cell-ECM interactions. Additionally, transmembrane glycoproteins are also known to be involved in cell adhesion.

The cell culture procedure also requires detaching the already attached cells and brings them back into a suspension by addition of proteases that may digest the ECM to liberate cells from matrix into the suspension. As mostly the CAM depend on Calcium ions, a chelating agent (e.g. EDTA) are added to trypsin solution for detachment of cells.

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.

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

Materials

Sr No	Chemicals/Materials	Source/Manufacturer
1.	Thiazolyl Blue Tetrazolium Bromide (MTT)	HiMedia, Mumbai, India
2.	Dulbecco's Modified Eagle Medium (DMEM) (high glucose)	HiMedia, Mumbai, India
3.	McCoy's 5A medium	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.	Mouse anti-FSHR primary monoclonal antibody	R&D Systems Inc., USA
12.	Goat anti-mouse (whole IgG) FITC tagged secondary antibody,	Sigma Aldrich, Mumbai India

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

Instruments

Sr No	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	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

Cell culture

SKOV3 human epithelial ovarian cancer cell line was kindly gifted by Zydus-Cadila Research Centre, Ahmedabad, India. Caov3 human ovarian adenocarcinoma cells were purchased from ATCC, USA. OVCAR3 human epithelial ovarian adenocarcinoma cell line was generously gifted by Dr. Khandan Keyomarsi, Professor, Department of Experimental Radiation Oncology, Division of Radiation Oncology, The University of Texas MD Anderson Cancer Centre, Houston, TX, USA.

The SKOV, Caov3 and OVCAR3 cells were grown and maintained in McCoy's 5A medium (Himedia), DMEM (Himedia) and DMEM (ATCC) respectively. The medium of all the cell line 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 at 37 °C temperature.

7.3 Methods

7.3.1 General Methods and Preparations

- ***Preparation of complete media***

To prepare complete media, McCoy's 5A medium or 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.

- ***Subculturing of cells***

The SKOV3, Caov3 and OVCAR3 cells were grown and maintained in McCoy's 5A medium (Himedia), DMEM (ATCC) and DMEM (Himedia) respectively. 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 subcultured twice every week by taking 10^4 cells in T-75 flasks.

Following procedure was followed for the subculturing;

1. The culture flask was taken to the sterile area in laminar air flow unit and culture media was removed.
2. 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.
3. 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.
4. Cells were observed under the inverted microscope until cell layer was detached (usually within 5 minutes).
5. 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.
6. Cells count was performed on haemocytometer.
7. 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.
8. The flask was closed and cells were incubated at 37 °C, 5% CO_2.

- ***Cell counting using hemocytometer***

Preparing haemocytometer

1. Haemocytometer was cleaned using 70% ethanol.
2. The coverslip was affixed using gentle pressure and small circular motions.

Preparing cell suspension

1. Cell suspension to be counted was mixed thoroughly by gentle agitation of the flask containing the cells so as to disperse the cell into individual.
2. Before the cells started settling down about 1 mL of cell suspension was sampled using a serological pipette and placed in microcentrifuge tube.
3. 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 dye and mixed gently.

Cell Counting

1. Some cell suspension containing trypan blue was drawn out using the micro pipette 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.
3. 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 Figure 7.1. At one time 16 squares were focused.
4. 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.
5. Counting of cells was continued for all other remaining three set of 16 corner squares.
6. 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.
7. 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.

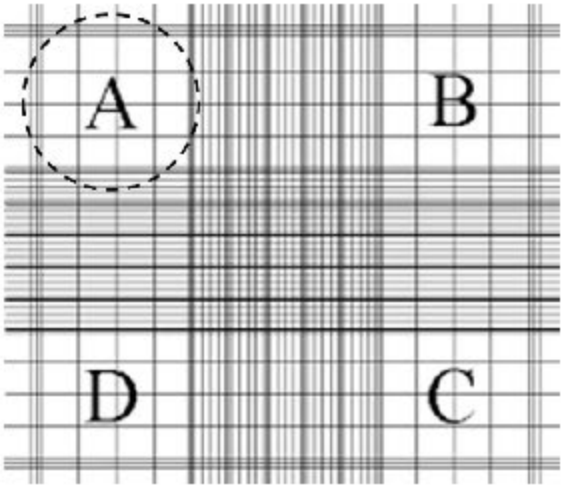


Figure 7.1 Haemocytometer diagram indicating the 16 corner squares which should be used for counting.

7.3.2 Determination of Follicle Stimulating Hormone Receptor (FSHR) Expression

Expression of, Follicle Stimulating Hormone Receptors, (FSHR) in all three ovarian cancer cell lines were determined by confocal microscopy and flowcytometry. It was determined by indirect labelling technique that involves two stages. First is cell incubation with a primary antibody and second is incubation with a compatible secondary antibody which has the fluorescent dye i. e FITC conjugated. The fluorescence than can be observed and estimated using confocal microscopy and flowcytometry techniques.

Method

7.3.2.1 Confocal microscopy

1. All the cell lines (SKOV3, OVCAR3 and Caov3) were grown in a tissue culture flask up to 60-70 % confluency in McCoy's 5A medium and DMEM respectively supplemented with 10% FBS and incubated at 37 °C, 5 % CO_2 atmosphere.
2. After achieving the said confluency, from all the tissue culture flasks media were removed and cells were washed with PBS once. The cells were then trypsinized and harvested in respective media for counting.
3. The cells were stained by trypan blue dye to differentiate between live and dead cells (1:1) and live cells were counted using Haemocytometer chamber.

4. After counting the cells were seeded at density of 2 x 10^5 cells /well in a 6 well plate (Corning, New York) and grown on sterilized glass cover slips in 2 ml of respective media supplemented with 10% FBS and incubated at 37 °C, 5 % CO_2 atmosphere for 24 hr to allow attachment as well as growth of the cells.
5. The cells were then fixed by adding 1 ml of 3.7% w/v paraformaldehyde (PFA) solution to each well and incubated for 3-5 min.
6. PFA was then removed and cells were then washed with PBS thrice after fixation.
7. The cells were incubated with mouse anti-FSHR monoclonal primary antibody (mouse anti-FSHR mAb) (1µg/million cells; dilution in PBS) for 1 hr at room temperature in dark.
8. Cells were then washed thrice with PBS to remove unconjugated primary antibody and then incubated with FITC labelled goat anti-mouse secondary antibody (1:200) for 1 hr at room temperature in dark.
9. After specified incubation time, cells were washed with PBS thrice to remove excess unconjugated secondary antibody and counterstained with DAPI (1 µg/ml DAPI added to each well and kept for 10 min at room temperature/RT in dark).
10. Cells were washed thrice with PBS to remove excess of DAPI.
11. Cells were mounted on a glass slide and visualised under confocal laser scanning microscope (CarlZeiss LSM 710, Germany). The results were then analysed using Zen lite software.

7.3.2.2 Flow cytometry analysis

1. All the cell lines (SKOV3, OVCAR3 and Caov3) were grown in a tissue culture flask up to 60-70 % confluency in McCoy's 5A medium and DMEM respectively supplemented with 10% FBS and incubated at 37 °C, 5 % CO_2 atmosphere.
2. After achieving the said confluency, from all the tissue culture flasks media were removed and cells were washed with PBS once. The cells were then trypsinized and harvested in respective media for counting.
3. The cells were stained by trypan blue dye to differentiate between live and dead cells (1:1) and live cells were counted using Haemocytometer chamber.
4. After counting the cells were seeded at density of 1 x 10^6 cells /well in a 6 well plate (Corning, New York) in 3 ml of respective media supplemented

with 10% FBS and incubated at 37 °C, 5 % CO_2 atmosphere for 24 hr to allow attachment as well as growth of the cells.

5. Cells were then trypsinized, harvested and cell suspension was transferred to eppendorf tubes. The cell pellet was obtained after centrifugation at 800 x g for 5 min at 4 °C and the supernatant trypsin was discarded.
6. The cells were washed twice with PBS and then fixed using 3.7 % PFA.
7. Cells in eppendorf tube were incubated with mouse anti-FSHR monoclonal primary antibody (1µg/million cells) (mouse anti-FSHR mAb) for 1 hr with gentle shaking in between at room temperature in dark.
8. Cells were then washed thrice with FACS buffer and then incubated with FITC labelled goat anti-mouse secondary antibody (1:200) for 1 hr room temperature in dark. Cells were washed with FACS buffer thrice and finally suspended in FACS buffer for analysis (1).

7.3.3 Immunoreactivity of prepared Fab' fragments and Immunoliposomes

The immunoreactivity of Fab′ fragments and Immunoliposomes were determined by flowcytometry method. Indirect labelling technique involves two stages. First is cell incubation with a primary antibody and second is incubation with a compatible secondary antibody which has the fluorescent dye i. e FITC conjugated. The intensity of fluorescence is then estimated by flowcytometry. For the purpose of determining reactivity of Fab′ fragments and Immunoliposomes towards their receptor (FSHR), Caov3 (highly FSHR expressing compared to OVCAR3 and non-expressing SKOV3) cells have been used in the study.

Method

1. The Caov3 cells were grown in a tissue culture flask up to 60-70 % confluency in DMEM supplemented with 10% FBS and incubated at 37 °C, 5 % CO_2 atmosphere.
2. After achieving the said confluency, from all the tissue culture flasks media were removed and cells were washed with PBS once. The cells were then trypsinized and harvested in respective media for counting.
3. The cells were stained by trypan blue dye to differentiate between live and dead cells (1:1) and live cells were counted using Haemocytometer chamber.

4. After counting the cells were seeded at density of 1 x 10^6 cells /well in a 6 well plate (Corning, New York) in 3 ml of respective media supplemented with 10% FBS and incubated at 37 °C, 5 % CO_2 atmosphere for 24 hr to allow attachment as well as growth of the cells.
5. Cells were then trypsinized, harvested and cell suspension was transferred to eppendorf tubes. The cell pellet was obtained after centrifugation at 800 x g for 3-5 min and the supernatant trypsin was discarded.
6. The cells were washed twice with PBS and then fixed using 3.7 % PFA. The cells were treated as follows and after the specified treatment (Table 7.1) cells were washed with FACS buffer thrice and finally suspended in FACS buffer for analysis and analysed using FACS diva software.

Table 7.1 Various treatments to cells for determination immunoreactivity of Fab' fragments and Immunoliposomes

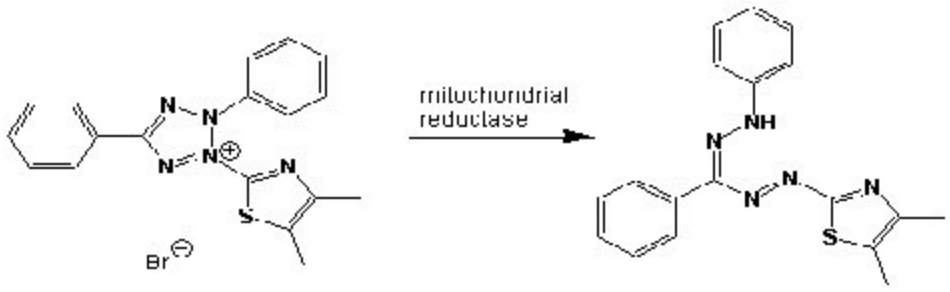
Tube No.	Treatments
1	Control - No treatment to adjust background fluorescence of cells
2	Secondary control - Cells were incubated with FITC labelled goat anti-mouse secondary antibody incubated for 1 hr at RT in dark.
3	Positive control- Cells were first incubated with mouse anti-FSHR monoclonal primary antibody (1µg/million cells) for 1 hr at RT, washed with FACS buffer three times and then incubated with FITC labelled goat anti-mouse secondary antibody (1:200) for 1 hr at RT in dark.
4	Cells were first incubated with Fab' (1µg/million cells) for 1 hr at RT, washed with FACS buffer three times and then incubated with FITC labelled goat anti-mouse secondary antibody (1:200) for 1 hr at RT in dark.
5	Cells were first incubated with Fab' (5µg/million cells) for 1 hr at RT, washed with FACS buffer three times and then incubated with FITC labelled goat anti-mouse secondary antibody (1:200) for 1 hr at RT in dark.

6	Cells were first incubated with Fab′ (10µg/million cells) for 1 hr at RT, washed with FACS buffer three times and then incubated with FITC labelled goat anti-mouse secondary antibody (1:200) for 1 hr at RT in dark.
7	Cells were first incubated with Immunoliposomes equivalent to (1µg of Fab′ /million cells) for 1 hr at RT, washed with FACS buffer three times and then incubated with FITC labelled goat anti-mouse secondary antibody (1:200) for 1 hr at RT in dark.
8	Cells were first incubated with Immunoliposomes equivalent to (5µg of Fab′ /million cells) for 1 hr at RT, washed with FACS buffer three times and then incubated with FITC labelled goat anti-mouse secondary antibody (1:200) for 1 hr at RT in dark.
9	Cells were first incubated with Immunoliposomes ′ equivalent to (10µg of Fab′ /million cells) for 1 hr at RT, washed with FACS buffer three times and then incubated with FITC labelled goat anti-mouse secondary antibody (1:200) for 1 hr at RT in dark.

7.3.4 In vitro cytotoxicity assay (MTT study)

The MTT assay is a sensitive and quantitative colorimetric assay technique to determine cell viability and cytotoxicity of drugs and the formulations. The activity of mitochondrial enzymes of live cells can reduce yellow coloured MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) dye to purple coloured formazan crystals (figure 7.2), giving a purple colour. So, *in vitro* cytotoxicity of the anticancer drugs and their formulations can be estimated by MTT assay. As those agents result in cell toxicity which leads to mitochondrial dysfunction and cell death that affects the cell viability. The extent of formazan formed is directly proportional to the viable cell number of cell lines. Dimethyl sulfoxide is generally added to solubilize the insoluble purple coloured formazan crystals and produce

purple coloured solution which can be read using microtiter plate reader (Biorad, California) at wavelength 570 nm keeping reference absorbance at 630 nm as blank.



MTT Br^- — Formazan

Yellow Coloured Dye — Purple coloured crystals

Figure 7.2 Formation of formazan crystals

Method

The MTT assay was performed to assess cell cytotoxic potential of marketed formulation Taxol®, blank liposomes and the prepared formulations, ICs (PTX-DMβCD inclusion complex), PLs (PEGylated liposomes loaded with PTX in bilayer), DLPLs (Double loaded PEGylated liposomes loaded with PTX) and ILs (immunoliposomes), in ovarian cancer cell lines SKOV3 (FSHR negative) and Caov3 (FAHR positive). The cells were seeded at density of 5000 cells/well in a 96 well plate (Corning, New York) in 200 µl of DMEM supplemented with 10% FBS and incubated at 37 °C, 5 % CO_2 atmosphere for 24 hr to allow attachment as well as growth of the cells. Liposomal formulations and Taxol® were diluted with serum free DMEM to prepare various concentrations of PTX. After 24 hr of seeding, the cells were treated with formulations and the treatment was kept for 4 hr at 37 °C, 5 % CO_2. After 4 hr of incubation the treatment was removed and cells were washed once using phosphate buffer saline. Fresh complete DMEM medium containing 10% FBS was then added to each well. After 24 hr & 48 hr, the media was again removed and 100 µl of MTT reagent (1 mg/mL) solution was added to each well and kept for 4 hr at 37 °C, 5 % CO_2 atmosphere. The medium in each plate was then replaced with 100 µl of dimethyl-sulfoxide (Himedia, Mumbai) and intensity of colour of the dissolved formazan crystals was measured using microtiter plate reader (Biorad, California) at wavelength 570 nm keeping reference absorbance at 630 nm as blank. Cells treated

with DMEM complete media and 0.1% Triton X-100 were used as negative and positive control, respectively. Vehicle related toxicity of Taxol ® was considered too. Cell viability was given relative to that of the negative control. The IC_{50} values of PTX were calculated graphically from concentration vs viability curves, considering the optical density of control well as 100% viable.

7.3.5 In vitro cell uptake study

Method

7.3.5.1 Preparation of 6-coumarin loaded liposomes

The 6-coumarin loaded liposomes were prepared by thin film hydration method as done for preparation of PLs described in chapter 5. These acted as a non-targeted formulation. For preparing targeted coumarin ILs, Fab′ fragment was conjugated to coumarin PLs by thioether linkage as described in chapter 6. (All the preparation steps were carried out in dark)

7.3.5.2 Cell uptake study 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 or 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 labelled 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.

Method

Coumarin loaded liposomes were evaluated in Caov3 and SKOV3, ovarian cancer cell lines expressing FSHR and not expressing FSHR respectively for uptake efficiency using confocal microscope. Cells were seeded at a density of 1 X 10^5 cells/well on flame sterilized 0.17 mm thick glass cover slips in 6 well plates. After 24 hr of seeding, cells were treated with coumarin loaded liposomal formulations (PEGylated and immunoliposomes). After 6 h of incubation at 37°C (5% CO_2), cells were washed with cold PBS and immediately fixed using ice cooled 4% paraformaldehyde solution for 10 min. Cells were stained by cell nuclei stain, 4',6-diamidino-2-phenylindole-DAPI, for next 10 min. Cover slips were mounted on slides after washing with PBS three times and confocal microscopy was performed using confocal laser scanning microscope.

7.3.5.3 Cell uptake study 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 (2). 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 experiments 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 nay experiment which are: set up control (instrument), specificity control (gating) and biological comparison control (3). '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 biologically relevant conditions, is required to set up positive/negative boundaries.

Method

The liposomes were evaluated in Caov3 and SKOV3, ovarian cancer cell line expressing FSHR and not expressing respectively, for uptake efficiency using FACS technique. Cells were seeded at a density of 6 x 10^5 cells/well in 6 well plates. After 24 h of seeding, cells were treated with coumarin loaded liposomal formulations (non-targeted PEGylated and immunoliposomes). After 6 hr of incubation, cells were washed with cold PBS and trypsinized. Cell suspension was allowed to settle down before centrifugation and cells were re-suspended in FACS buffer for analysis (1).

7.3.6 Wound scratch assay

Wound healing assay was performed as per the earlier reports (4) (5). Caov3 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 DLPLs and ILs formulation in incomplete media at 10 nM, 1nM, 0.1 nM and 0.01 nM drug concentrations. For comparison purpose, another well was treated with Taxol® at 0.1nM concentration tested against equimolar amount of DLPLs 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.

7.3.7 Cell death analysis using flow cytometry

1. Caov3 and SKOV3 cell lines were grown in a tissue culture flask up to 60-70 % confluency in McCoy's 5A medium and DMEM respectively supplemented with 10% FBS and incubated at 37 °C, 5 % CO_2 atmosphere.
2. After achieving the said confluency, from all the tissue culture flasks media were removed and cells were washed with PBS once. The cells were then trypsinized and harvested in respective media for counting.
3. The cells were stained by trypan blue dye to differentiate between live and dead cells (1:1) and live cells were counted using Haemocytometer chamber.
4. After counting the cells were seeded at density of 1 x 10^6 cells /well in a 6 well plate (Corning, New York) in 3 ml of respective media supplemented with 10% FBS and incubated at 37 ° C, 5 % CO_2 atmosphere for 24 hr to allow attachment as well as growth of the cells.
5. After 24 hr the seeded cells were treated with 1mL of 2 nM solution of Taxol®, DLPLs, and ILs for a period of 24 and 48 hr.
6. After specified time periods treatment was removed and cells were washed with sterile PBS twice. The cell then trypsinized, harvested and cell suspension was transferred to eppendorf tubes. The cells were centrifuged at

800 x g for 5 min to remove trypsin and pellet down the cells. The cells were washed with sterile PBS once.

7. To pellet down the cells centrifugation was done at 800 x g for 5 min and cells were washed once with sterile PBS.
8. The cell pellet was suspended again in sterile PBS and RNAse solution (100 µg/ ml of sterile PBS) was added in the cells. The cells were incubated for 30 min at 37 °C.
9. After 30 min cells were pellet down by centrifugation at 800 x g for 5 min and supernatant was removed. The cells were suspended again in FACS buffer.
10. To the cells propidium iodide (um) was added in dark and cells were incubated at room temperature for 30 min or at 37 °C for 10 min.
11. Cells were washed with FACS buffer thrice and finally suspended in FACS buffer for analysis.
12. The cells were then washed with PBS and incubated with PBS containing 0.5 mg/mL for 30 min at 37 °C. The volume was then made up to 1mL and 50 mL of PI (50 mg/mL) was added and analysed for cell cycle analysis using flow cytometer.

7.3.8 Cell cycle analysis

1. Caov3 and SKOV3 cell lines were grown in a tissue culture flask up to 60-70 % confluency in McCoy's 5A medium and DMEM respectively supplemented with 10% FBS and incubated at 37 °C, 5 % CO_2 atmosphere.
2. After achieving the said confluency, from all the tissue culture flasks media were removed and cells were washed with PBS once. The cells were then trypsinized and harvested in respective media for counting.
3. The cells were stained by trypan blue dye to differentiate between live and dead cells (1:1) and live cells were counted using Haemocytometer chamber.
4. After counting the cells were seeded at density of 1 x 10^6 cells /well in a 6 well plate (Corning, New York) in 3 ml of respective media supplemented with 10% FBS and incubated at 37 °C, 5 % CO_2 atmosphere for 24 hr to allow attachment as well as growth of the cells.
5. After 24 hr the seeded cells were treated with 1mL of 2 nM solution of Taxol®, DLPLs, and ILs for a period of 24 and 48 hr.

6. After specified time periods treatment was removed and cells were washed with sterile PBS twice. The cell then trypsinized, harvested and cell suspension was transferred to eppendorf tubes. The cells were centrifuged at 800 x g for 5 min to remove trypsin and pellet down the cells. The cells were washed with sterile PBS once.
7. The cells were fixed using 70% ice cold ethanol at 4 °C and kept for 15 min.
8. After fixation ethanol was removed by centrifugation at 800 x g for 5 min and cells were washed once with sterile PBS.
9. The cell pellet was suspended again in sterile PBS and RNAse solution (100 µg/ ml of sterile PBS) was added in the cells. The cells were incubated for 30 min at 37 °C.
10. After 30 min cells were pellet down by centrifugation at 800 x g for 5 min and supernatant was removed. The cells were suspended again in FACS buffer.
11. To the cells propidium iodide (um) was added in dark and cells were incubated at room temperature for 30 min or at 37 °C for 10 min.
12. Cells were washed with FACS buffer thrice and finally suspended in FACS buffer for analysis.
13. The cells were then washed with PBS and incubated with PBS containing 0.5 mg/mL for 30 min at 37 °C. The volume was then made up to 1mL and 50 mL of PI (50 mg/mL) was added and analysed for cell cycle analysis using flow cytometer.

Moreover, periods of time (gaps) are located between the end of cellular division and DNA synthesis start (G 1 phase) as well as between the end of DNA synthesis and mitosis start (G 2 phase). The mitotic phase is distinguished from other cycle phases (called together interphase). To reach the mitotic phase, cells have to double their whole components, at the same time that their genetic material doubles. Constituent synthesis is generally continuous, with a varying rate during interphase [6]. The growth cycle is considered as distinct from the nuclear cycle and its regulation mechanism seems to be different [7], but these two cycles are closely dependent and have to converge in a synchronous way towards mitosis; otherwise, there is an unbalanced growth [8]. DNA amount in cells is often the single parameter measured for cell cycle studies by flow cytometry. Analyses are performed with fluorescent molecules that bind specifically and stoichiometrically to DNA, in order to obtain a linear relationship between cellular fluorescence intensity and DNA amount [9].

Some dyes possess an intercalative binding mode, such as propidium iodide or ethidium bromide, whereas others present an affinity for DNA A-T rich regions: Hoechst 33342, Hoechst 33258 and DAPI, or G-C rich regions: mithramycin and chromomycin A3. The cell cycle is divided into four phases: G1, S, G2, and M. The G1 phase decides the cell cycle progression at a number of restriction sites. The signals from environment regulate the entry into cell cycle. The free edges around the cell at low density under the influence of mitogenic growth factors such as epidermal growth factor (EGF), fibroblast growth factors (FGFs), or platelet-derived growth factor (PDGF) after their interaction with cell surface receptors. High cell density inhibits the cell proliferation after cell contact through induction of change in shape.

7.4 Result and Discussion

7.4.1 Determination of Follicle Stimulating Hormone Receptor (FSHR) Expression

The expression of FSHR in all the cells was determined and confirmed by confocal microscopy and FACS using mouse anti-FSHR monoclonal antibody and FITC labelled goat anti-mouse secondary antibody.

7.4.1.1 Confocal microscopy

It has been demonstrated that FSHR is expressed in high amount in Caov3 cells while SKOV3 cells do not express these receptors or expresses it to a very low extent. As the immunoliposomes were developed by conjugating anti-FSHR antibody fragment, the expression of target receptor, FSHR was confirmed through confocal microscopy after incubation with mouse anti-FSHR monoclonal antibody (mAb) (primary antibody) followed by FITC labelled goat anti-mouse secondary antibody in the three-cell line viz. SKOV3, OVCAR3 and Caov3 cells (Figure 7.3). It can be seen that significantly higher binding of primary antibody was there in case of Caov3 cells followed by OVCAR3 cells, while lowest fluorescence and hence lowest binding was observed in SKOV3 cells confirming that these cells expresses FSHR to the lowest level. This study demonstrated that FSHR is expressed to the highest extent on the Caov3 cell line and antibody used is specific to the FSHR. Our results are in accordance with the results obtained by Zhang et al., have reported higher FSHR

expression levels of Caov3 cells than OVCAR3 cells and no expression was detected in SKOV3 cells (6).

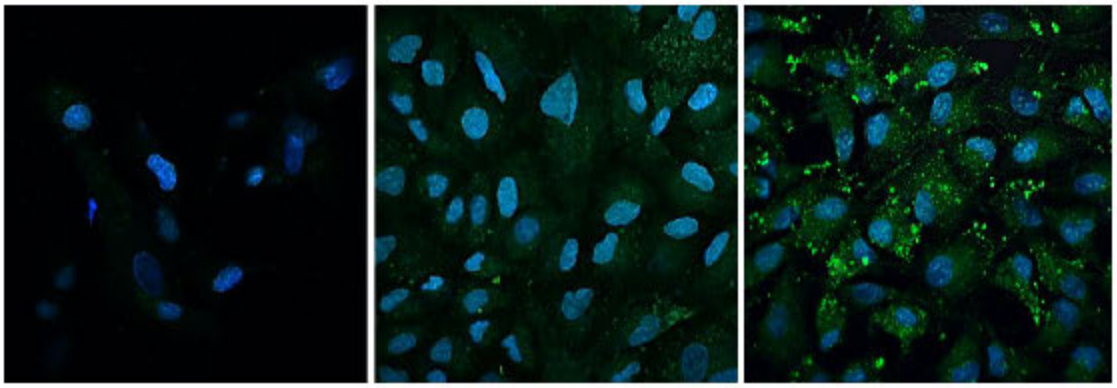


Figure 7.3 Specific binding of FITC labelled goat anti-mouse secondary antibody to the primary mAb (anti-FSHR mAb) indicating expression of FSHR in Caov3 cells, OVCAR3 cells and in SKOV3 cells

7.4.1.2 Flow cytometry analysis

The results of the confocal microscopy were further confirmed by flowcytometry/FACS. The cells were treated with both primary followed by fluorescently labelled secondary antibodies. The results of flowcytometry for treated cell are shown in figure 7.4 in comparison with non-treated control cells. The relative mean fluorescence intensities for Caov3, OVCAR3 and SKOV3 cells were 86.20 ± 2.2%; 43.68 ± 2.8%; 15.23 ± 1.8% respectively as shown in figure 7.5. Thus, among the three-cell line, Caov3 cell line was most suited for evaluating the optimized immune-liposomal formulation for cellular uptake studies due to its ubiquitous expression of FSHR and SKOV3 served as negative control cell not expressing the receptor of interest.

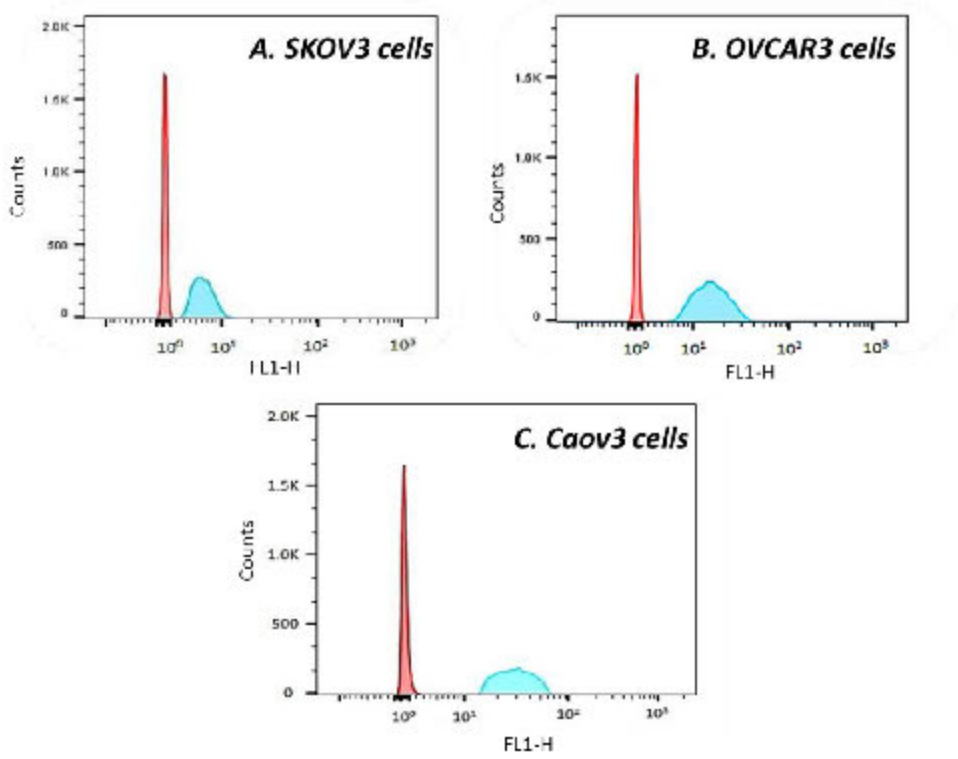


Figure 7.4 FACS analysis of FITC labelled goat anti-mouse secondary antibody to the primary mAb (anti-FSHR mAb) indicating expression of FSHR in (A) SKOV3 cells (B) OVCAR3 cells and (C) Caov3 cells.

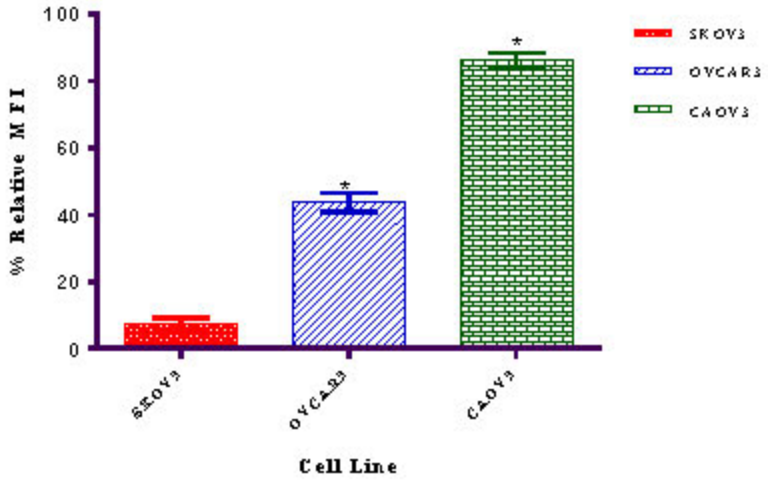


Figure 7.5 Comparison of % relative mean fluorescence intensities of SKOV3 cells, OVCAR3 cells and Caov3 cells. (* indicates significantly high expression of FSHR in OVCAR3 and Caov3 cells P<0.01, as compared to SKOV3 cells).

7.4.2 Immunoreactivity of prepared Fab' fragments and Immunoliposomes

For preparation of immunoliposomes, intact antibody was first digested with pepsin followed by reduction of $F(ab)_2$ fragments by dithiothreitol to obtain Fab′-SH fragment of mouse anti-FSHR primary monoclonal antibody which was then conjugated to liposomes. Due to these chemical and enzymatic modifications, it was speculated that the antibody might have loose its immunoreactivity. Thus, to confirm the performance of the antibody during *in vitro* use; against tumour cells overexpressing FSHR and *in vivo* in animal model, immunoreactivity of digested Fab′ (Fab′-SH) and antibody conjugated immunoliposomes (ILs) was carried out.

Herein, for the experiment we have used indirect method of determining reactivity of primary antibody by estimating binding of compatible fluorescent dye (FITC) labelled secondary antibody to primary antibody. Use of primary antibody which is fluorescently tagged or labelled with enzyme may allow direct detection of the target molecule (7). However, chances of poor recovery or inactivation of antibody during estimation/detection process and cost were the main constrains in not using labelled primary antibody. Use of secondary antibody provides for amplification of signal to many folds as the number of labelled molecules that binds to target molecules is very high (8). Further, secondary antibodies can be designed for specific fraction of immunoglobulin classes. We have used FITC labelled goat anti-mouse secondary antibody that binds specifically to mouse anti-FSHR primary monoclonal antibody (9) .

Cell were first treated with increasing concentrations of Fab′ and immunoliposomes for an hour, washed and then allowed to react with compatible FITC labelled secondary antibody. We observed a rightward shift in MFI with increase in concentration of Fab′ (figure 7.6) and ILs (figure 7.7) in treatment to cells indicating retention of immunogenic potential of Fab′ and conjugated liposomes. At 1 µg concentration tested the %RMFI for mAb, Fab′ and ILs was found to be 203.62, 248.63 and 480.26 respectively as compared to control cells. A significant difference was observed in RMFI of cells treated with ILs and Fab′. The RMFI of ILs at 1 µg was found more than that of Fab′ at 10 µg treatment to cells (table 7.2) (figure 7.8). This can be due to the surface conjugation of more than one number of Fab′ antibody fragment on ILs rather than a single one as more number of mAb are attached on the surface for conjugation with secondary antibody. If a single Fab' fragment could have

been conjugated, this would have been utilized for bonding with the cell receptor and would have been unavailable for reacting with secondary antibody. However, this is not the case and hence ILs exhibit higher MFI than Fab′ fragment. Further, the lower value for Fab' fragment would be due to the fact that Fab′ acts as a single moiety and those that get linked to the receptors will not be available at all for conjugating to the secondary antibody, a complete different scenario as that observed in ILs. It was further confirmed from the FACS data of secondary control, treatment with secondary antibody is devoid of non-specific reaction to cell surface and fluorescence was due to binding with primary antibody only. The RMFI of ILs at 5 and 10 µg treatment was found be the same with no-significant difference. The increase in RMFI for Fab′ at 1 µg concentration as compared to positive control cells indicates that the modified fragment is equally immunoreactive to that of intact antibody and thus the use of fragment for conjugation to liposomes will lead to efficient interaction with the overexpressing FSHR and will lead to accurate detection of *in vitro* and *in vivo* performance. Further, from the comparison of positive control cell group and secondary control, it can be concluded that the antibody used for conjugated to ILs and added as such in Caov3 have remained intact and also maintained the binding characteristic to the receptor.

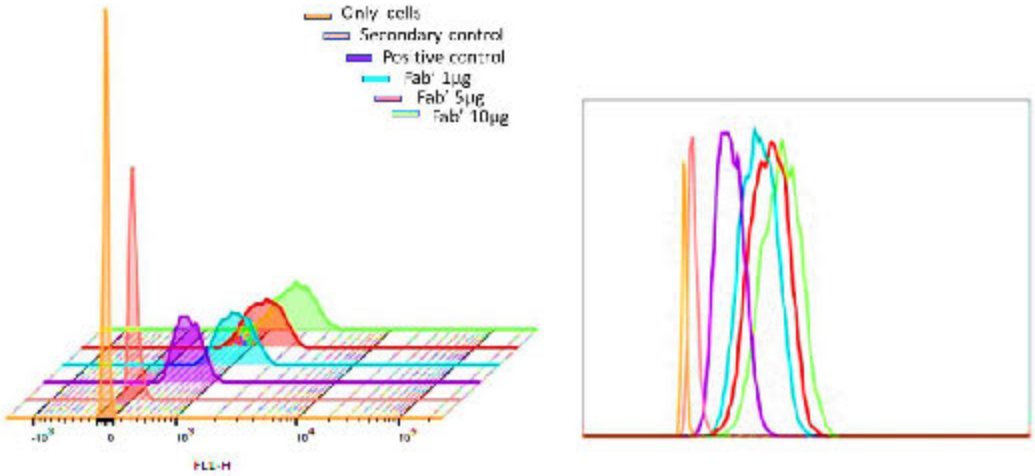


Figure 7.6 Immunoreactivity of Fab' fragments in Caov3 cell line: FACS analysis of cells treated at different concentrations of Fab' (left) and its 2D overlay (right)

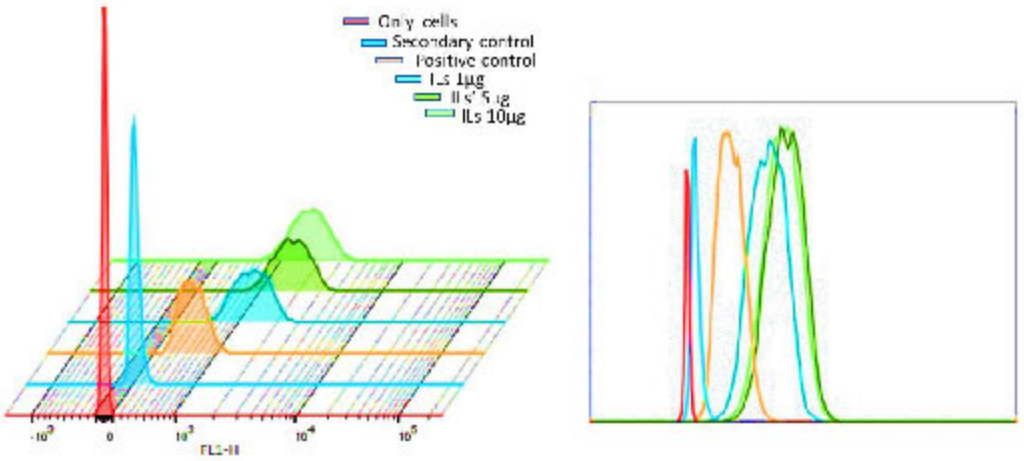


Figure 7.7 Immunoreactivity of immunoliposomes in Caov3 cell line: FACS analysis of cells treated at different concentrations of ILs (left) and its 2D overlay (right)

Table 7.2 Comparison of % relative mean fluorescence intensity of Caov3 cells treated with different concentration of Fab' fragment and ILs to determine immunoreactivity towards FSHR.

Experiment	Relative MFI (%)*	SD
Only cells	100	0
Secondary control	108.56	3.15
Positive control	203.62	12.67
Fab' 1 µg	248.63	15.32
Fab' 5 µg	315.91	16.87
Fab' 10 µg	364.46	17.71
ILs 1 µg	480.26	18.92
ILs 5 µg	668.69	22.63
ILs 10 µg	680.52	21.55

**Values are mean ± SD, n=3.*

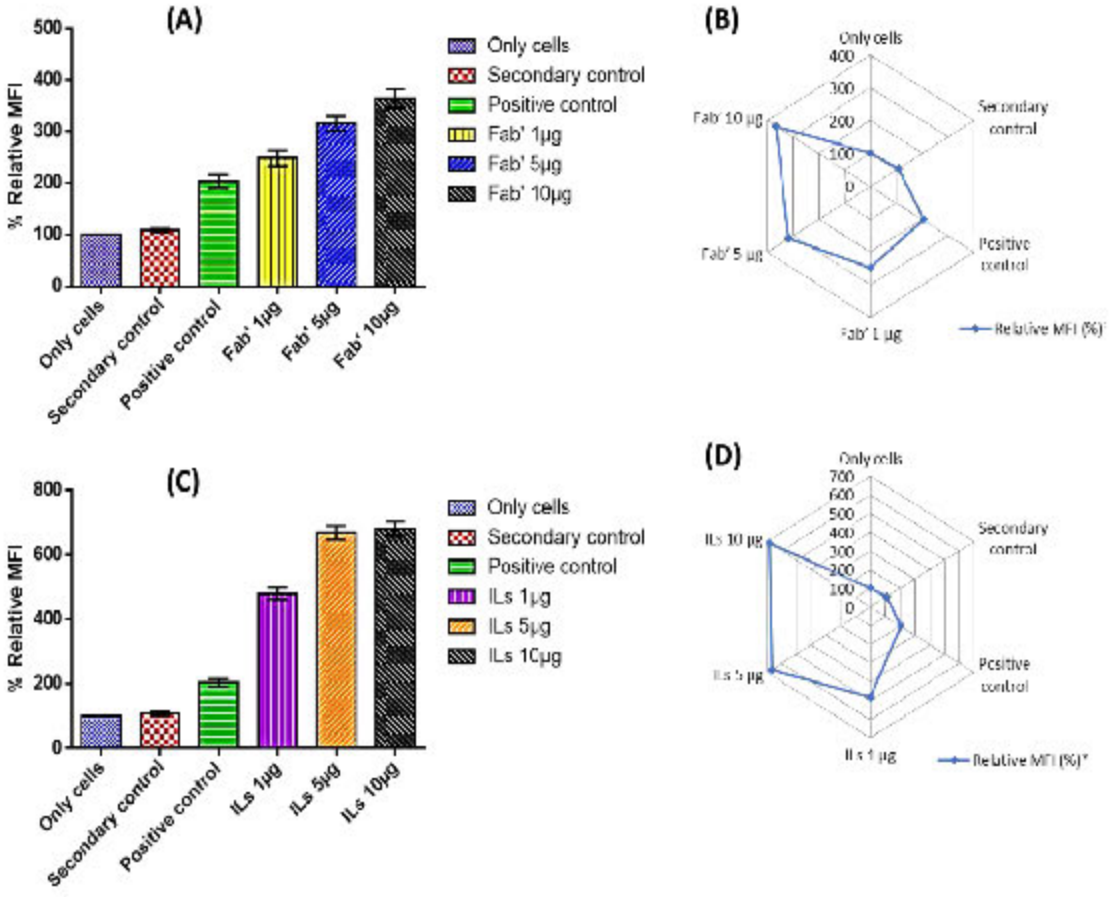


Figure 7.8 Graphs representing % RFMI of Caov3 cells treated with different concentration of (A) Fab' fragments and (B) radar representation of FACS values (C) ILs and (D) radar representation of FACS values

7.4.3 In vitro cytotoxicity assay (MTT study)

The MTT assay was performed to assess cell cytotoxic potential of marketed formulation Taxol®, blank liposomes and the prepared formulations, ICs, PLs, DLPLs and ILs, in ovarian cancer cell lines SKOV3 (FSHR negative) and Caov3 (FSHR positive). Cells were incubated in serum-free medium with different concentrations (0.001–10 µM) of ICs, Taxol®, PLs, DLPLs, ILs and blank liposomes for 24 hr and 48 hr, respectively. To determine the toxicity due to use of carrier, blank liposomes were prepared having different lipid concentration that were same as the lipid concentration used to prepare PLs. As evident from the % viability plot, blank PLs does not confer any cytotoxicity to both the cell line and the viability of the cells was observed to remain around 100%. Thus, all the cytotoxicity observed was due to formulations only.

The cytotoxicity to cells is based on the toxicity of the treatment confers to the cells. The highest toxicity in cells will be observed for a group with highest concentration of test formulation. Further, the increase in contact time or longer the time, higher will be toxic effect.

For assessment of effect of complexation on the viability of cells, inclusion complexes were screened. At the end of 24 hr and 48 hr, the IC_{50} values were found to be highest compared to all the treatment groups in both the cell line. The low effect on the viability of the cells may be due to complexation of drug with cyclodextrin, which imparted hydrophilicity to the drug leading to a decrease in easy uptake by cells. However, it can be concluded that the complexation has not impacted the cytotoxic potential of the drug, from the lower IC50 values of liposomal formulation compared to PTX solution.

The MTT assay showed that the percentage viability of SKOV3 and Caov3 cells was inhibited in a concentration and time dependent manner with IC_{50} (inhibitory concentration required to inhibit 50% of cell growth). An IC_{50} of 4.3 ± 0.27; 2.3 ± 0.16 and 1.9 ± 0.08 µmol/L for Taxol®, PLs and DLPLs respectively was observed in SKOV3 cells after 24hr. Similarly, after 48 hr, IC_{50} for the three formulations were found to be 0.72 ± 0.08; 0.43 ± 0.06 and 0.17 ± 0.05 respectively. Figure 7.9 and 7.10 shows the % cell viability result at 24 and 48 hr after treatment of SKOV3 cells with various formulations. Here, the % cell viability for ICs was found higher compared to Taxol® at all tested concentration and time. Incorporation of PTX to DLPLs decreased IC_{50} 2.26 folds and 1.2 folds as compared to Taxol® and PLs respectively after 24 hr. Whereas, IC_{50} was 4.2 folds and 2.5 folds lower compared to that of Taxol® and PLs respectively after 48 hr. Thus, it can be concluded that although during both the time point tested the IC_{50} for DLPLs was observed lower compared to Taxol® and PLs, the effect was more marked at the later time point. This may be because of the higher loading in liposomes which presented higher concentration of drug to the cells due to prolonged release effect which can partially be correlated to the release profile of DLPLs. Among the treatment groups, the lowest IC_{50} value was observed for ILs group, 1.83 ± 0.05 µmol/L and 0.162 ± 0.09 µmol/L respectively at the end of 24 hr and 48 hr respectively. This value is close to that observed with DLPLs, which indicates that ILs are not so effective in its cytotoxic effect in SKOV3 cells. This may be due to the lack of FSHR receptor on these cells and hence receptor mediated targeting potential of the ILs would not come into play

for increasing the therapeutic potential of the formulation. Further, at the end of 24 hr it can be stated that the IC_{50} value of ILs were 2.35, 1.26 and 1.04 folds lower compared to Taxol®, PLs and DLPLs respectively. Similarly, at the end of 48 hr, the IC_{50} value for ILs was found to be 4.44, 2.65 and 1.05 folds lower than the above three treatment groups.

For assessment of impact due to receptor targeting of formulation on cytotoxicity, effect of various formulations was also evaluated on receptor positive Caov3 cell line. An IC_{50} of 0.95 ± 0.12; 0.52 ± 0.08 and 0.39 ± 0.07 µmol/L for Taxol®, PLs and DLPLs respectively was observed after 24 hr. Similarly, after 48 hr, IC_{50} for the three formulations were found to be 0.108 ± 0.01; 0.078 ± 0.012 and 0.035 ± 0.005 respectively. Figure 7.11 and Figure 7.12 shows the % cell viability result at 24 and 48 hr after treatment of Caov3 cells with various formulations. Incorporation of PTX to DLPLs decreased IC_{50} 2.4 folds and 1.82 folds as compared to Taxol® and PLs respectively after 24 hr. Whereas, IC_{50} was 3.08 folds and 2.23 folds lower compared to that of Taxol® and PLs respectively after 48 hr. The lowest IC_{50} value was observed for ILs group, 0.117±0.015 µmol/L and 0.01±0.002 µmol/L at the end of 24 hr and 48 hr respectively. As observed in case of receptor negative SKOV3 cells wherein there was not much difference in IC_{50} values of DLPLs and ILs, herein a marked decrease in IC_{50} value was observed. This may be due to the antibody mediated uptake by FSHR receptor present on these cells and hence higher uptake of ILs compared to the other formulation in the cell line. At the end of 24 hr, it can be stated that the IC_{50} value of ILs were 8.09, 4.45 and 3.37 folds lower compared to Taxol®, PLs and DLPLs respectively. Similarly, at the end of 48 hr, the IC_{50} value for ILs was found to be 10.8, 7.8 and 3.5 folds lower than the above three treatment groups (Figure 7.13).

It can be summarized that in both the cell line, the PLs, DLPLs and ILs were more effective than Taxol®. When comparing the release profile for all the formulation, in case of liposomes though less amount of drug or incomplete release of drug occurs at the end of 24 hr, the cytotoxic potential was found higher. This may be due to the mechanism of uptake that in case of liposomes occurs by endocytosis, and thus increase in intracellular accumulation of cytotoxic drug occurs in case of lipid based formulations.

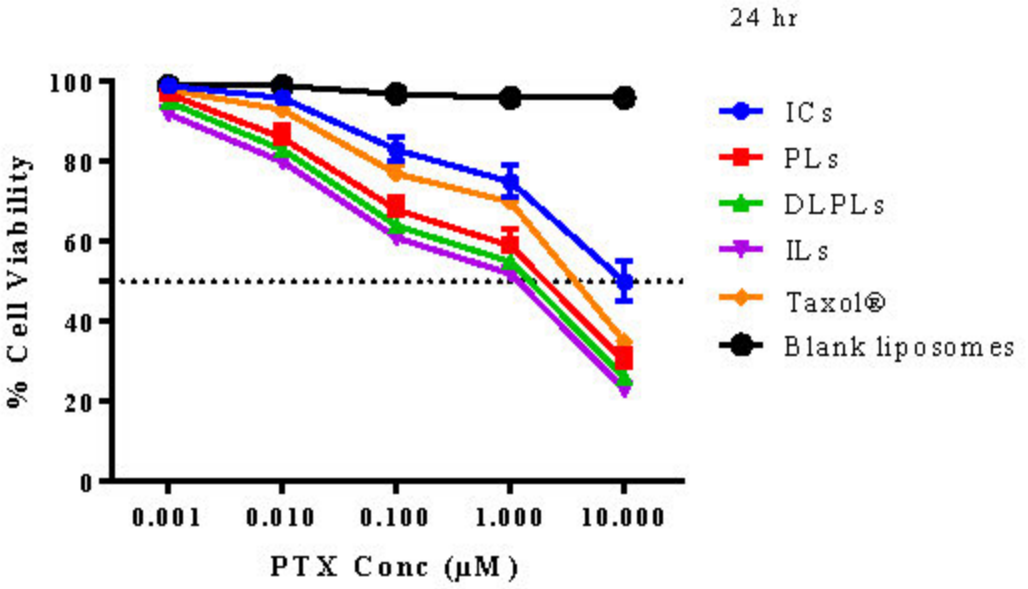


Figure 7.9 % cell viability at various concentrations of Taxol®, ICs, PLs, DLPLs, ILs and blank liposomes at 24 hr in SKOV3 cell line.

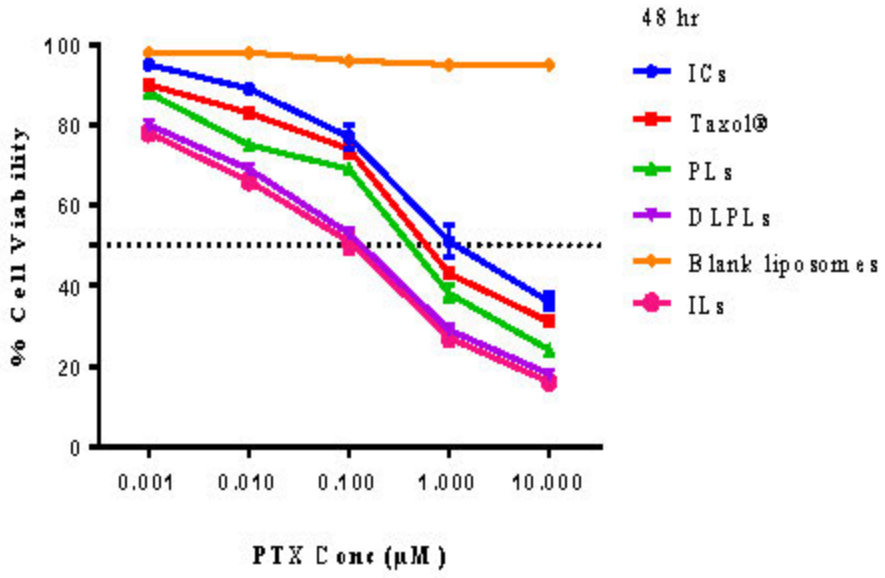


Figure 7.10 % cell viability at various concentrations of Taxol®, ICs, PLs, DLPLs, ILs and blank liposomes at 48 hr in SKOV3 cell line.

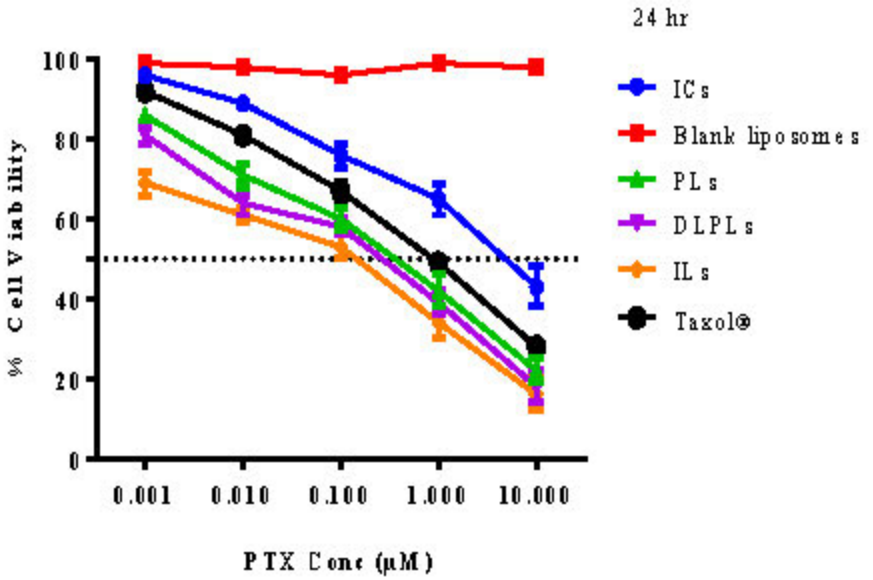


Figure 7.11 % cell viability at various concentrations of Taxol®, ICs, PLs, DLPLs, ILs and blank liposomes at 24 hr in Caov3 cell line.

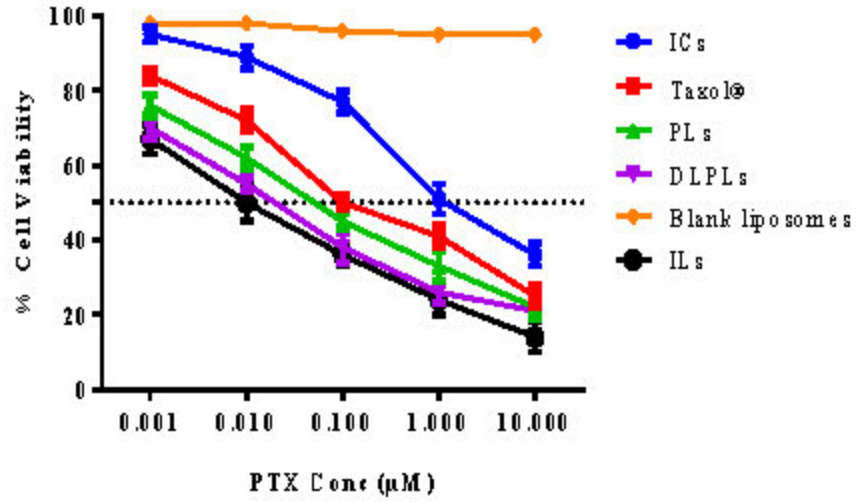


Figure 7.12 % cell viability at various concentrations of Taxol®, ICs, PLs, DLPLs, ILs and blank liposomes at 48 hr in Caov3 cell line

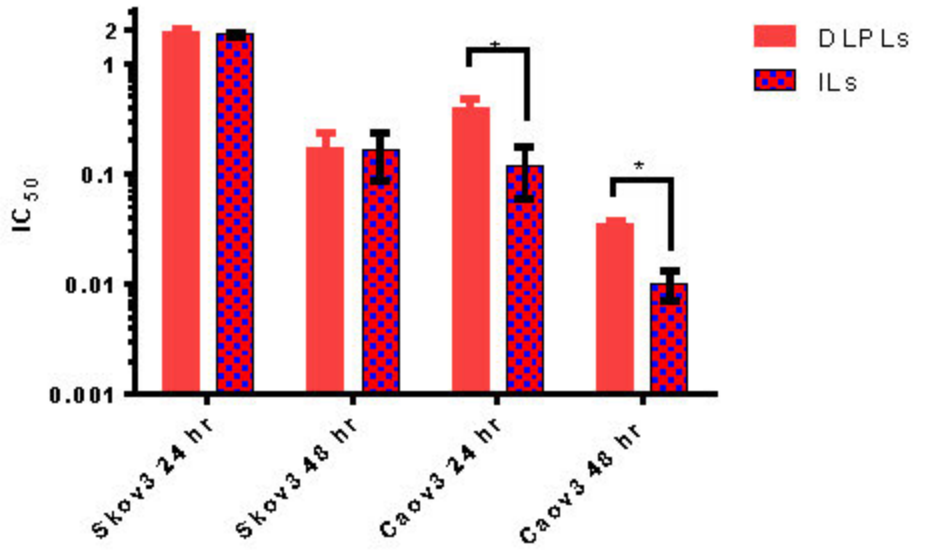


Figure 7.13 Comparative IC50 values for SKOV3 and Caov3 cell line after treatment with DLPLs and ILs at the end of 24 hr and 48 hr

7.4.4 In vitro cell uptake study

7.4.4.1 Preparation of 6-coumarin loaded liposomes

The coumarin loaded liposomes bearing the same composition as the final formulation for PLs and ILs were prepared for comparing the uptake of formulations in SKOV3 and Caov3 cell line.

7.4.4.2 Cell uptake study using confocal microscopy

To prove, whether immune-tagging improves cell specific uptake of liposomes in FSHR expressing cells, cell uptake studies were carried out using confocal microscopy. For demonstrating the cell specific uptake of immunoliposomes, cellular uptake studies of coumarin loaded liposomes was carried out in FSHR expressing cell line (Caov3) and FSHR non-expressing cell line (SKOV3) (10). As it can be noticed, uptake of immunoliposomes was there in SKOV3 cells however it was significantly lower as compared to that in Caov3 cells. This demonstrates that cell specific uptake of immunoliposomes occurs in FSHR-expressing ovarian cancer cells as liposomes surface could be specifically recognized by FSHR (figure 7.14) (11, 12).

To further demonstrate the immune-targeting potential of liposomes, plain (non-targeted) and anti-FSHR antibody targeted coumarin loaded liposomes were prepared and the uptake in Caov3 cell lines was evaluated over time period of 15 min, 30 min, 45 min and 60 min (figure 7.15). As it can be seen the cellular uptake increases in both the cases, however, immunoliposomes show higher uptake potential due to the antibody mediated endocytosis that plays major role in the cellular uptake of immunoliposomes. Non-targeted liposomes are endocytosed at slower pace and low level uptake of non-targeted liposomes may be due to the presence of PEGylation (13). While in case of immunoliposomes, surface decoration with antibody is able to induce endocytosis and thus eliminating the negative effect of PEGylation on cellular uptake. This demonstrates the targeting potential of the liposomes.

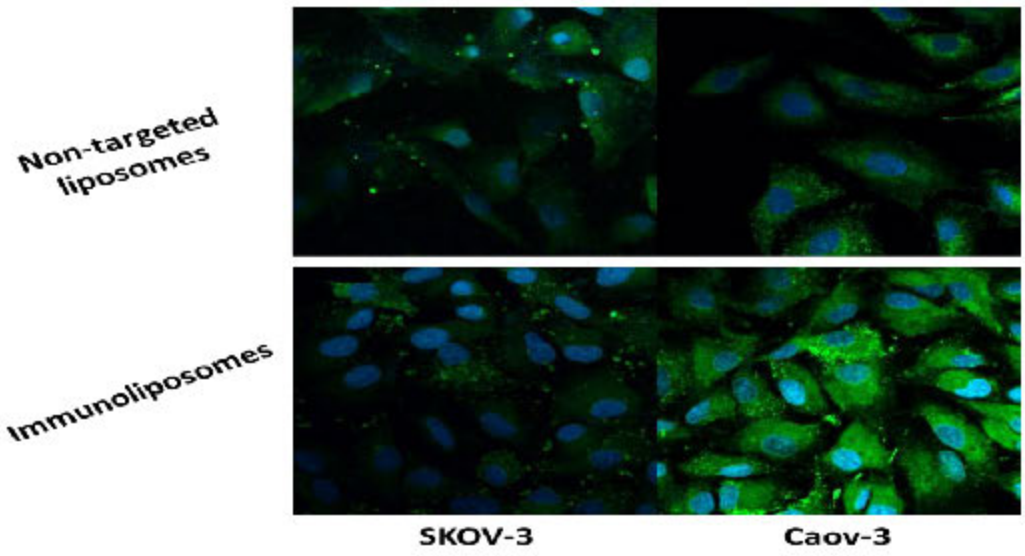


Figure 7.14 Cellular uptake studies of coumarin loaded non-targeted PLs and ILs carried out in FSHR expressing cell line (Caov3) and FSHR non-expressing cell line (SKOV3)

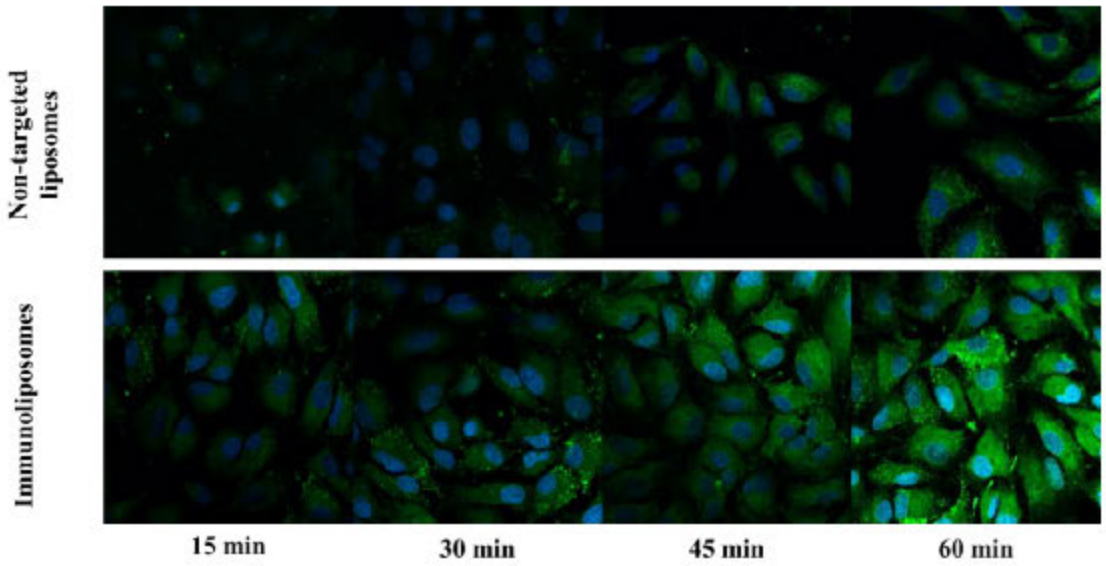


Figure 7.15 Specific uptake of coumarin 6–loaded non-targeted PLs and immunoliposomes (ILs) in FSHR-expressing Caov3 cells incubated with 10 µg/mL of PLs and ILs for 15, 30, 45, and 60 min.

7.4.4.3 Cell uptake study using flow cytometry

Further to evaluate the importance of the cellular uptake of non-targeted and non-targeted liposomes over period of time, time dependent cell uptake studies were carried out using confocal microscopy as well as FACS analysis (figure 7.16 and 7.17).

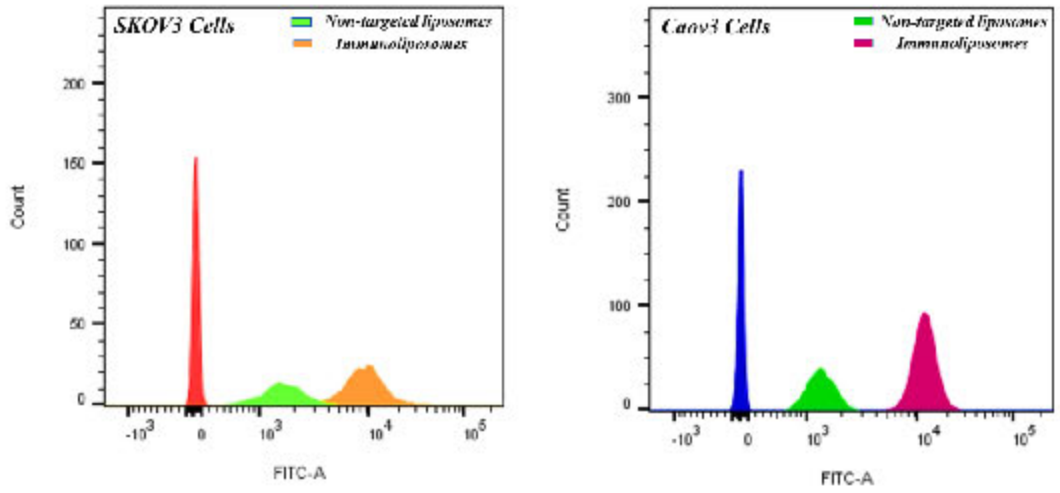


Figure 7.16 FACS study for coumarin loaded non-targeted PLs and ILs carried out in FSHR non-expressing cell line (SKOV3) and FSHR expressing cell line (Caov3).

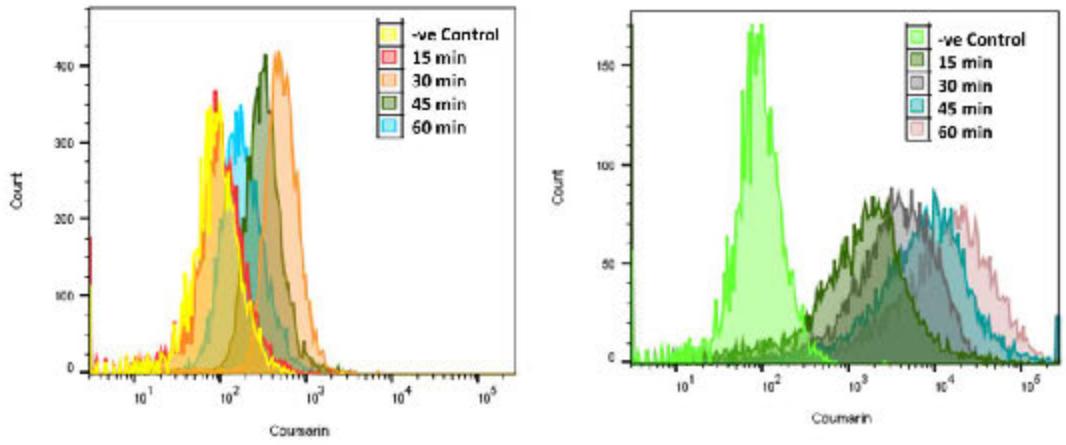


Figure 7.17 Specific uptake of coumarin 6–loaded non-targeted PLs and immunoliposomes (ILs) in FSHR-expressing Caov-3 cells incubated with 10 µg/mL of PLs and ILs for 15, 30, 45, and 60 min.

7.4.5 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 DLPLs and ILs, as evidenced from the Figure 7.18(A), a concentration dependent inhibition of wound recovery was observed. Figure 7.18(B) shows the % wound recovery for the different concentration of treatment with DLPLs and ILs. The recovery observed was concentration dependent ($p<0.01$); lowest for the well with

highest concentration of PTX in ILs i.e. for 10 nM it was 27 ±8 % while the recovery was highest for well treated with 0.01 nM PTX concentration 69 ± 3%. Also, at equimolar concentration of treatment given to the cells by DLPLs and ILs, the % recovery of the wound was 1.3 times and 2.3 times lower than Taxol® respectively indicating the superior performance of liposomal formulation in inhibiting cell proliferation (Figure 7.18(C)). Wound scratch study suggests a marginal superiority of the DLL formulation compared to the marketed one at equimolar concentration, testifying the potential *in-vivo* anti-angiogenic activity.

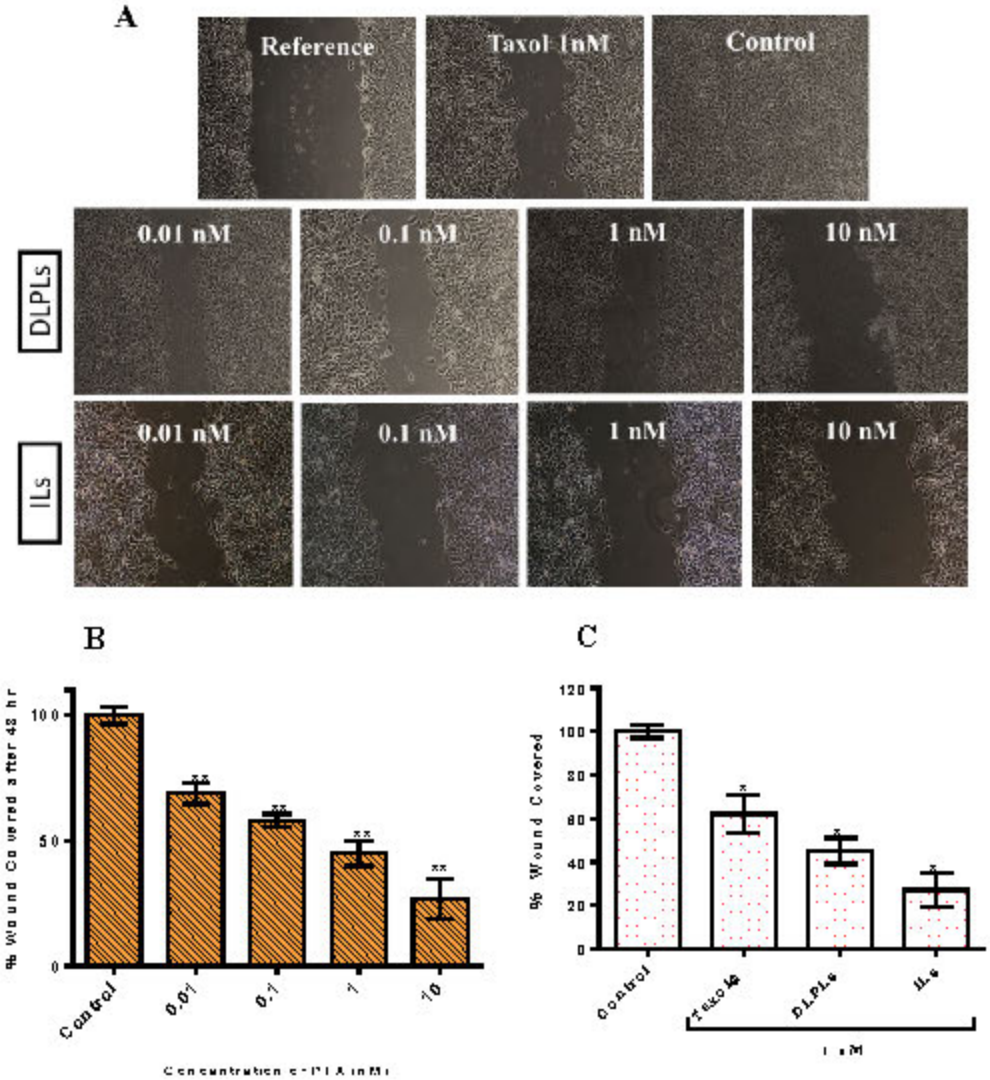


Figure 7.18 (A) Wound healing images in presence of TXT and various concentration of DLPLs. (B) Percent wound covered after 48 hr in comparison to untreated wound at 0 hr for ILs at various concentrations. ** P < 0.01 vs untreated as control. (C) Percent wound covere

7.4.6 Cell death analysis using flow cytometry

Flow cytometry is a valuable tool for the simultaneous assessment of necrosis and apoptosis in a single population of cells. Necrosis is detected by measuring the permeability of the plasma membrane to a normally impermeable fluorescent dye, such as the DNA-binding dye propidium iodide (PI). The quantitation of cell death after treatment with various formulations was carried out by FACS after staining with propidium iodide (Figure 7.19 – 7.23). A total of 8000 events were collected and analysed in FACS software. Figure 7.24 shows representative data of cell death in SKOV3 and Caov3 cells after 24 hr. As can be inferred from the data that there is a rightward shift of the FACS plot due to the higher amount of the PI taken up by dead cells giving fluorescent signals. From the values obtained for SKOV3 cells and Caov3 cells, the highest amount of cell death was seen in ILs group for Caov3 cells (Table 7.3). Further, there was a non-significant increase in the cell death for Taxol® in the two cell-lines (Figure 7.25). It was also noted that there was an initial higher percent of cell death in the untreated cells and was considered for measuring the statistical significance between the values obtained for Caov3 cells in comparison to SKOV3 cells.

Table 7.3 Percentage of cell death after treatment of Caov3 cells with various formuations at equimolar concentration

Treatment	*SKOV3*		*Caov3*	
	24 hr (Mean±SD)	***48 hr (Mean±SD)***	***24 hr (Mean±SD)***	***48 hr (Mean±SD)***
Untreated cells	0.56 ± 0.12	1.24 ± 0.36	0.98 ± 0.23	2.24±0.92
Taxol® 2nM	6.21 ± 0.36	8.42 ± 1.05	13.67 ± 1.36	15.84±2.15
DLPLs 2nM	7.14 ± 1.26	9.82 ± 1.54	15.44 ± 2.69	19.75±3.98
ILs 2nM	7.51 ± 1.34	11.60 ± 2.66	22.23 ± 3.51	33.79±4.23
Values are mean±SD, n = 3				

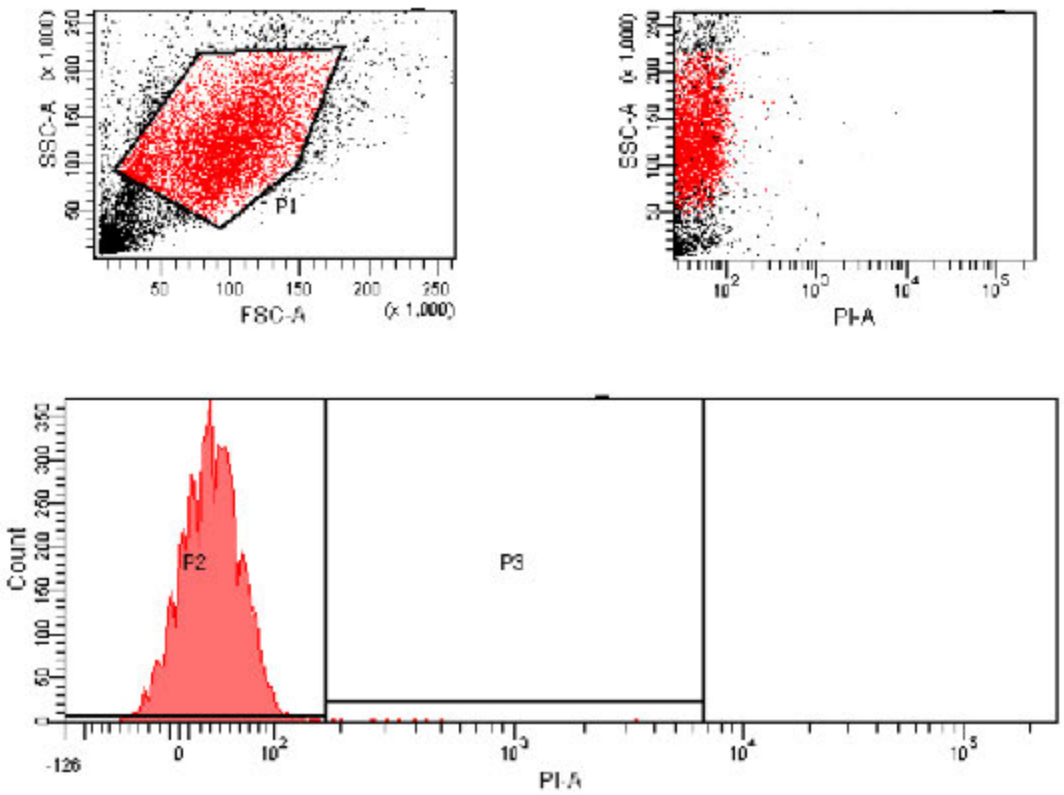


Figure 7.19 FACS for untreated Caov3 cells

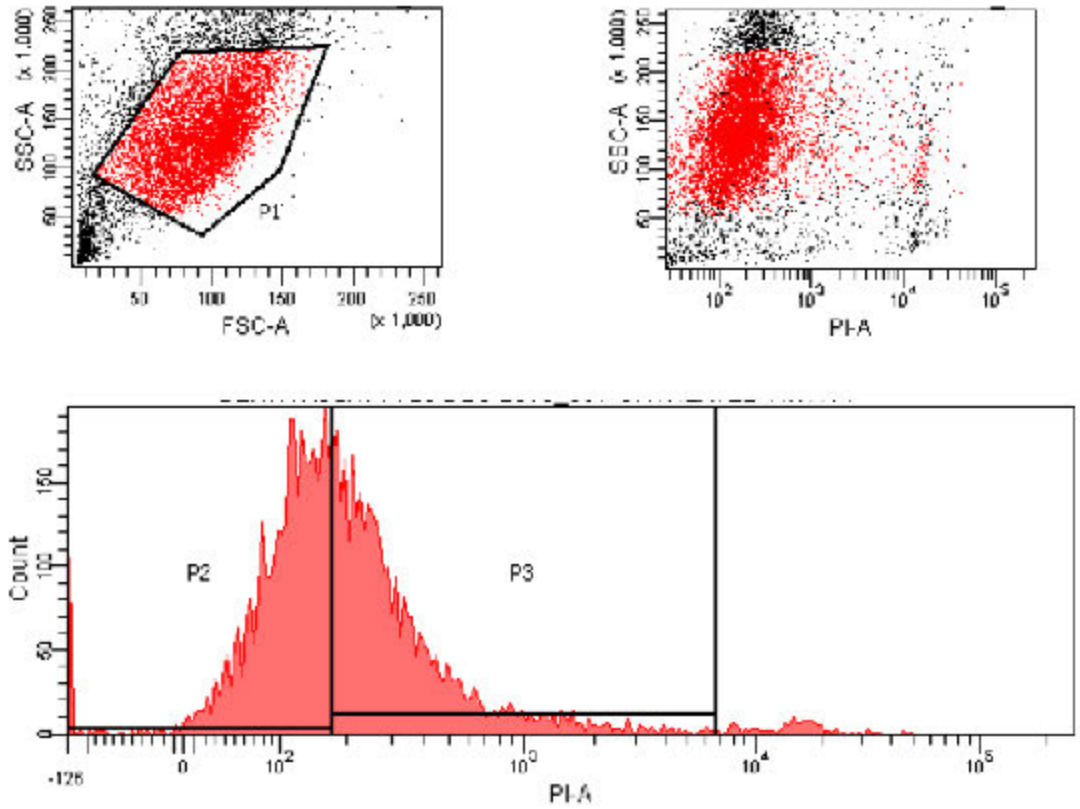


Figure 7.20 FACS for Caov3 cells treated with PI

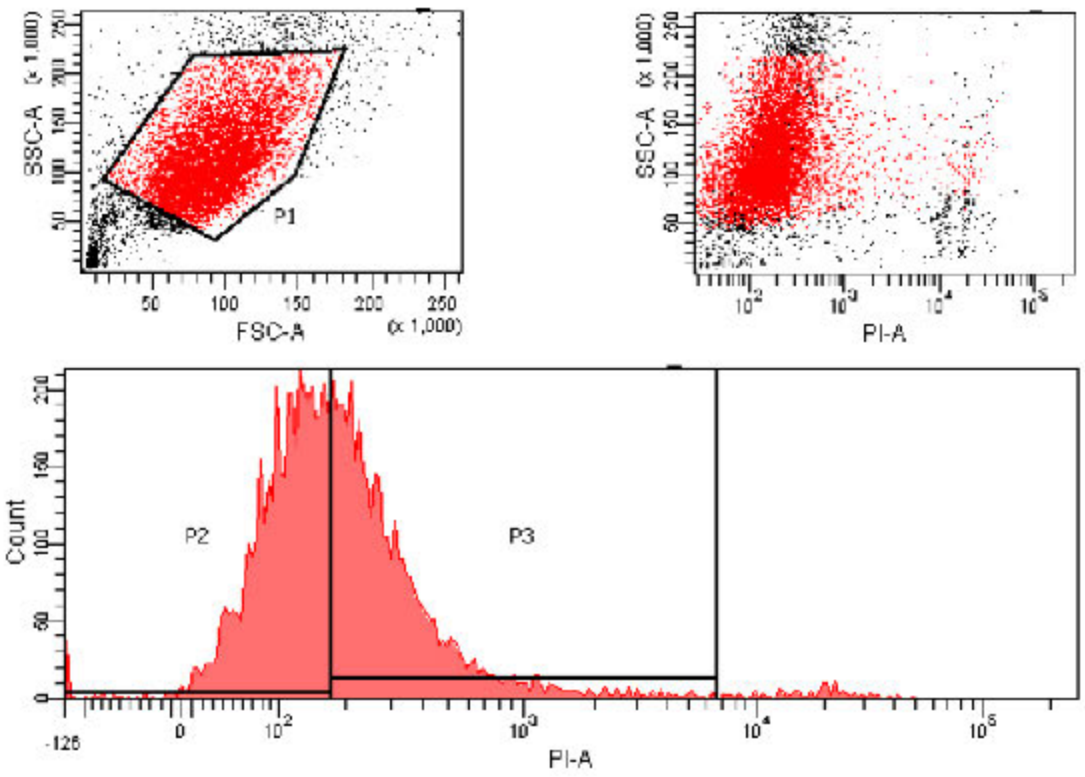


Figure 7.21 FACS for Caov3 cells treated with 2nM Taxol®

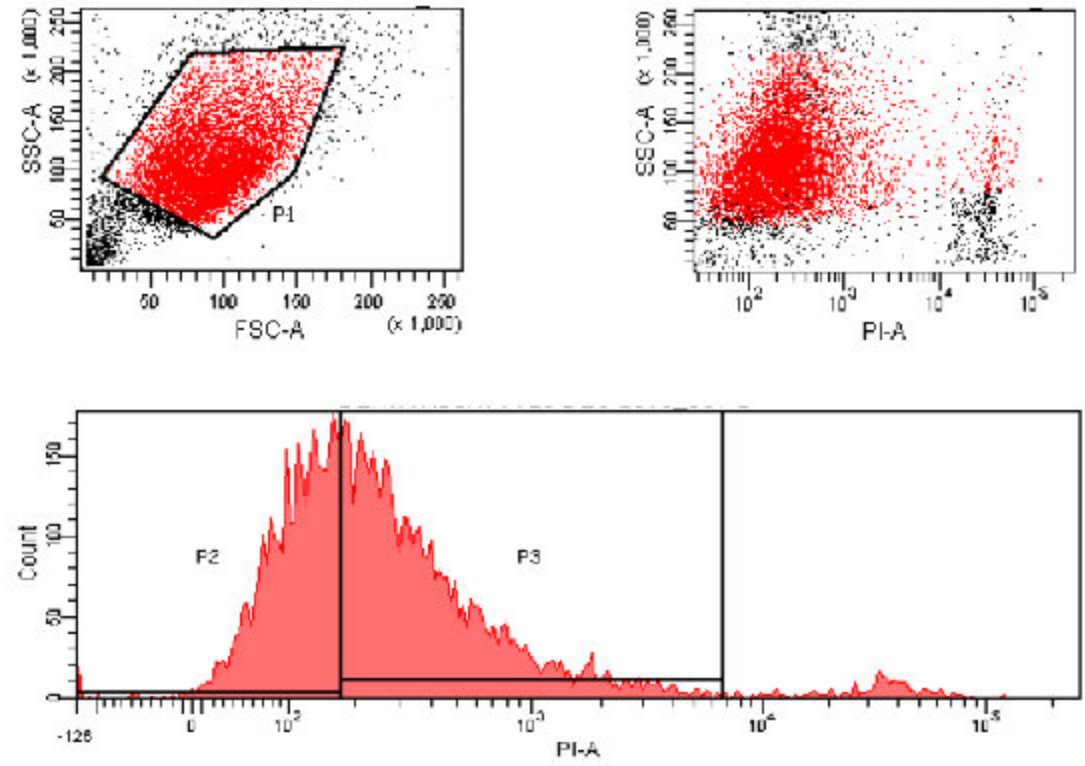


Figure 7.22 FACS for Caov3 cells treated with 2nM DLPLs

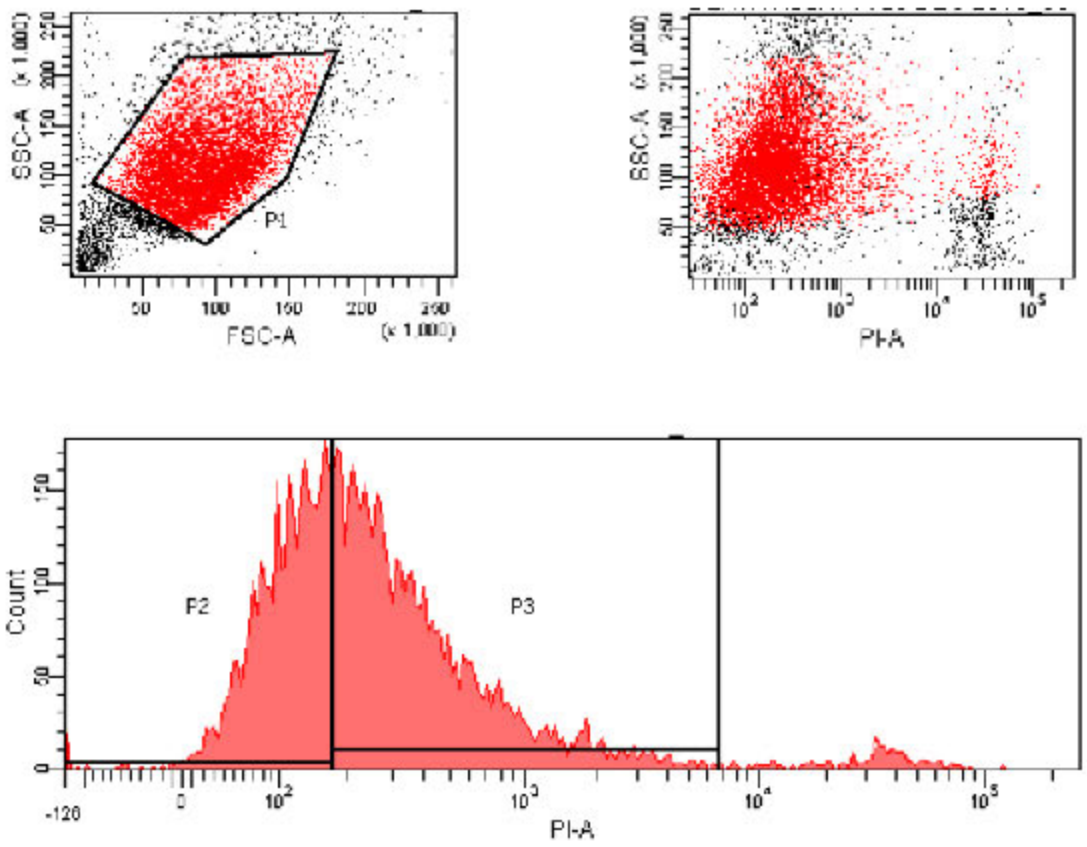


Figure 7.23 FACS Caov3 for cells treated with 2nM ILs

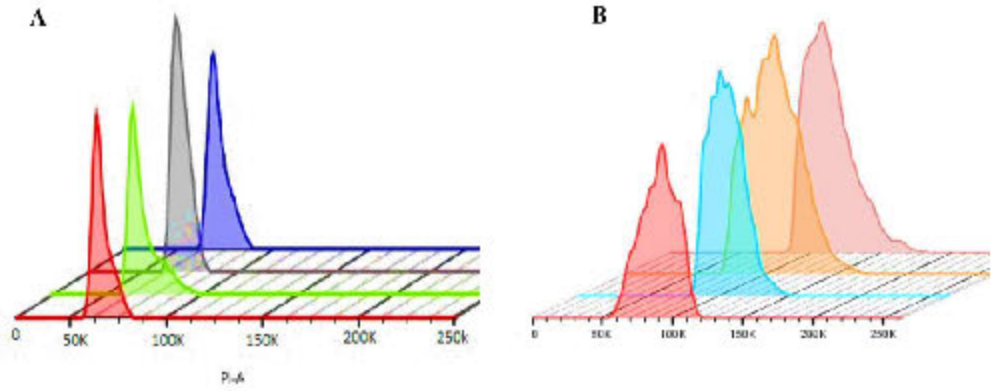


Figure 7.24 (A) % SKOV3 and (B) Caov3 cell death after treatment with 2 nM Taxol, DLPLs and ILs for a period of 24 hr. (representative data)

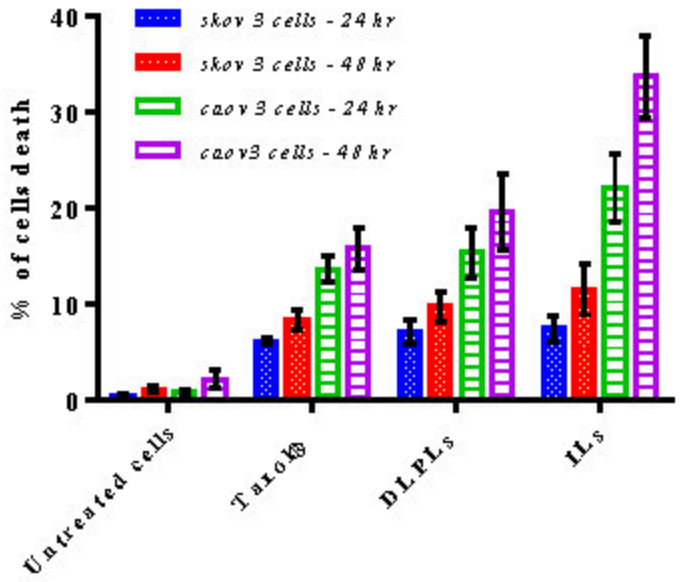


Figure 7.25 % cell death after 24 and 48 hr treatment with different formulations in SKOV3 and Caov3 cells

We observed about 6.21±0.36%, 7.14±1.26% and 7.51±1.34% of cell death in SKOV3 cells after 24 hr of treatment with Taxol®, DLPLs and ILs respectively as compared to 0.56±0.12% of untreated cells. Thus, no significant difference was found between the three treatment groups at the end of 24 hrs. After 48 hr, 8.42±1.05%, 9.82±1.54 and 11.60± 2.66% of cells were found dead compared to untreated cells (1.24±0.36%). Thus, an increase of approximately, 2% each for Taxol® and DLPLs and 6% for ILs group in the amount of cell death was observed. No significant difference in cell death between the Taxol® and DLPLs after 48 hr of treatment was observed.

Similarly, for Caov3 cells, we observed about 13.67±13%, 15.44±2.69% and 22.23±3.51% of cell death after 24 hr of treatment with Taxol®, DLPLs and ILs respectively. Whereas, after 48 hr of treatment 15.84±2.15%, 19.75±3.98% and 33.79±4.23% of cell death was found. There was an insignificant increase in the amount of apoptotic cells in the Taxol® treated cells after 24 hr and 48 hr respectively. Further, at the end of 24 hr there was insignificant difference in apoptotic cell between the Taxol® and DLPLs group also.

The increase in the amount of cells that underwent cell death when treated with ILs for Caov3 cells is due to presence of FSHR on cell surface leading to higher

uptake of the formulation. The low percentage of apoptosis in SKOV3 cells using ILs also substantiate that the cell line very transiently if at all expresses FSHR.

7.4.7 Cell cycle analysis

Paclitaxel causes mitotic arrest by stabilizing the microtubules and thus preventing its disassembly. This is accompanied by increase in cell stress and finally the cells undergo apoptosis (Moos and Fitzpatrick 1998). We have performed flow cytometry to evaluate the effectiveness of the various formulation of PTX on the cell cycle in two cell lines. The main cell cycle step that indicates arrest of cells is G2/M. Each treatment was done at concentration of PTX equivalent to 2nM (Figure 7.26-7.33).

To study the effect of formulation on cell cycle, skov3 and caov3 cells were treated with 2 nM of Taxol®, DLPLs and ILs. The treatment was done for a period of 24 hr and 48 hr and data for cell cycle analysis at both the time points were acquired to study the population of cells in various stages of cell cycle. The percentage of cells in various stages that are gated are presented in figure 7.34 to figure 7.37 and are represented graphically in figure 7.38 and figure 41.

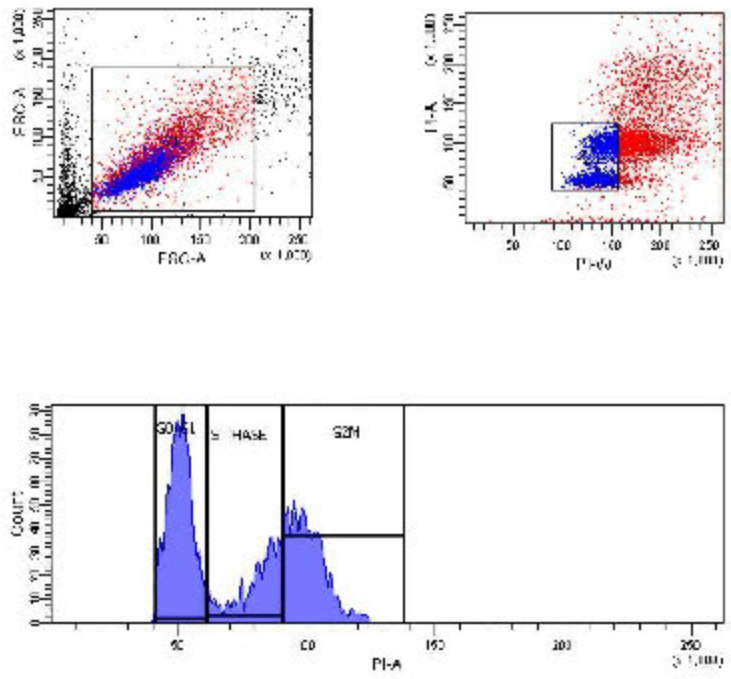


Figure 7.26 Cell cycle analysis of control sample cells at 24 hr

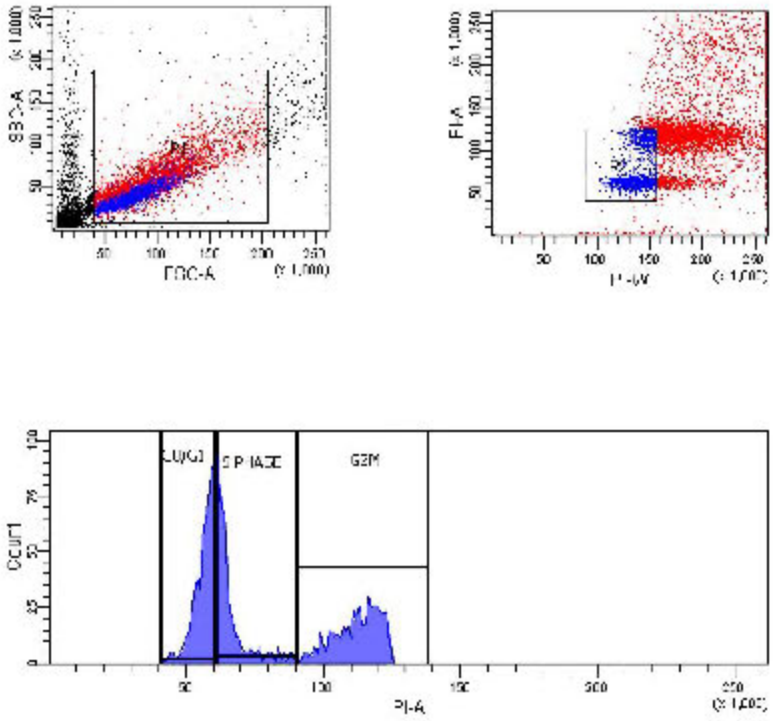


Figure 7.27 Cell cycle analysis of Caov3 cells for Taxol® at 24 hr

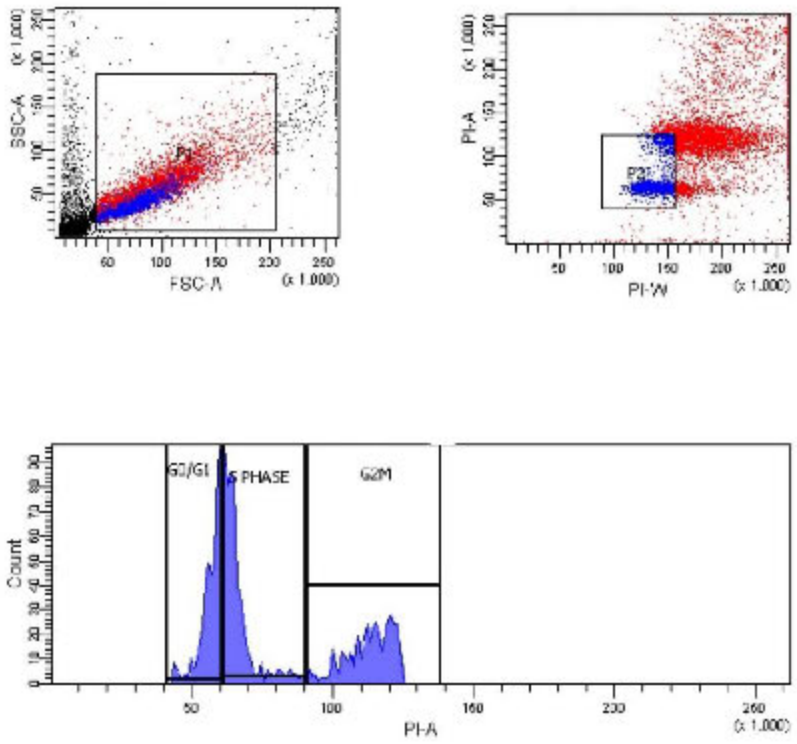


Figure 7.28 Cell cycle analysis for Caov3 cells for DLPLs at 24 hr

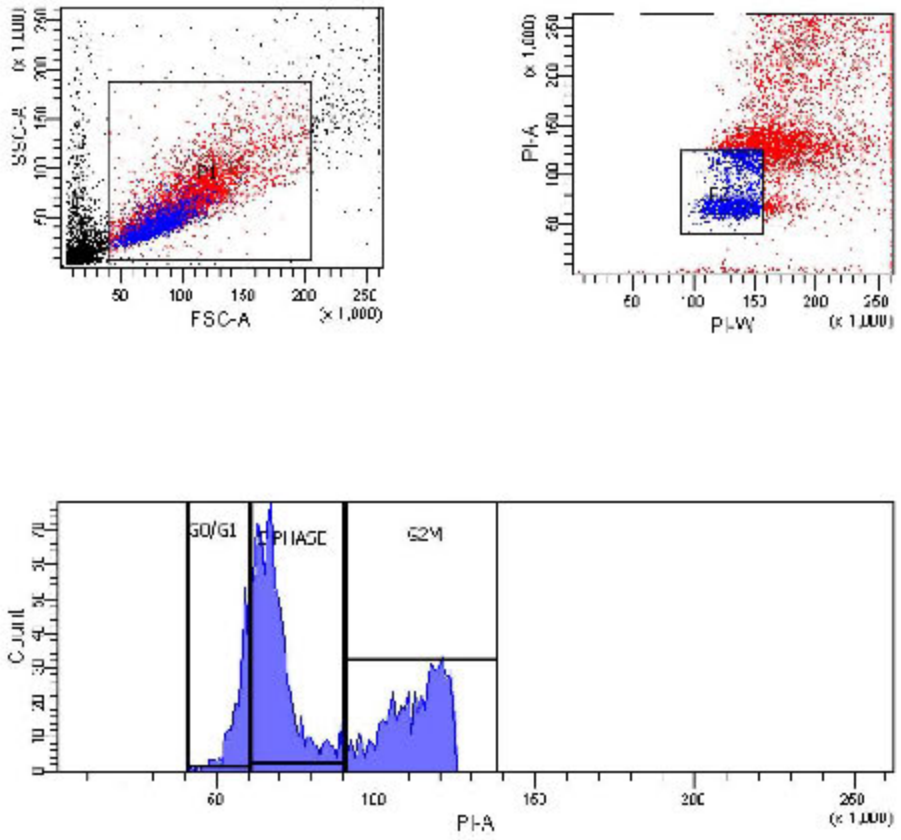


Figure 7.29 Cell cycle analysis for Caov3 cells for ILs at 24 hr

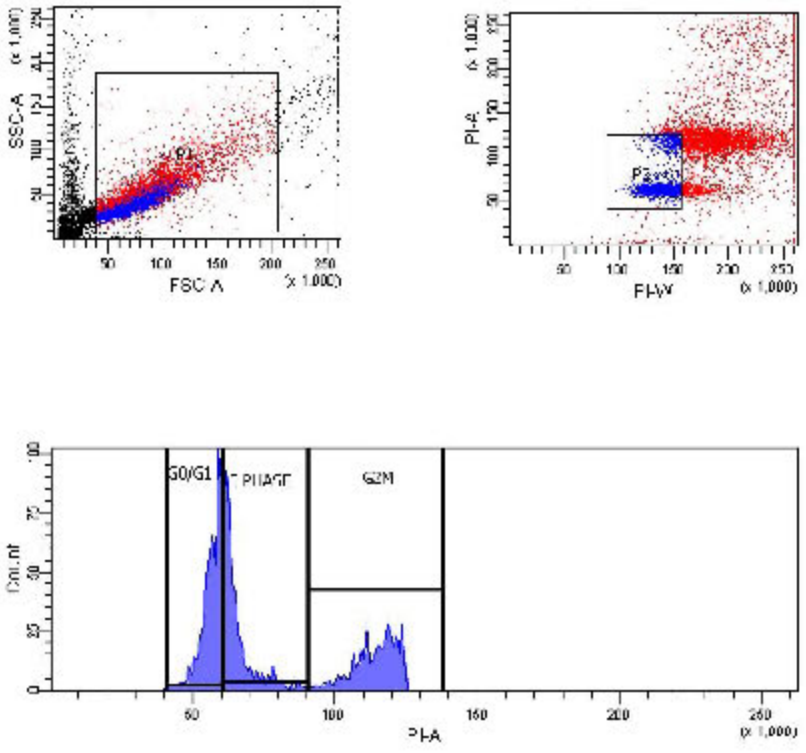


Figure 7.30 Cell cycle analysis for Caov3 cells for control cells at 48 hr

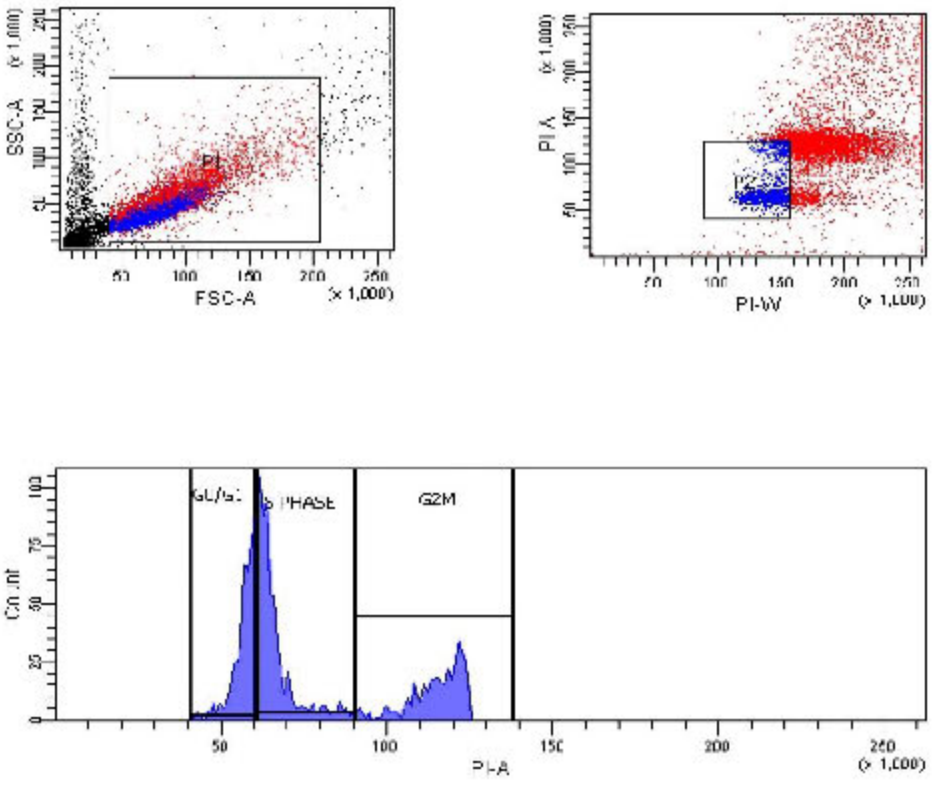


Figure 7.31 Cell cycle analysis for Caov3 cells for Taxol® at 48 hr

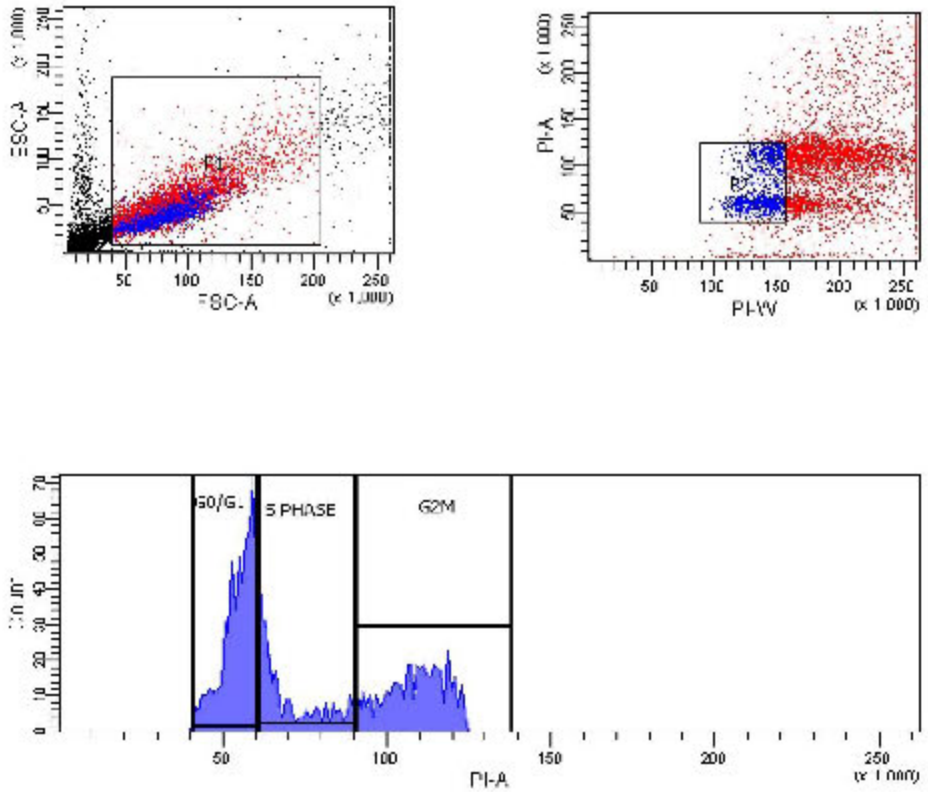


Figure 7.32 Cell cycle analysis for Caov3 cells for DLPLs at 48 hr.

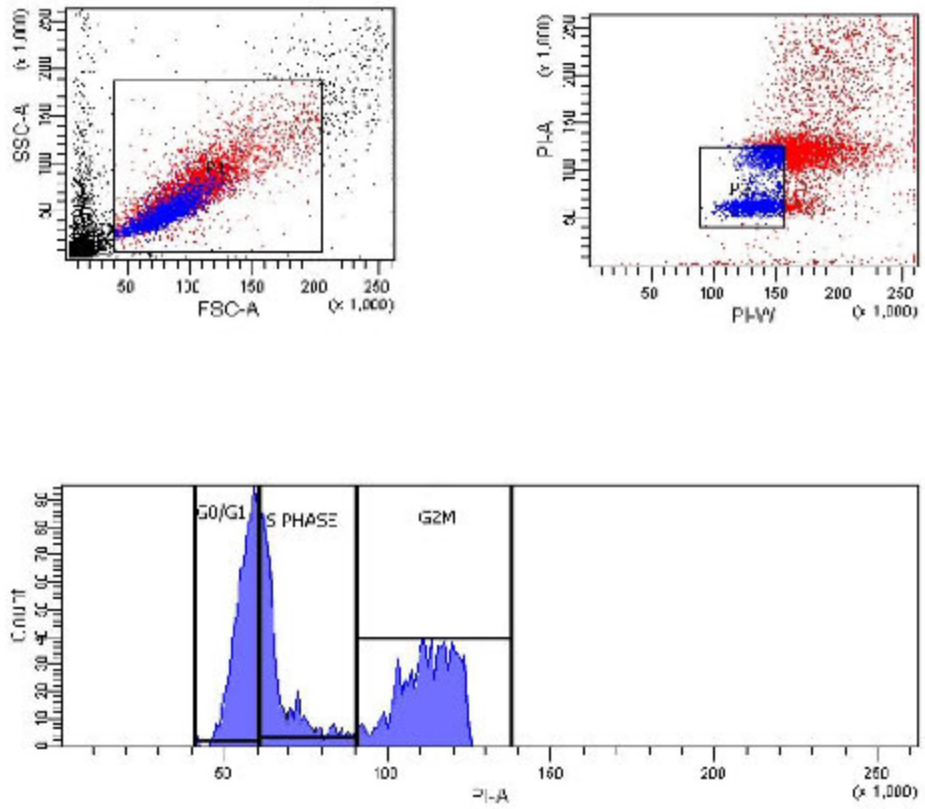


Figure 7.33 Cell cycle analysis for Caov3 cells for ILs at 48 hr

The statistics of the cells after treatment with the three formulation at end of 24 hr and 48 hr in skov3 cells can be summarized as follows (% of cells are approximated to whole value): For skov3 cell line, for Taxol® the amount of cells in G2/M increased by 16 % at the end of 24 hr and 6 % at the end of 48 hr. In case of prepared liposomal formulation, the effect was more pronounced leading to an increase to 21% and 11% for DLPLs and 20% and 12% for ILs respectively at the end of 24 hr and 48 hr. In all the cases increase in G2/M stage population was accompanied by a decline in cell population in G0/G1 stage. Further, an increase in the cell death was observed indicated by the cells in subG1 stage of cell cycle. The percentage of cells in S phase in the three-treatment group varied to a low extent. The similar performance of DLPLs and ILs may be due as stated previously, the absence of receptors for FSH.

Evaluation of cell cycle in the caov3 cell line, which is a receptor positive cell line indicated superior results of arrest of cells in G2/M phase. for Taxol® the amount of cells in G2/M increased by 13 % at the end of 24 hr and 6 % at the end of 48 hr. In case of prepared liposomal formulation, the effect was more pronounced leading to an

increase to 22% and 13% for DLPLs and 28% and 16% for ILs respectively at the end of 24 hr and 48 hr. Here also, in all the cases increase in G2/M stage population was accompanied by a decline in cell population in G0/G1 stage. Further, an increase in the cell death was observed indicated by the cells in subG1 stage of cell cycle. The percentage of cells in S phase in the three-treatment group varied to a low extent. One thing is to be noted is that of the three-formulations tested in two cell line only the performance of non-targeted liposomes i.e. DLPLs were similar in both the cell line leading to almost around 22% and 12% of arrest in G2/M stage, however, for targeted liposomal formulation i.e. ILs, the amount of cells in G2/M was highest for cao3 cells at the end of 24 hr and 48 hr indicating that the targeted formulation was able to better get internalized in the cells due to receptor mediated uptake.

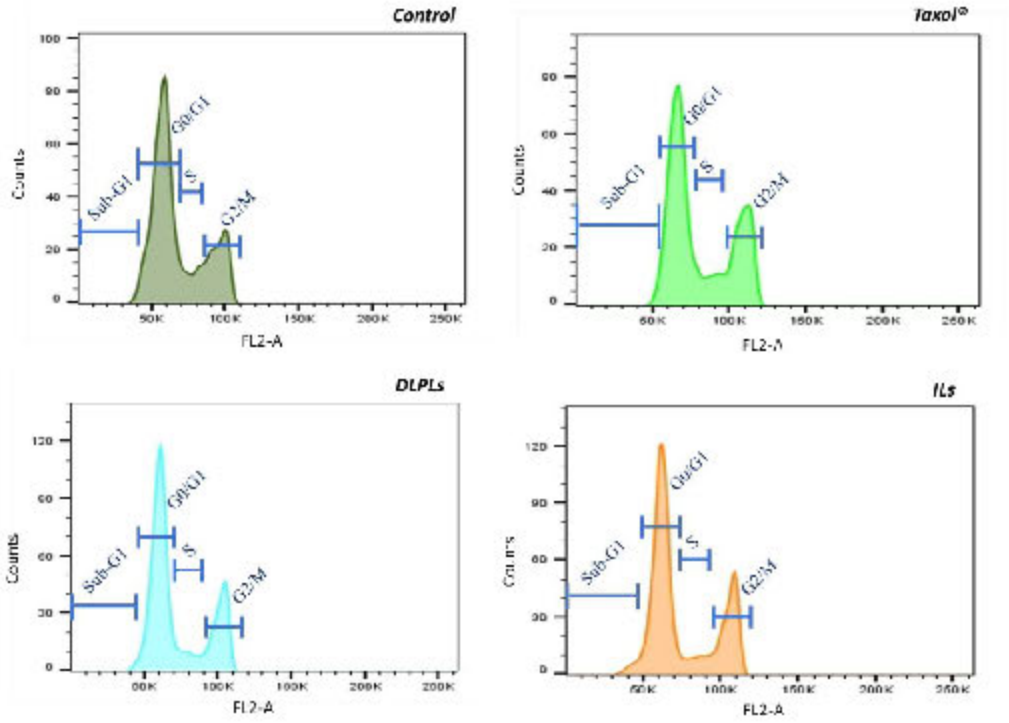


Figure 7.34 Cell cycle analysis of Taxol®(PS), PLs and ILs in Skov3 cell line after 24 hr.

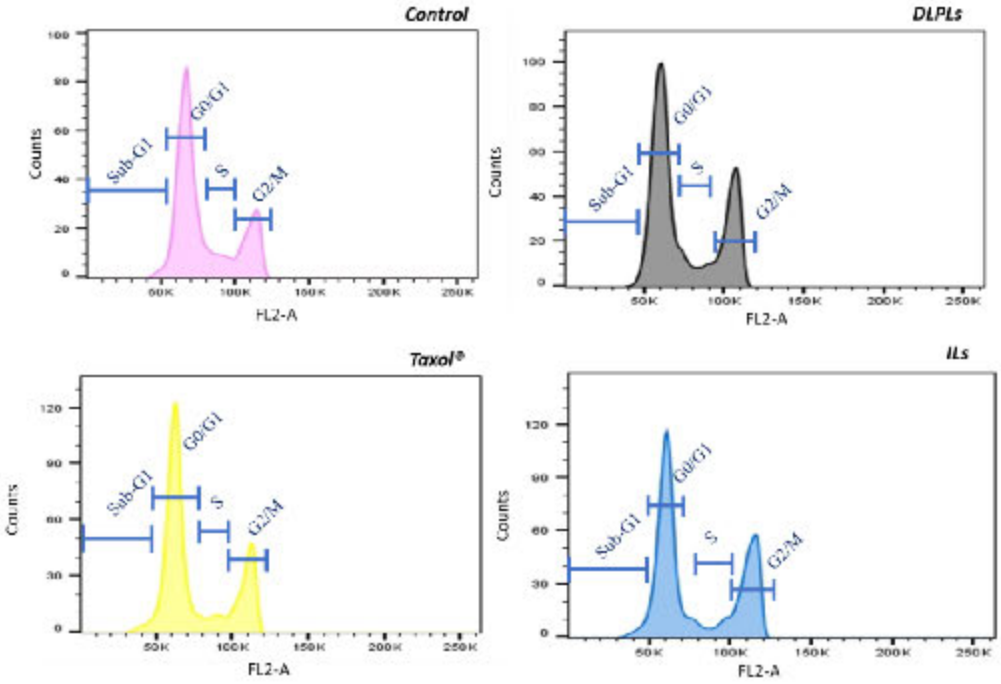


Figure 7.35 Cell cycle analysis of Taxol®(PS), PLs and ILs in Skov3 cell line after 48 hr.

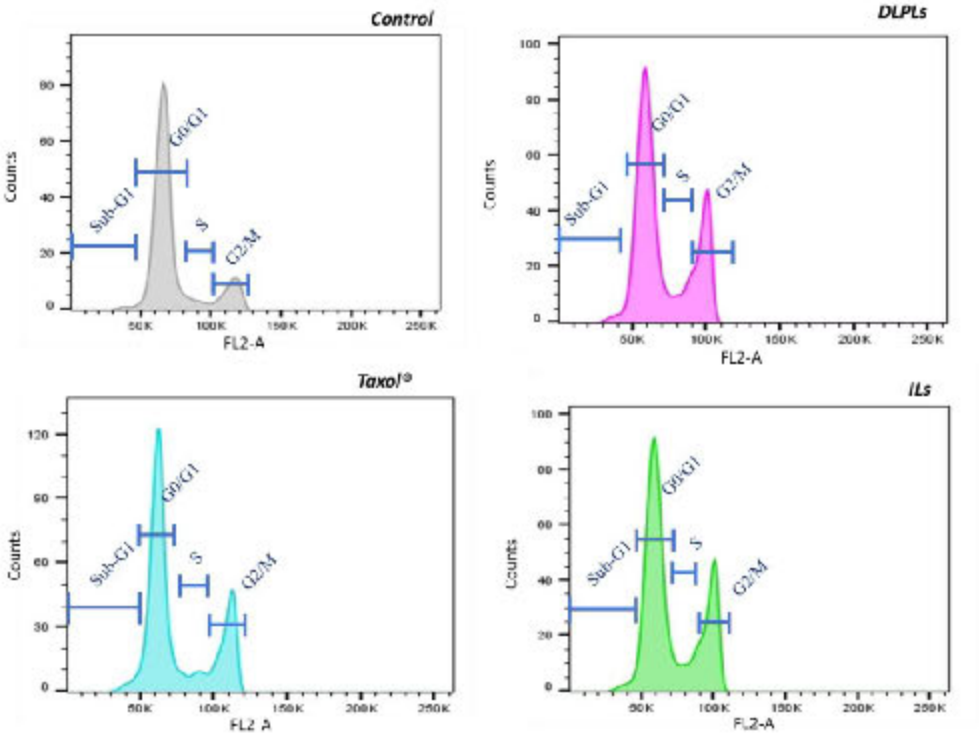


Figure 7.36 Cell cycle analysis of Taxol®(PS), PLs and ILs in Caov3 cell line after 24 hr.

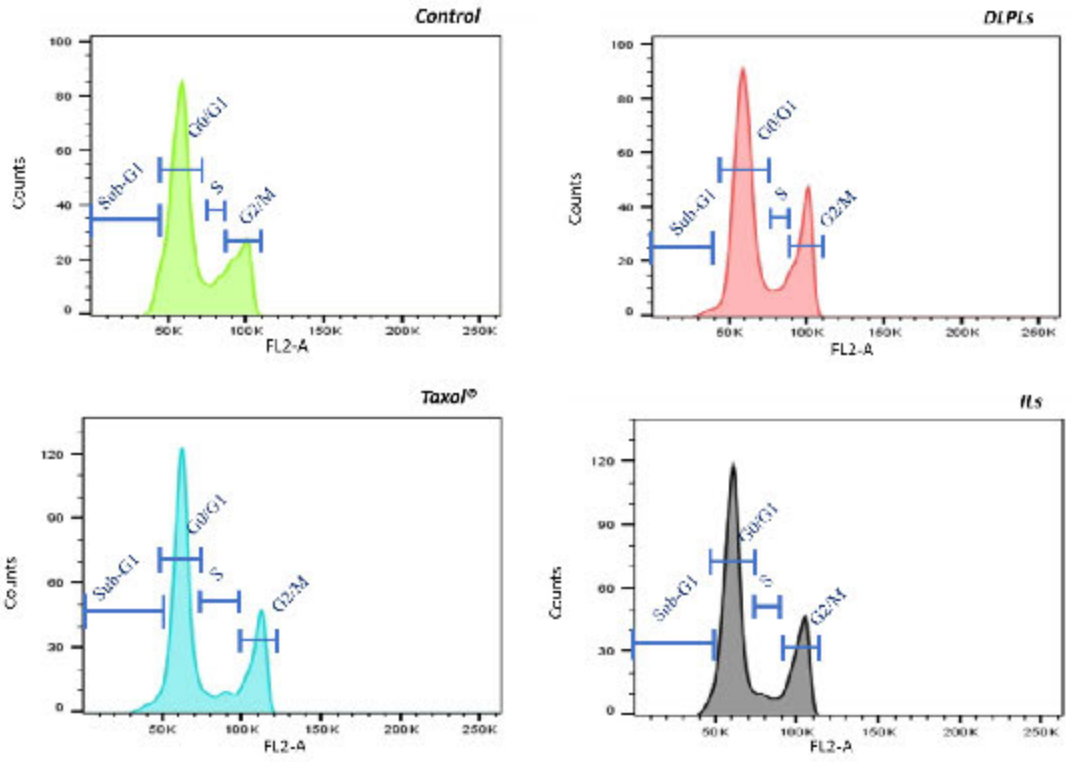


Figure 7.37 Cell cycle analysis of Taxol®(PS), PLs and ILs in Caov3 cell line after 48 hr.

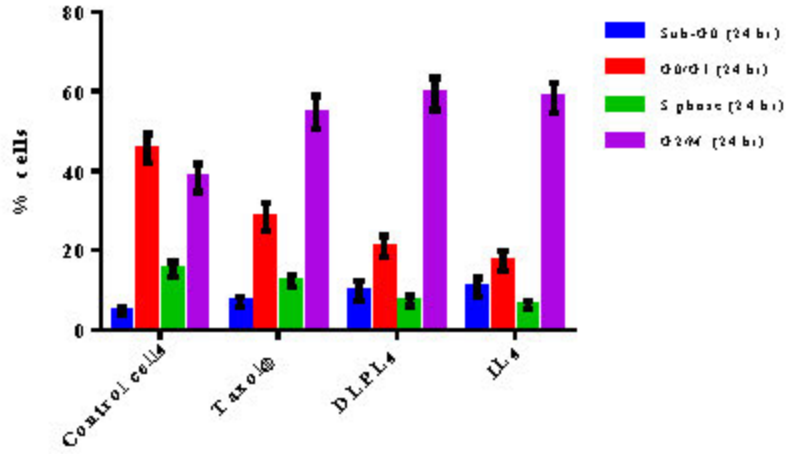


Figure 7.38% Cell in various phase of cell cycle after 24 hr treatment of skov3 cells with Taxol®(PS), DLPLs and ILs.

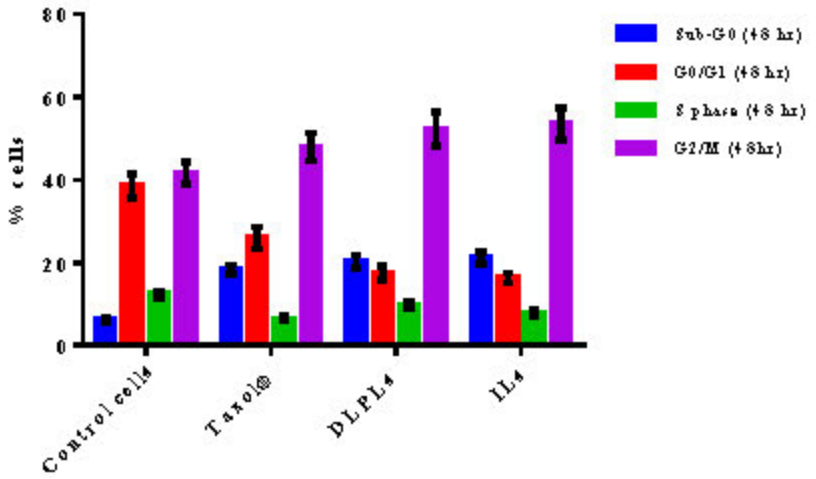


Figure 7.39 % Cell in various phase of cell cycle after 48 hr treatment of skov3 cells with Taxol®(PS), DLPLs and ILs.

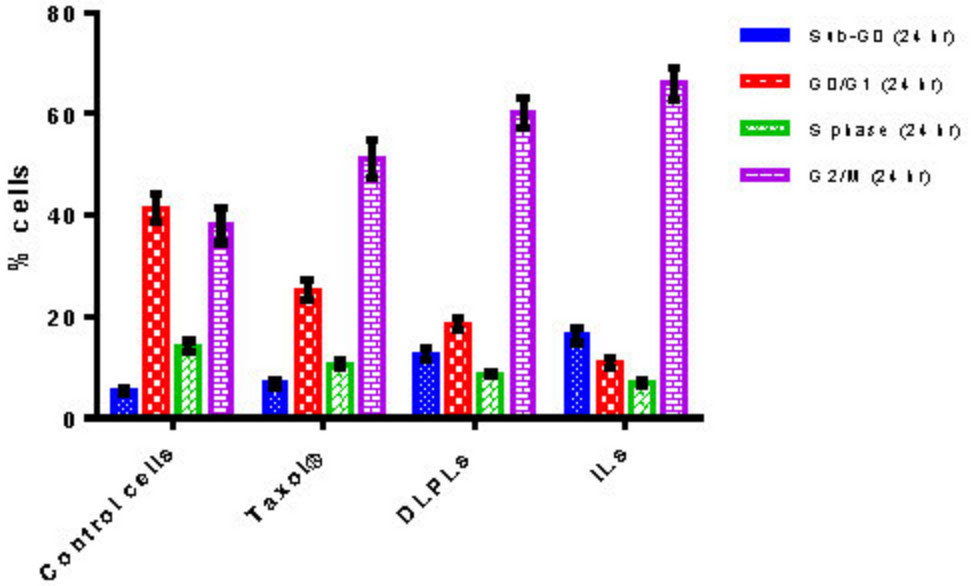


Figure 7.40 % Cell in various phase of cell cycle after 24 hr treatment of caov3 cells with Taxol®(PS), DLPLs and ILs

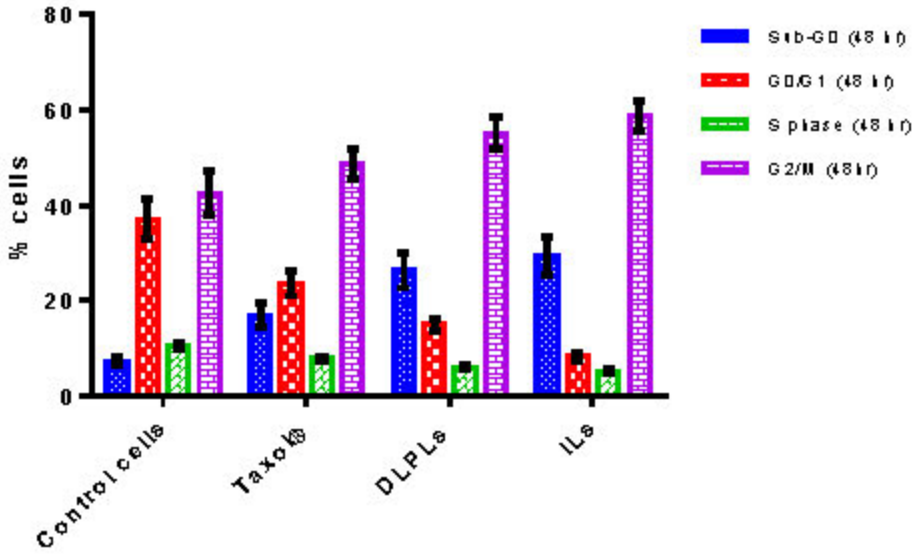


Figure 7.41 % Cell in various phase of cell cycle after 48 hr treatment of Caov3 cells with Taxol®(PS), DLPLs and ILs

7. 5 References

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