6.1 Introduction

The cell cytotoxicity of the excipients used in formulation of solid lipid nanoparticles was investigated using MTT (3-(4,5dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) dye reduction assay method on the Human epithelial colorectal adenocarcinoma (Caco-2) cell line. Different cofncentrations of SLNs in a wide range were tested for cell toxicity in order to estimate any cell death at high SLNs concentrations. **MTT assay** is a colorimetric assay for determining the viable cell count depending on the mitochondrial dehydrogenase activity measurement. The MTT (3-(4,5dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole dye) is taken up and reduced inside the living cells which have mitochondrial dehydrogenase (reductase) enzyme activity intact. This reduction produces a purple colored formazan compound that gives a direct estimate of number of viable cells when measured spectrophotometrically (1, 2).

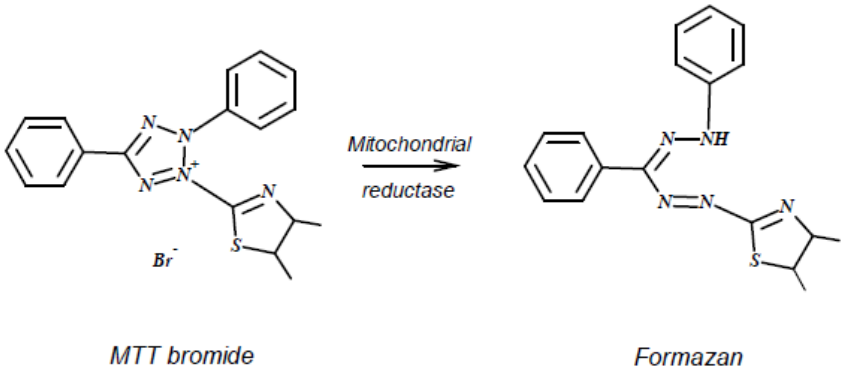

Figure 6.1 MTT dye reduction by mitochondrial reductase enzyme of viable cells

Formazan formed so is water insoluble, so solubilization of formazan precipitates is done using chemicals like dimethyl sulfoxide (DMSO), an acidified ethanol solution or a surfactant (sodium lauryl sulfate) 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 by culture. This in turn can be useful in determining the cytotoxicity of various toxins or chemicals of medical interest as these agents will cause reduction in the viable cell count by killing the

cells of culture or by inhibiting the proliferation of cells when cells are incubated with them.

Another cell line study carried out was the **identification of endocytic pathways for solid lipid nanoparticles of Darunavir and Atazanavir sulfate into the Caco-2 cells**. Nanoparticles present in the external environment of cells can interact with the intestinal cells which can lead to uptake of the nanoparticles through various mechanisms. Understanding the interaction of nanoparticles with the intestinal epithelium as a portal of entry into the body is essential in the area of drug delivery as this interaction dictates the release of drug in cytoplasm of intestinal cells or the transport of intact nanoparticles through the intestinal cells and entry into lymphatic system. There has been a recent explosion of interest in exploring the uptake mechanism of nanosized systems. The nanoparticles are already been reported to be taken up intact by the M-cells of peyer's patches in the intestinal region associated with the lymphatic tissue (3). In addition, nanoparticles have been reported to be transported via various endocytic pathways (4). Endocytosis, in general, includes four major categories: clathrin- mediated endocytosis, caveolae mediated endocytosis, macropinocytosis and phagocytosis (5). The various physicochemical characteristics of nanoparticles influencing their interaction with the epithelium comprise of nanoparticles material (6), size (7), surface charge (8) and surface chemistry (9). Almost an infinite number of possible combinations of these parameters of nanoparticles are possible and their effect on membrane interactions needs to be studied. Moreover, the specific endocytic pathway involved in uptake of nanoparticles can be expected to affect nanoparticles intracellular localization and trafficking. Understanding the endocytic mechanisms is then crucial for the development of nanoparticles for clinical therapies. More than one type of endocytosis are often involved in uptake of nanoparticles (4). Such identification of the specific type of endocytosis pathway is possible by use of various pharmacologic inhibitors for endocytosis that includes various chemicals and biological agents. These inhibitors block a specific endocytic pathway to confirm whether it is employed by the nanoparticles to enter cells. All endocytic pathways are energy dependent processes and can be inhibited by low temperature and an ATPase inhibitor like sodium azide (10). With respect to specific endocytosis mechanism, examples of inhibitors includes use of hypertonic

sucrose (0.4-0.5 M) (4) and chlorpromazine (50-100 µM) (11) for clathrin mediated endocytosis, filipin (12) and nystatin (13) for caveolae mediated endocytosis while amiloride, cytochalasin D and rottlerin for macropinocytosis (14). Hence, for developed Darunavir and Atazanavir sulfate SLNs, uptake study was done at 37ºC to determine whether active uptake of nanoparticles is involved or not. Moreover, in order to reveal specific endocytosis pathways involved in uptake of developed nanoparticles, study was also conducted in presence of chlorpromazine, nystatin and amiloride as specific inhibitors for clathrin mediated endocytosis, caveolae mediated endocytosis and macropinocytosis respectively.

The **apperant permeability of Darunavir loaded SLNs and Atazanavir sulfate loaded SLNs** were also determined using Caco-2 cells. Caco-2 permeability study is a method used for predicting the *in-vivo* absorption of drugs across the gut wall by measuring the rate of transport of a compound across the caco-2 cell line. The caco-2 cell line forms confluent monolayers of tight junctions after differentiation of 14-21 days which exhibits similar properties to intestinal epithelial cells. Intestinal absorption of orally administered drugs occurs via three major pathways: passive diffusion, carrier mediated and vesicular transport. The Caco-2 cells encompass all of these pathways. Caco-2 cells grown on permeable filters (Figure 6.2) have become the golden standard for *in-vivo* prediction of intestinal drug permeability and absorption. In present study, the permeable filter used was polycarbonate membrane present in the Transwell® inserts (Nunc, Denmark). Moreover, for permeability assessment, the prime requirement is the integrity of the cells upon differentiation. To evaluate it, Lucifer yellow is widely used. Lucifer yellow travels across caco-2 cell monolayers only through passive paracellular diffusion (through spaces between cells) and has low permeability. Therefore it is not able to pass across cell monolayers when tight junctions between cells are maintained. Permeability of Lucifer yellow of <= 5-12 nm/s has been reported to be indicative of well established caco-2 monolayers (15).

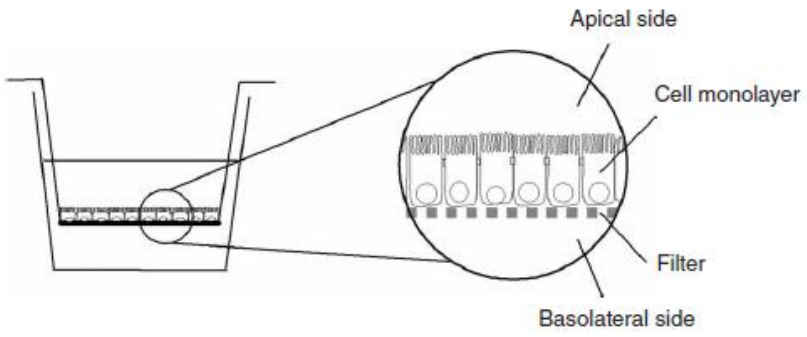

Figure 6.2 Diagram of Caco-2 monolayer grown on a permeable filter support

In order to enhance the uptake and binding of formulated SLNs to the HIV infected cells, a peptide reported to bind selectively to CD4 molecules ($CD4^+$ receptors are present on HIV host cell) was grafted on surface of nanoparticle. The binding of the grafted nanoparticles to these $CD4^+$ receptor containing cells was studied using **confocal microscopy** in Molt-4 cells (expressing high $CD4^+$ receptors) using Caco-2 cells (not expressing $CD4^+$ receptors) as control.

6.2 Materials

Human epithelial colorectal adenocarcinoma cell line (Caco-2) and Human T cell leukemia cell line (Molt-4) were procured from National centre for cell science (NCCS), Pune. Dulbecco's MEM medium (DMEM), RPMI 1640, Trypsin-EDTA solution, Fetal bovine serum (FBS), Hank's balanced salt solution (HBSS), penicillin-streptomycin solution and trypan blue were purchased from Himedia, Mumbai. 12 well Transwell inserts were purchased from Nunc, Denmark. 3-(4,5dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI), Lucifer yellow, coumarin, Chlorpromazine, nystatin and amiloride were purchased from Sigma-aldrich, Germany. sodium chloride, hydrochloric acid, sodium lauryl sulphate, sodium dihydrogen phosphate, disodium hydrogen phosphate, sodium pyruvate, sodium bicarbonate, dimethyl sulphoxide (DMSO), sodium hydroxide and chloroform were purchased from S.D.Fine chem Ltd. (Mumbai, India). Distilled water was prepared in the laboratory.

6.3 Subculturing of the cell lines

6.3.1 Subculturing of Caco-2 cells (2, 16)

Caco-2 (human epithelial colorectal adenocarcinoma cells) was procured from NCCS, Pune. Caco-2 cells of passages between 33 and 40 were used for the study. The cells were maintained as monolayer culture in 25 cm^2 tissue culture flasks. Dulbecco's MEM medium (containing 1.5 mM L-glutamine) supplemented with 10% fetal bovine serum (FBS), 1mM sodium pyruvate, 1.5 g/L sodium bicarbonate and 1% penicillin-streptomycin solution was used as culture medium. Cells were subcultured as a monolayer at 37ºC in a humidified atmosphere containing 5% CO_2 and medium was replenished every 3rd day.

Following procedure was followed for subculturing:

1. Culture medium was removed from the Tissue culture flask containing Caco-2 cells.
2. 2 ml of Trypsin-EDTA solution was added to flask and shaken 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.
3. Cells were observed under the inverted microscope until cell layer was detached (usually within 5 minutes). Note: To avoid clumping do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach.
4. 10 ml of complete growth medium was added to flask and cells were aspirated gently by pipette.
5. Subcultures were incubated at 37ºC with 5% CO_2.

6.3.2 Subculturing of Molt-4 cells

Molt-4 (Human T cell leukemia cell line) was procured from NCCS, Pune. The cells were maintained as monolayer culture in 25 cm^2 tissue culture flasks. RPML 1640 (AL162S, HiMedia) (containing 2mM L-Glutamine, 1.5 g/L sodium bicarbonate and 1mM sodium pyruvate) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin solution was used as culture medium. Cells were subcultured as a monolayer at 37ºC in a humidified atmosphere containing 5% CO_2 and medium was

replenished every 3rd day. Further, the subculturing of Molt-4 cell line was done as per same procedure done for Caco-2 cell line.

6.4 Cell counting using haemocytometer (16)

6.4.1 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. The phenomenon of Newton's rings was easily seen when the coverslip is correctly affixed, thus the depth of the chamber is ensured.

6.4.2 Preparing cells 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 1ml of cell suspension was sampled using a serological pipette and placed in microcentrifuge tube.
- Using a 100µl 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 gently.

6.4.3 Cell counting

- Using the micropipette, 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 corners square was focused as indicated by the circle in the diagram below:

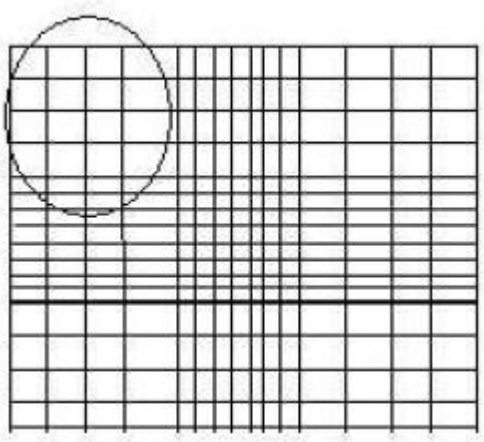

Figure 6.3 Haemocytometer diagram indicating the 16 corners which should be used for counting

- 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 cell positioned on the right hand or bottom boundary line were counted. Dead cells stained blue with trypan blue can be counted separately for a viability count.
- Counting of cells was continued for all other remaining set of 16 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 number 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 10^4x 2.
  Where 10^4 is conversion factor (conversion of 0.1 mm^3 to ml) and 2 is dilution factor.

6.5 Cell cytotoxicity study

6.5.1 Preparation of phosphate buffer saline pH 7.4 (17)

2.5 g of sodium dihydrogen phosphate, 2.523 g of disodium hydrogen phosphate and 8.2 g of sodium chloride were weighed accurately and dissolved in sufficient water to produce 1000 ml. The prepared buffer was autoclaved before use.

6.5.2 Preparation MTT solution

1 mg/ml MTT solution was prepared in growth medium. This stock solution was filtered through 0.2 µ membrane filter and stored at 2-8ºC in dark until use.

6.5.3 Preparation of stock solutions

Solid lipid nanoparticles batch (Dar-SLN2) and its placebo form (Dar-SLN2 batch without drug) were prepared by emulsification solvent evaporation technique and lyophilized as mentioned in Chapter 4. 10 mg drug equivalent lyophilized powder was suspended in medium to give concentration of 10 mg/ml (A). From this, dilutions were done to get drug concentrations 5 mg/ml (B) and 1 mg/ml (C). From C, 50µl was taken and diluted to 100 µl to get 500 µg/ml (D). From D, 20µl was taken and diluted to 100 µl to get 100 µg/ml (E). From E, 10µl was taken and diluted to 100 µl to get 10 µg/ml (F). All formulation dilutions were made in incomplete growth medium (DMEM alone). 20µl of the stocks (A-F) were diluted upto 200µl cell media in the wells individually so as to get final concentrations 1000 µg/ml (A'), 500 µg/ml (B'), 100 µg/ml (C'), 50 µg/ml (D'), 10 µg/ml (E') and 1 µg/ml (F'). Placebo SLNs were prepared similar to the formulation dilutions done. All the dilutions were carried out in laminar air flow hood and all the materials and equipments used were sterilized appropriately before use. The schematic representation of preparation of stock solutions is given in Figure 6.4.

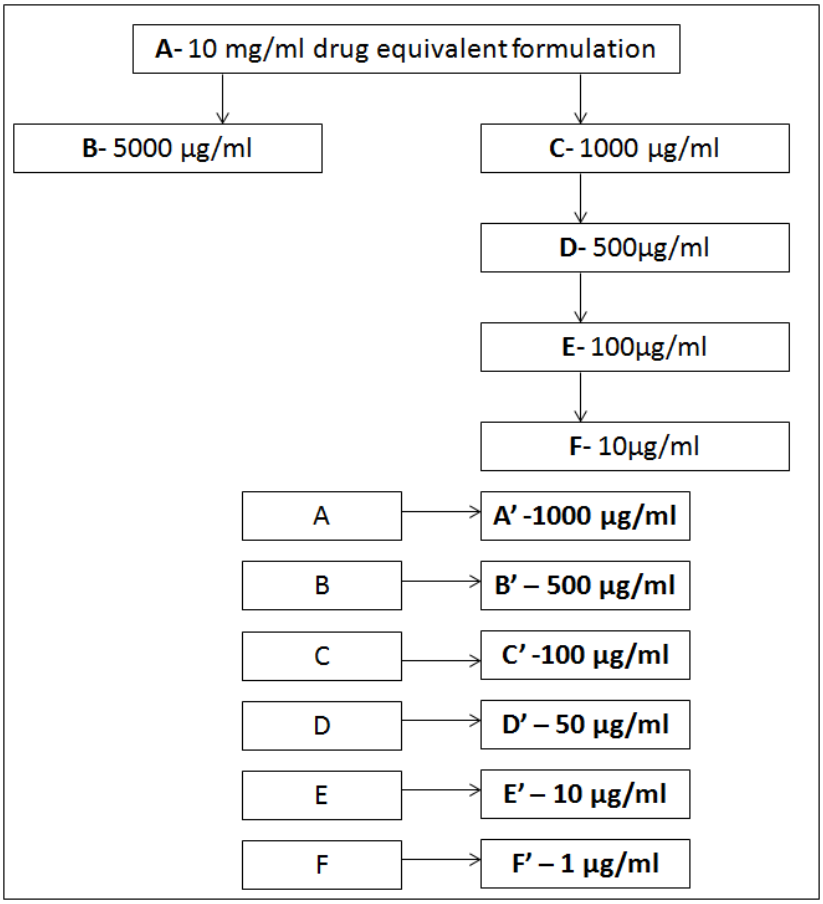

Figure 6.4 Schematic representation of preparation of various samples for MTT assay

6.5.4 MTT assay protocol

6.5.4.1 Plating out cells

- Subconfluent monolayer culture in one T-25 flask of Caco-2 cells 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 x 10^3 cells/ml in complete medium
- Then cell suspension was transferred to 96-well plate (Column 1 was kept as control i.e. without cells) with a multichannel pipette to produce cell concentration of 5 x 10^3 cells per well. Lid was placed over the plate.

- Cells were incubated in the incubator at 37ºC and 5 % CO_2 exposure for 1-2 days such that the cells are in the exponential phase of growth at the time when formulations are added.

6.5.3.2 Addition of formulations

- Medium from the wells was removed using multichannel pipette and discarded.
- Column 1 of each well plate was fed with 200 µl of fresh growth medium. These wells were used as blank wells and its absorbance was used as blank in microplate reader (Control). Wells in second column were fed fresh complete media (Absorbance of this well to be considered as 100 %). 20 µl of prepared samples A-F were fed in other columns and to it, 180 µl fresh complete media was added so that the final concentrations in wells become A'-F' as mentioned in Figure 6.4. Two such plates were prepared; one for drug loaded and other for placebo solid lipid nanoparticles.
- Lid was placed on plates and plates were returned to incubator (37ºC and 5% CO_2 exposure). The cells were incubated for 6 hr exposure period.

6.5.3.3 Estimation of surviving cell numbers

- At the end of exposure period, medium was removed from all the wells. After washing the wells with PBS pH 7.4, 200 µl of fresh complete media and 100 µl of prepared MTT solution (1 mg/ml) were added in each well.
- Both the plates were wrapped in aluminium foil and incubated for 4 hr.
- Medium along with MTT were removed from the wells. Cell lysis and solubilization of formazan crystals was done by adding 200 µl of DMSO to all the wells.
- Absorbance was recorded at 570 nm with a reference filter 655 nm immediately in ELISA plate reader. Blank wells (column 1) were taken as blank in plate reader.

6.5.3.4 Analysis of MTT assay

- Determination of the cell viability was done by using the following equation:

$$\%\, Viability = \frac{Mean\ absorbance\ of\ sample}{Mean\ absorbance\ of\ control}\ x\ 100$$

Where absorbance of sample and control cells represent the amount of formazan determined for cells treated with different formulations and for control cells (nontreated), respectively.

- Graph of concentrations of drug loaded and drug free formulation versus % cell viability was plotted.

6.6 Internalization pathway for SLNs into the Caco-2 cells

6.6.1 Preparation of 0.5 M NaOH (17)

2 g Sodium hydroxide was accurately weighed and dissolved in sufficient double distilled water. The final volume was made with double distilled water up to 100 ml.

6.6.2 Internalization pathway study (11)

- Subconfluent monolayer culture in one T-25 flask of Caco-2 cells 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 5 x 10^3 cells/ml.
- Then cell suspension (400 µl) was transferred to 24-well plate with a pipette to produce cell concentration of 2 x 10^3 cells per well.
- Cells were incubated in the incubator at 37ºC and 5 % CO_2 exposure for 24 hr to allow the cells to attach to the surface.
- Medium from the wells was removed and discarded.
- Following samples were added.
  a. CONTROL Group wells (n=6) - 400µl complete media was added.
  b. Group A wells (n=6) - 400µl complete media containing chlorpromazine (10µg/ml) was added.

c. Group B wells (n=6) - 400µl complete media containing nystatin (25µg/ml) was added.
d. Group C wells (n=6) - 400µl complete media containing Amiloride (550µM) was added.

The wells were incubated for 1 hr at 37°C.

- After 1 hr, 100 µl of formulations (lyophilized form of optimized Darunavir loaded SLNs- Dar-SLN2 and optimized Atazanavir sulfate loaded SLNs- ALN-23 dispersed in PBS 7.4) were added to the culture medium such that the drug concentration in final volume (500 µl) becomes 10 µg/ml. The plate was kept in incubator for 1 hr at 37°C.
- After 1 hr, the culture medium was removed, the cell monolayer was rinsed thrice with ice cold PBS (pH 7.4). The cells were lysed using 200µl NaOH solution (0.5 M) for 30 min at room temperature.
- The lysate was centrifuged at 3000 rpm, 10 min to settle the cell components. The supernatant was taken and drug amounts were quantified by HPLC.

6.6.3 Extraction of drugs from above supernatant

- For Darunavir: 200 µl of chloroform was added to the obtained supernatant to dissolve the nanoparticles. The mixture was allowed to settle down and chloroform layer was separated. The procedure was repeated 3 times by adding chloroform and all the chloroform extracts were collected. 0.5 ml DMSO was added to the chloroform extract into a 5 ml beaker and the chloroform was allowed to evaporate to precipitate lipid (drug will remain solubilized in DMSO while lipid is not soluble in DMSO). The resultant was centrifuged at 10,000 rpm for 10 min at 4ºC using sigma centrifuge (3K30, osterode, Germany), filtered using 0.2 µm polycarbonate filters. Drug amount present in resultant solution was quantified by developed HPLC method after proper dilution with the mobile phase.

  For Atazanavir sulfate: 200 µl of solvent mixture of methanol: dichloromethane was added to the supernatant obtained to dissolve the nanoparticles. The resultant was filtered using 0.2 µm polycarbonate filters and drug was quantified using developed HPLC method after proper dilution with the mobile phase.

- The concentration of drug obtained in control group (A) was taken as 100 % and the relative uptake efficiency were calculated as per following equation.

$$\%\ Relative\ cell\ uptake\ efficiency = \frac{drug\ amount\ in\ sample\ group}{drug\ amount\ in\ control\ group}\ x\ 100$$

% Reduction in cell uptake = 100 – (% relative cell uptake efficiency)

6.7 Intestinal permeability study (18, 19)

- Caco-2 cells were grown on Transwell® inserts (Nunc, Denmark) with (0.4µ pore diameter, 1.13 cm^2 area).
- The inserts were washed twice using transport buffer (Hank's balanced salt solution (HBSS) containing 25mM of HEPES, pH 7.4**)** and equilibrated for 30 min.
- The integrity of the monolayers was checked by monitoring the permeability of the paracellular leakage marker, Lucifer yellow across the monolayers.
- The cell monolayers were considered tight enough for the transport experiments when the apparent permeability coefficient (P_{app}) for Lucifer yellow was less than 0.5×10^{-6} cm/s. All transport studies were conducted at 37°C.
- 1.5ml of transport buffer was added to basolateral side.
- 0.5 ml of transport buffer containing sample (150µl sample containing 0.1 mg drug diluted to 0.5 ml with transport buffer) was added to apical side. For Darunavir, the samples taken were Darunavir solution (1% DMSO in transport buffer) and Darunavir loaded SLNs. For Atazanavir sulfate, the samples taken were Atazanavir sulfate solution (1% DMSO in transport buffer) and Atazanavir loaded SLNs.
- After the incubation of 0.5, 1, 2, 3 and 4 hr, aliquot of 100 µl was withdrawn from the receiver chambers and was immediately replenished with an equal volume of pre-warmed HBSS.
- The concentrations of the drug in the transport medium were analyzed by developed HPLC methods.
- The apical-to-basolateral permeability coefficient (P_{app} in cm/s) was calculated according to following equation:

$$\boldsymbol{P_{app} = \frac{dQ/dt}{A * C * 60}}$$

Where, dQ/dt = the amount of drug in basolateral compartment per min (mg/min)

A = the monolayer area (cm^2) (= 1.13 cm^2)

C = the concentration of drug added in apical compartment initially (=0.2mg/ml)

6.8 Confocal microscopy

6.8.1 Formulation of coumarin-6 loaded SLNs

6-coumarin was used as a hydrophobic dye for the study. 6-coumarin loaded SLNs were prepared by emulsification solvent evaporation technique as mentioned for Darunavir loaded SLNs. Darunavir was replaced by 6-coumarin in optimized batch of Darunavir loaded SLNs (Dar-SLN2). The unbound 6-coumarin was removed by centrifugation at 4000 rpm for 10 min using sigma centrifuge at 20ºC (settled portion). Peptide was grafted to the surface of prepared 6-coumarin loaded SLNs as per procedure mentioned in chapter 4, section 4.3.2.1. 6-coumarin loaded SLNs without peptide grafting was used as control.

6.8.2 Protocol

Cellular binding of peptide grafted SLNs was monitored by confocal microscopy. The study was conducted in two different cell lines- Molt-4 cell line expressing high $CD4^+$ and Caco-2 cell line as negative control (no $CD4^+$ expression). The cells, at density of 5 x 10^5 cells/ well, were seeded onto 6 well plates with a glass cover slip at the bottom of each well. The cells were incubated in incubator for 24 hr at 37ºC and 5 % CO_2 exposure. After 24 hr, medium from the wells was removed and discarded. Peptide grafted solid lipid nanoparticles and non-peptide grafted SLNs were diluted to lipid concentration of 100 nM in complete media, added to wells and incubated. After 4 hr of incubation, cells were washed with cold PBS immediately and fixed using ice cooled 4% paraformaldehyde solution for 10 min. Cells were stained by cell nuclei stain, 2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI), for next 10 min. Cover slips were mounted on slides after washing with PBS three times and proceeded for

confocal microscopy using confocal laser scanning microscope (LSM 710, Carl-Zeiss Inc., USA).

6.9 Results and discussion

6.9.1 Cell cytotoxicity study

Mean absorbance values and % viability of Caco-2 cell line observed for control (no drug treatment), placebo solid lipid nanoparticles and Darunavir loaded solid lipid nanoparticles is shown in Table 6.1. The viability of Caco-2 cells in presence of different samples is shown graphically in Figure 6.5. There was no significant difference (P value > 0.5) between absorbances of placebo and drug loaded SLNs. The results show that % cell viability greater than 95 % at all concentrations used irrespective of drug loading indicating the non-cytotoxicity of excipients used in formulation of SLNs up to the high concentration of 10 µg/ml Darunavir equivalent formulation.

Table 6.1 MTT assay results of control sample, placebo SLNs and Darunavir loaded SLNs

Concentration of Darunavir	Concentration of lipid (µg/ml)	Placebo SLNs		Darunavir loaded SLNs	
		Mean absorbance ± SD	% cell viability (%)	Mean absorbance ± SD	% cell viability (%)
Control- Mean absorbance = 0.620 ± 0.033 and % cell viability = 100 %					
A’	6000	0.601 ± 0.010	96.93 ± 1.61	0.617 ± 0.036	99.51 ± 5.81
B’	3000	0.614 ± 0.034	99.08 ± 5.43	0.580 ± 0.010	93.60 ± 1.56
C’	600	0.593 ± 0.077	95.69 ± 12.37	0.610 ± 0.054	98.38 ± 8.71
D’	300	0.615 ± 0.031	99.30 ± 4.89	0.591 ± 0.072	95.32 ± 11.61
E’	60	0.583 ± 0.065	94.13 ± 10.38	0.598 ± 0.042	96.55 ± 6.67

F'	6	0.604 ± 0.071	97.52 ± 11.35	0.589 ± 0.052	95.10 ± 8.28

*Values are represented as mean±SD, n=3.

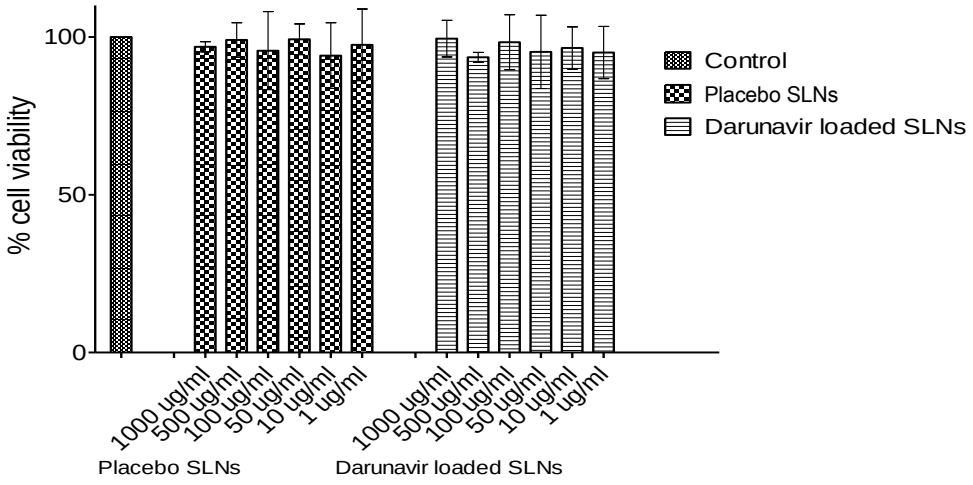

Figure 6.5 Viability of Caco-2 cells in presence of various samples of placebo SLNs and Darunavir loaded SLNs

6.9.2 Internalization pathway for SLNs into the Caco-2 cells

The results of endocytic uptake of formulated solid lipid nanoparticles in presence or absence of specific endocytic inhibitors is shown in below Table 6.2. The relative uptake efficiency of Darunavir and Atazanavir in presence of endocytic inhibitors is graphically depicted in Figure 6.6 and Figure 6.7 respectively.

Table 6.2 Relative Caco-2 cell uptake efficiency of Darunavir and Atazanavir sulfate in absence or presence of various endocytic inhibitors

Group	Treatment	% relative cell uptake of Darunavir ± SD	% Reduction in uptake of Darunavir ± SD	% relative cell uptake of Atazanavir sulfate ± SD	% Reduction in uptake of Atazanavir sulfate ± SD
-	CONTROL- no treatment	100 %	-	100 %	-
A	Chlorpromazine	70.65 ± 6.5 %	29.35 ± 6.5 %	82.37 ± 5.2 %	17.63 ± 5.2 %

B	Nystatin	58.42 ± 5.3 %	41.58 ± 5.3 %	43.23 ± 6.7 %	56.77 ± 6.7 %
C	Amiloride	99.65 ± 5.2 %	0.35 ± 5.2 %	98.23 ± 8.2 %	1.77 ± 8.2 %

*n=6

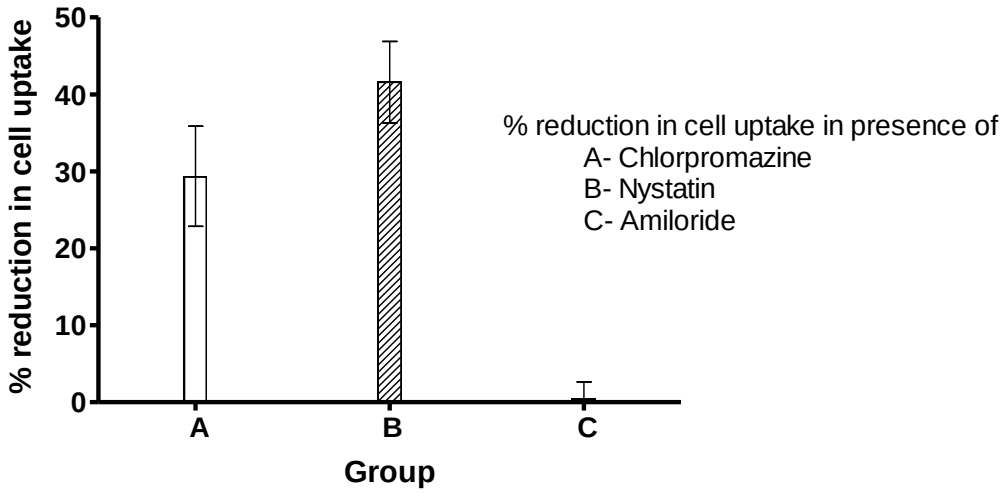

Figure 6.6 % relative reduction in cell uptake of Darunavir in presence of various endocytic inhibitors (n=6)

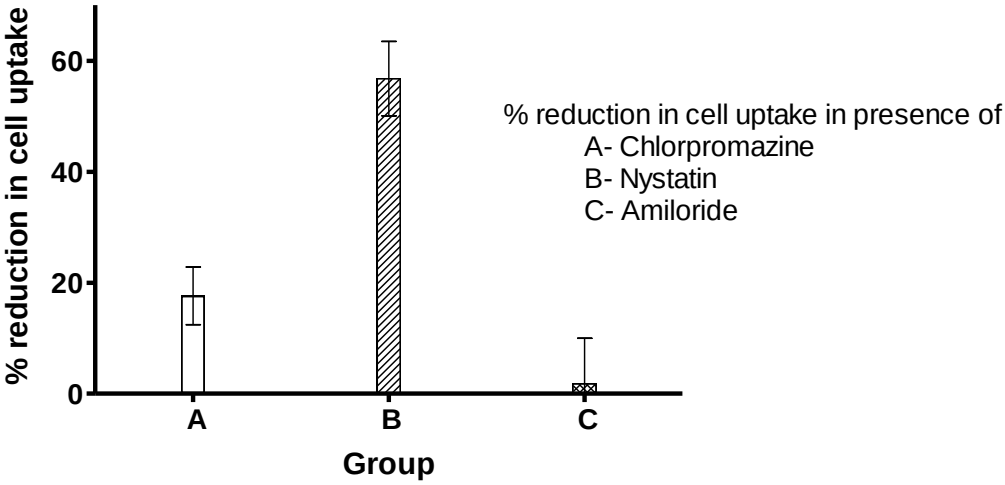

Figure 6.7 % relative reduction in cell uptake of Atazanavir sulfate in presence of various endocytic inhibitors (n=6)

In case of Darunavir loaded SLNs, cellular uptake of SLNs was reduced by 29.35 % in presence of Chlorpromazine, decreased over 41.58 % in presence of Nystatin while there was no significant difference in cell uptake in presence of Amiloride. In case of

Atazanavir sulfate loaded SLNs, similar results were obtained. Cellular uptake of ATZ SLNs was reduced by 17.63 % and 56.77 % in presence of Chlorpromazine and Nystatin respectively while no significant decrease in uptake was obtained in presence of Amiloride. It is reported that Chlorpromazine and Nystatin inhibits clathrin and caveole mediated endocytosis (13). Hence, the decrease in cellular uptake of SLNs in presence of these inhibitors indicated that the Caco-2 uptake of SLN was mediated via clathrin and caveoli mediated endocytosis and not via macropinocytosis since amiloride (which inhibits macropinocytosis) presence had no significant effect on uptake efficiency of SLNs.

Among the three endocytic pathways explored in the above study, highest decrease in cellular uptake was obtained by use of caveole mediated endocytic inhibitor-Nystatin. Thus, it could be deduced that, among these pathways, SLNs preferably use caveole dependent endocytic pathways. This is supported by the reported data that the anionic nanoparticles are more likely to use caveole-dependent endocytosis (14) and our developed nanoparticles are anionic in nature. It is also reported that majorly more than one pathways have been utilized by different nanoparticles for the endocytic uptake (13, 20).

6.9.3 Intestinal permeability study

Table 6.3 Drug transfer across Caco-2 cell line for Darunavir loaded SLNs and plain Darunavir solution

Time (min)	Plain Darunavir solution		Darunavir loaded SLNs	
	Amount of drug transferred (µg) ± SD	% permeation	Amount of drug transferred (µg) ± SD	% permeation
30	0.67 ± 0.04	0.67 ± 0.04	4.32 ± 1.24	4.32 ± 1.24
60	1.56 ± 0.22	1.56 ± 0.22	12.01 ± 3.87	12.01 ± 3.87
90	3.85 ± 0.88	3.85 ± 0.88	20.18 ± 4.55	20.18 ± 4.55
120	7.09 ± 2.11	7.09 ± 2.11	26.19 ± 5.14	26.19 ± 5.14
180	7.87 ± 3.06	7.87 ± 3.06	36.15 ± 8.14	36.15 ± 8.14

240	11.23 ± 4.94	11.23 ± 4.94	45.33 ± 10.11	45.33 ± 10.11
dQ/dt	0.046 µg/min	-	0.188 µg/min	-
Paap	3.44 x 10^{-6} cm/sec	-	1.39 x 10^{-5} cm/sec	-

*n=3

The gastrointestinal permeability of Darunavir loaded SLNs was assessed by calculating the *in-vitro* permeability coefficients in Caco-2 cell model of gastrointestinal barrier. The results are given in Table 6.3. The permeability coefficient P_{app} for plain Darunavir solution was found to be 3.44 x 10^{-6} cm/sec. Even in presence of DMSO which is a known permeation enhancer, the P_{app} of plain Darunavir solution was significantly lower than that of the nanoparticles (21). The permeability coefficient for Darunavir loaded SLNs was found to be 1.39 x 10^{-5} cm/sec which was 4.04 times more than plain drug solution. The lower permeability for plain drug solution was because of poor permeability characteristics of Darunavir. The permeability of Darunavir was enhanced by loading drug in SLNs which could be attributed to higher uptake of SLNs by endocytosis in Caco-2 cells.

Table 6.4 Drug transfer across Caco-2 cell line for ATZ loaded SLNs and plain ATZ solution

Time (min)	**Amount of drug transferred (µg)**	
	Plain ATZ solution	**ATZ loaded SLNs**
30	6.66 ± 2.02	18.55 ± 4.06
60	12.64 ± 3.82	36.84 ± 10.87
90	16.38 ± 4.46	46.86 ± 12.14
120	28.09 ± 9.24	81.42 ± 14.46
180	53.19 ± 11.17	126.25 ± 21.22
240	83.17 ± 15.62	214.92 ± 20.10
dQ/dt	0.35 µg/min	0.89 µg/min

Paap	1.31 x 10^{-5} cm/sec	3.3 x 10^{-5} cm/sec

*n=3

The results of permeability study for Atazanavir sulfate loaded SLNs and plain drug solution is shown in Table 6.4. The P_{app} values of less than 1 x 10^{-5} cm/sec suggest a poor permeability whereas P_{app} between 1-10 x 10^{-5} cm/sec suggests a moderate to good permeability (22). The P_{app} value of 1.31 x 10^{-5} cm/sec obtained for plain Atazanavir sulfate solution indicated a moderate permeability of ATZ. ATZ has log P value of 4.54 and it is lipophilic drug therefore it is permeable to Caco-2 cells but ATZ loaded SLNs showed a 2.52 fold increase in permeability suggesting a higher uptake of nanoparticles by Caco-2 cells.

6.9.4 Confocal study

The uptake of peptide grafted and non-peptide grafted SLNs was studied in two cell lines- Molt-4 and Caco-2 cell line in order to study the binding effect due to grafting of SLNs with peptide having CD4+ receptor affinity. The nuclei were stained using DAPI. After 4 hr of incubation, the nanoparticles were mainly observed in cytoplasm. The results of uptake in Molt-4 cell line (CD4 positive cell line) are shown in Figure 6.8 while uptake in Caco-2 cell line (CD4 negative cell line) are shown in Figure 6.9. The images showed a higher fluorescence obtained using peptide grafted SLNs in Molt-4 cell line in comparison to non-peptide grafted SLNs indicating the greater binding of peptide grafted SLNs to CD4 positive Molt-4 cell line. In contrast, both the nanoparticles (peptide grafted and non-grafted) showed similar fluorescence in CD4 negative Caco-2 cell line indicating no effect of peptide on binding with these cells. Moreover, for peptide grafted SLNs, fluorescence was lesser as compared to that obtained in Molt-4 cells which showed that the peptide grafted SLNs has higher affinity for CD4 positive Molt-4 cell line. Hence, the peptide grafted SLNs has higher affinity to bind with the HIV host cells compared to peptide non-grafted SLNs. This would lead to enhanced exposure of drug to the HIV host cells.

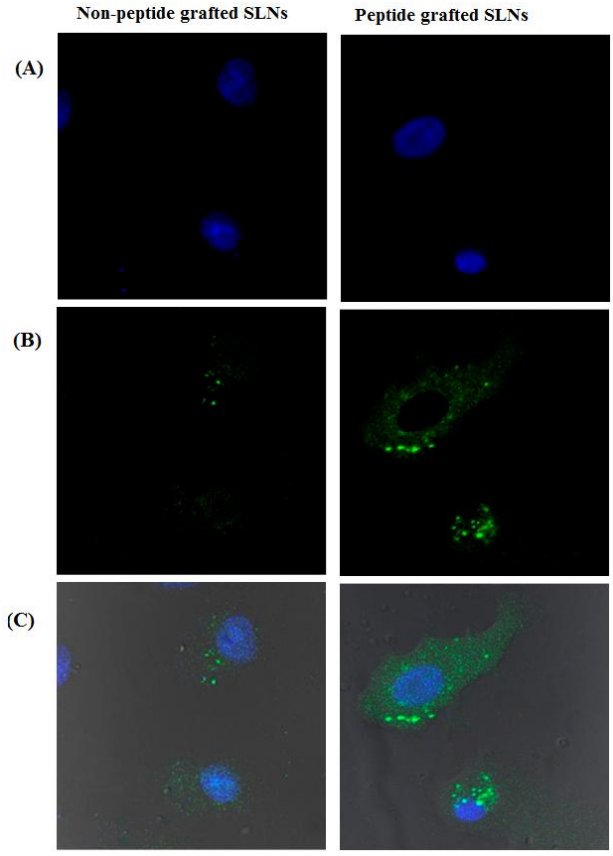

Figure 6.8 Cell uptake in Molt-4 cell line. (A) DAPI-nuclei staining (B) SLNs (C) Merged

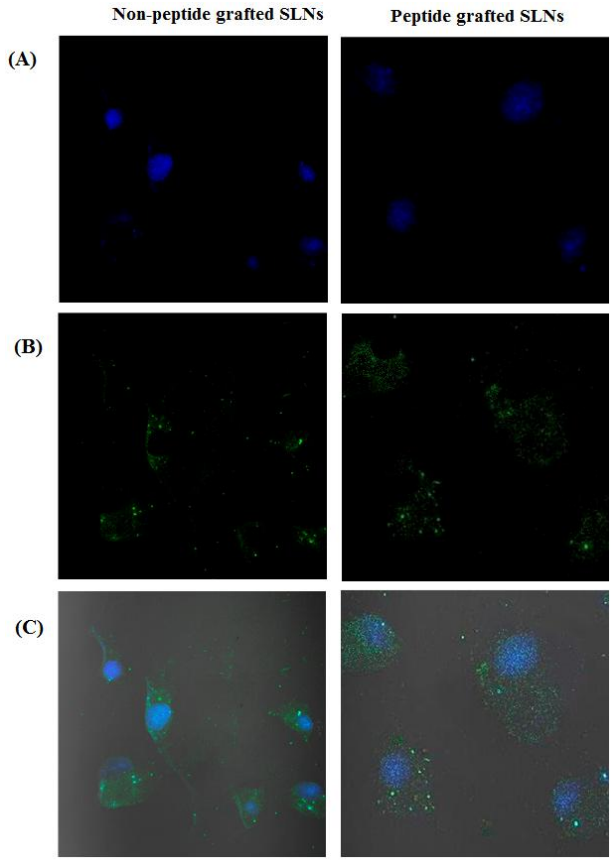

Figure 6.9 Cell uptake in Caco-2 cell line. (A) DAPI-nuclei staining (B) SLNs (C) Merged

6.10 References

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