# 7. CELL LINE STUDY

## Cell line Study - Procedure:

### 7.1 In vitro Cell Viability Study

The effect of pure drugs viz., ILO and VDN and their SMEDDS and niosomes formulations on cell proliferation was determined by MTT based colorimetric assay. Caco2 cells were plated into 96-well plates at density of $10^4$ cells per well and were left for seeding for 24 h in incubator (pre-treatment incubation). The cells were than incubated for 4 h with serially diluted samples of pure drugs and their respective formulations of SMEDDS and niosomes at concentrations varying from 1 – 50 µg/mL prepared in MEM media (treatment incubation). Composition of the optimized formulation is shown in table 7.1. Serial dilution was performed to get final concentration of 1 – 50 µg/mL.

Table 7.1 Optimized formulation composition of SMEDDS and Niosomes

| Formulation | | ILO SMEDDS (mg) | | VDN SMEDDS (mg) | | ILO Niosomes (mg) | VDN Niosomes (mg) |
|---|---|---|---|---|---|---|---|
| Drug | ILO | 24.00 | VDN | 40.00 | Drug | 12 | 10 |
| Oil | Capmul MCM C-8 | 74.30 | Capmul MCM C-8 | 162.90 | Cholesterol | 26.20 | 11.65 |
| - | Oleic acid | 37.10 | - | - | Span 60 | 91.90 | 46.98 |
| `Surfactant | C-EL | 638.50 | C-EL/T-20 | 500.00 | - | - | - |
| Co-surfactant | T-HP | 250.00 | PEG 200 | 337.09 | - | - | - |

Cells without any treatment were considered 100% viable and served as control. After treatment, the samples were discarded and 100 µL MTT reagent (1 mg/mL) prepared in MEM was added to each well. The plates were incubated for 4 h (after-treatment incubation). Followed by drying of the plates by dabbing against tissue paper, DMSO was added to

solubilize the formazan crystals formed from MTT and the plates were oscillated for 15 min to aid solubilization of formazan crystals. Thereafter, the absorbance was immediately measured at 570 nm by a microtiter plate reader (680-XR, Bio-Rad, France). %Cell viability was calculated with reference to absorbance of control cells using the equation 7.1 [1].

$$\%Viable\ cells = \frac{(Abs_{sample} - Abs_{blank})}{(Abs_{control} - Abs_{blank})} \times 100 \quad \text{...Equation 7.1}$$

**7.2 Intracellular uptake study**

Qualitative cellular uptake was carried out after proliferating the cells at a density of $10^6$ on a coverslip in a 6 well plate. The cells were later treated for 4 h with formulations prepared by replacing the drug with coumarin 6 dye. After treatment, the coverslips were washed thrice with phosphate buffer saline (pH 7.4). DAPI was used to stain the nuclei. Following staining, the coverslips were mounted using glycerin. The slides were observed using confocal microscopy (ZEISS LSM, Germany) and the images were processed for color channeling using ZEN lite – Blue edition software (ZEISS LSM, Germany) [2].

**7.3 Flow cytometry**

To further assess quantitative cellular uptake, the cells were seeded at a density of $1*10^6$ cells/well in a 6 well plate and were allowed to grow in complete media. After 24 h, the cells were treated with optimized formulations (niosomes and SMEDDS) prepared by replacing drug with coumarin 6 dye. Following 4 h of treatment, cells were washed with phosphate buffered saline pH 7.4. The cells were then trypsinized and resuspended in FACS buffer (9.8 mL PBS pH 7.4 + 0.1 mL FBS + 100 mg BSA) and were analyzed using fluorescence activated cell sorter FACS Caliber Flow (BD lifesciences, USA) at 10000 events per second. Cells without any treatment were used as negative control. The graphs were plotted for SSC (**S**ide **Sc**atter) vs. florescence parameter [3].

**7.4 In vitro permeability study**

To determine apical (AP) to basolateral (BL) permeability across Caco2 monolayer, Transwell® inserts of 12 mm diameter, 0.4 μm pore size (Corning, USA) were used. According

to standard protocol for permeability study, Caco2 cells were cultivated on filter support at a density of $3*10^5$ cells per well. The cells were maintained by changing media every alternate day for 21 days. The integrity of the monolayers was checked by monitoring the trans-epithelial resistance measurement using Millicell® ERS meter (Millipore, Bedford, Massachusetts, USA). After incubation, transepithelial permeation from AP to BL was carried out by placing 1 mL of 25 µg/mL pure drug (ILO or VDN) and their formulations (Niosomes and SMEDDS) prepared in HBSS pH 7.4 on the AP side, and 2.5 mL of HBSS on the BL side. Samples (200 µL) were withdrawn from BL compartments after 120 min incubation. Withdrawn samples were stored at −20 °C until analyzed by UPLC. The apparent permeability coefficient (Papp in cm/s) from apical-to-basolateral was calculated according to following equation 7.2:

$$P_{app} = \frac{dQ}{dt} \times \frac{V_R}{A \times C_o} \quad \ldots\ldots \text{ equation 7.2}$$

Where, Papp is the apparent permeability co-efficient (cm/s), dQ/dt is the cumulative flux in the AP to BL direction (µg/s), $V_R$ is the volume of the receptor compartment ($cm^3$), A is the diffusion area of the monolayer ($cm^2$), and $C_0$ is the initial concentration applied on the AP side (µg) [4].

**7.5 Actin Visualization**

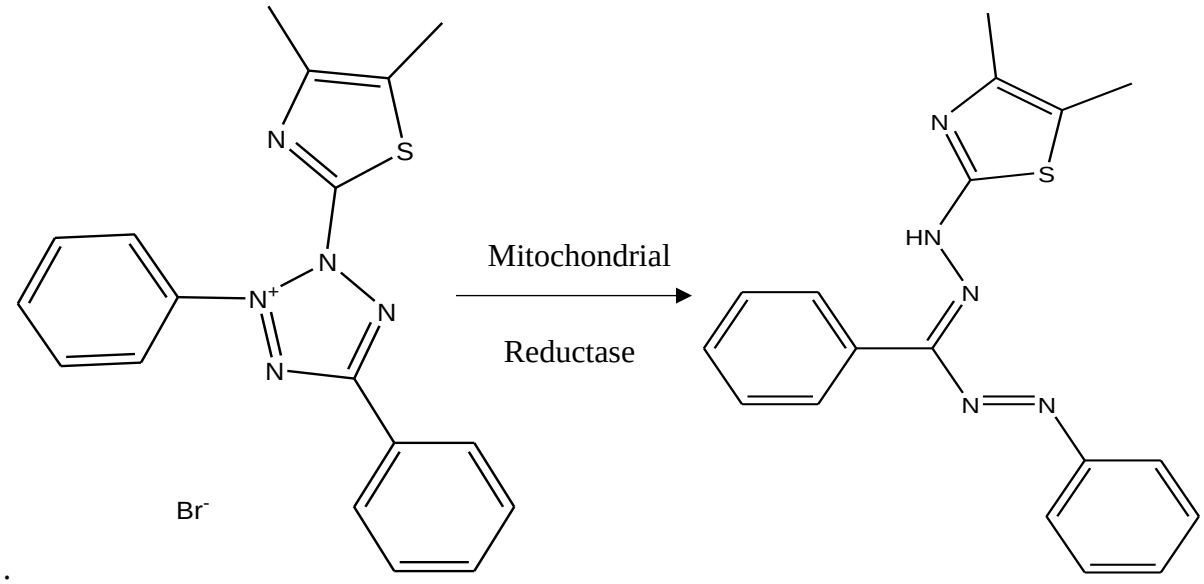
Caco-2 cells cultured on transwells for at least 21 days were incubated with optimized formulations of ILO and VDN (niosomes and SMEDDS, concentration 25 µg/mL)) for 2 h at 37°C. At the end of the in vitro permeability study, the monolayers were washed with PBS and fixed using 4% paraformaldehyde solution. The cells were than permeabilized using 0.2% Triton X-100 in PBS. For actin staining, the cells were incubated with Phalloidin Alexa 488 green dye for 2 h [5]. At the end of this incubation, the cells were washed three times with PBS and were visualized by EVOS FLoid Cell Imaging Station (Thermo Fisher Scientific, India).

## Results and Discussion

### 7.6 In vitro Cell Viability Study

With the foundation of various novel nano sized drug delivery systems, there has been a rise in the toxicological evaluation of such formulations [6]. This toxicological evaluation has also been revolutionized from *in vivo* to *in vitro* evaluation which provides rapid results on potential of new chemicals before they can be tested in animals or humans. *In vitro* cell culture is a potential means that provides an insight about the clinical relevance of formulations for studying cell toxicity mechanisms. Hence, *in vitro* evaluation has been used for many years as an alternative to *in vivo* evaluation owing to close correlation between them [7]. *In vitro* testing methods are used to check potentially toxic effects of chemicals on cell culture. *In vitro* cytotoxicity study data are now being considered by various regulatory agencies like Environment Protection Agency, National Institute of Health, National Institute of Cancer, Food and Drugs Administration etc [8]. This regulatory requirement necessitates the evaluation of cytotoxicity study of any formulation in *in vitro* cell culture.

Hence, in this research work, the possibility of adverse effects elicited by different formulations on cultured Caco2 cell monolayers were evaluated by determining the mitochondrial dehydrogenase activity of the cells by the MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazoliumbromide) assay. This is an established method for addressing cell functionality and is widely used as a criterion for cytotoxicity [9]. The MTT assay is a sensitive and quantitative colorimetric method to assess cell viability. It measures the formazan's dark purple color produced after mitochondrial succinate dehydrogenase's activity on yellow MTT. The yellow MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, colored (dark purple) formazan product (figure 7.1). This is then solubilized with DMSO and the solubilized formazan's optical density is measured spectrophotometrically. As reduction of MTT only occurs in metabolically active cells, the quantity of formazan is a measure of the viability of the cells. The MTT assay specifically detects mitochondrial metabolic activity and the ability of the cells for proliferation. As a result, it mirrors the activity of mitochondrial dehydrogenase, whose production decreases with increasing cellular distress.




.

Figure 7.1 MTT dye reduction by mitochondrial reductase enzyme

Figure 7.2 represents the concentration versus percent viability data of cells incubated with ILO, ILO SMEDDS, placebo SMEDDS, ILO Niosomes and placebo Niosomes as compared to control. Similarly, figure 7.3 is for VDN, VDN SMEDDS (T-20), VDN SMEDDS (C-EL), placebo SMEDDS (T-20), placebo SMEDDS (C-EL), VDN Niosomes and placebo Niosomes. The percent cell viability data values were found to be >80% for all the test samples at all the studied concentrations as observed in both the figures ($p$ value $> 0.05$). As the lipidic matrices used herein are biocompatible molecules, it might be the reason for less toxic effect on cells for the tested concentration. Thus, the results of MTT assay proved the nontoxic behavior of the formulation and the ingredients used therein. Any of the concentration tested did not cause toxicity on cells when compared to MEM control, confirming the compatibility of these formulations for endocytosis investigation by confocal microscopy, at the concentrations tested.

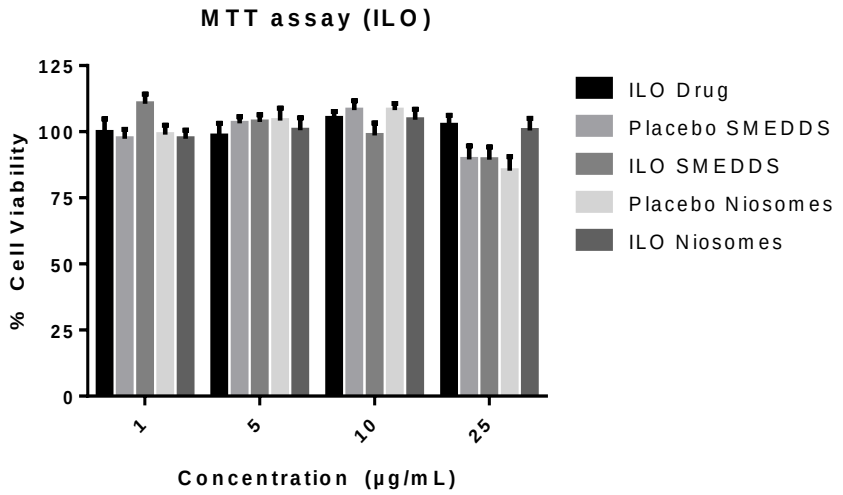



Figure 7.2 Caco-2 cell viability after 4 h incubation with drug and formulations of ILO

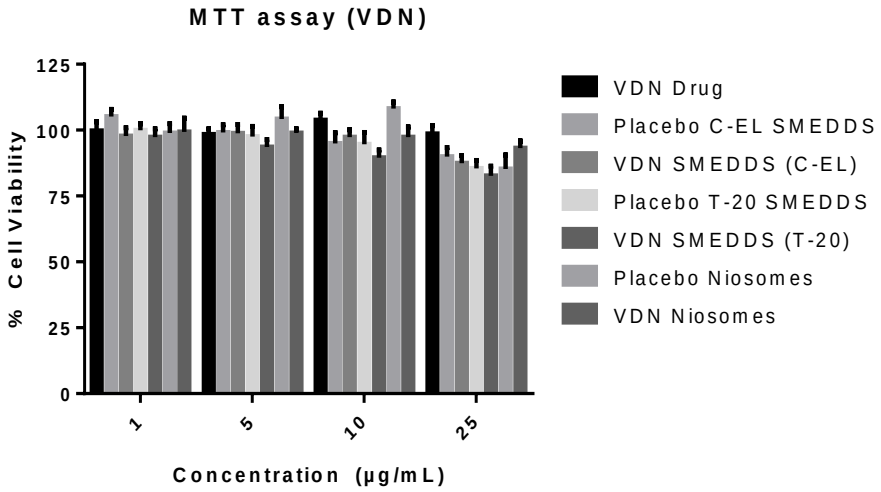



Figure 7.3 Caco-2 cell viability after 4 h incubation with drug and formulations of VDN

### 7.7 Intracellular uptake study

Cellular uptake studies were performed using confocal microscopy as it allows imaging of cells at a specific focal point creating sharper images after reducing the noise that arises from scattering. In case of cellular uptake studies of a fluorophore loaded formulations, the fluorescence concentrated in the cytosol and nucleus can be imaged separately to give insight on the actual uptake of the formulation [10]. Coumarin 6 was added as the fluorophore for

illumination, whereas for nucleus staining, DAPI dye was added. The drugs, ILO and VDN belong to BCS class II. This indicates the hydrophobic nature of drug. Hence, coumarin 6 was chosen as a hydrophobic model dye to mimic their nature [11].

Figure 7.4 shows control cells and cells incubated with respective formulations of ILO and VDN. Dense nuclei area is stained in blue by DAPI, whereas green color is due to formulation loaded with coumarin 6. No autofluorescence was seen as indicated by absence of green color in control Caco2 cells. On the contrary, abundance of green color in formulation treated cell indicates internalization of formulation by Caco2 cells [12]. Both lipid-based formulations, SMEDDS or Niosomes, did not appear in the nucleus area. Their perinuclear accumulation was not homogenous but occurred preferentially at one location where the distance from plasma membrane to nuclear membrane was the greatest [13].

The mechanism of endocytosis may be direct cytoplasmic delivery or mixing/exchange of phospholipids between target cells membrane and the lipid-based formulation [14]. As the lipid-based formulations have a strong chemical and structural similarity with the lipids of cell membrane, the structural lipids of our formulations might have merged with cell membranes and facilitate drug delivery into the interior of the cell [15]. It can be concluded that lipid-based drug delivery systems represent a good carrier for intracellular drug delivery. Due to added advantage of nano size, it provides higher surface area which facilitates the contact with the cell membrane.

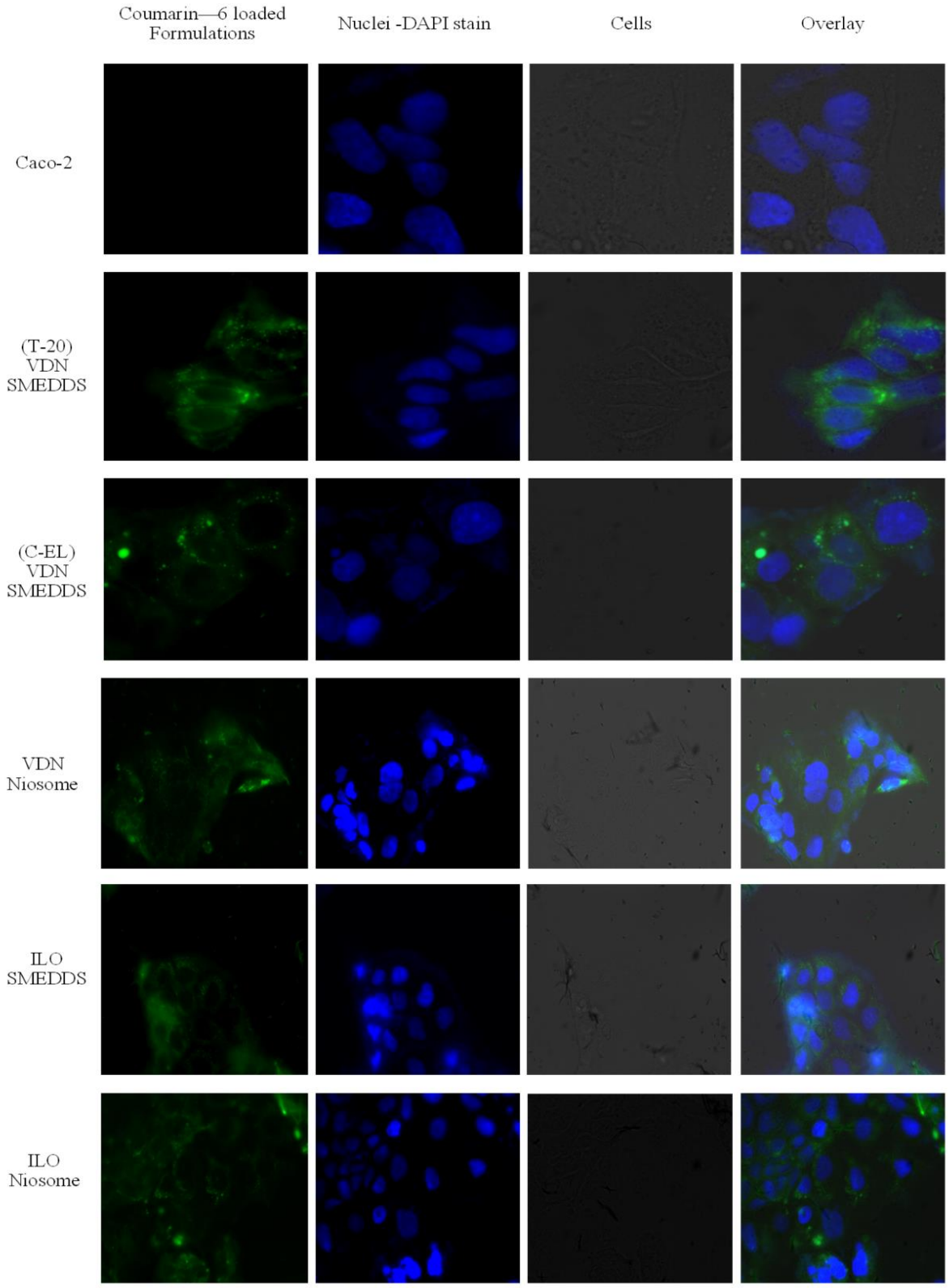



Figure 7.4 Study of cellular uptake of formulations in Caco-2 cell line after 4 h incubation

Comparing between the different formulations of SMEDDS and niosomes for both the drugs (ILO and VDN), it is clearly indicative that SMEDDS showed more fluorescence intensity than Niosomes at the end of 4 h treatment. Upon comparison between VDN SMEDDS prepared using different surfactants, T-20 and C-EL, more green colored intensity was observed in VDN SMEDDS (T-20). This might be owing to structural similarity of T-20 molecules to the lipid A of lipopolysachharides of lipid bilayer which gave an easy access inside the cellular structure [16].

However, as the confocal study is a qualitative study, any generalization cannot be given regarding superiority of SMEDDS over niosomes. Hence, quantitative parameters (% uptake) was studied using FACS cytometry [17].

**7.8 Flow cytometry**

Quantitative cell uptake study of the coumarin 6 fluorescent probe was performed by Fluorescence Activated Cell Sorting (FACS) in Caco-2 cells. FACS dot plot of cellular uptake of developed formulation are shown in figure 7.5.

Flow cytometry is a single cell analysis, using which cell's (Caco2) physical property can be characterized. In this technique, a single cell suspension passes through a laser set. The scattered laser light by the single cell than falls on the detector. The detector placed at ninety-degree angle measured side scattering (SSC). SSC is used to identify varying complexity of the cells [17].

Herein, we have used coumarin 6 dye loaded formulations which were than incubated with the Caco2 cell line. After treatment time period, the cells were than detached from the well plates and suspended in FACS buffer. This single cell suspension then passes through nozzle to form single-cell stream, which scatters laser light. The SSC was measured for a fixed no of cells (10,000 events) and the graph as shown in figure 7.5 was plotted.

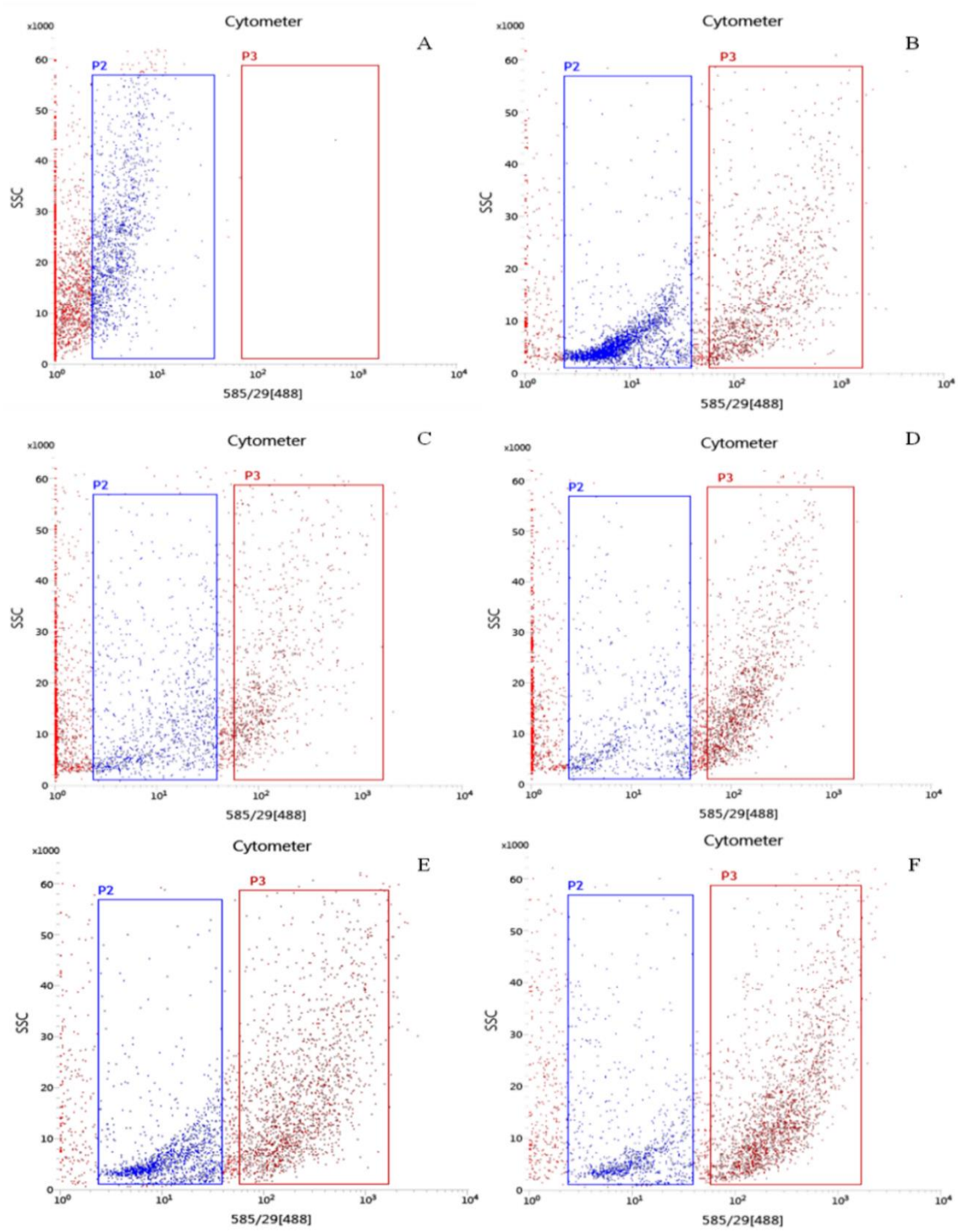



Figure 7.5 Comparative Dot plot analysis of uptake of coumarin 6 loaded formulations in Caco2 cell line (A: Control, B: ILO Niosomes, C: VDN Niosomes, D: ILO SMEDDS, E: C-EL VDN SMEDDS, F: T-20 VDN SMEDDS)

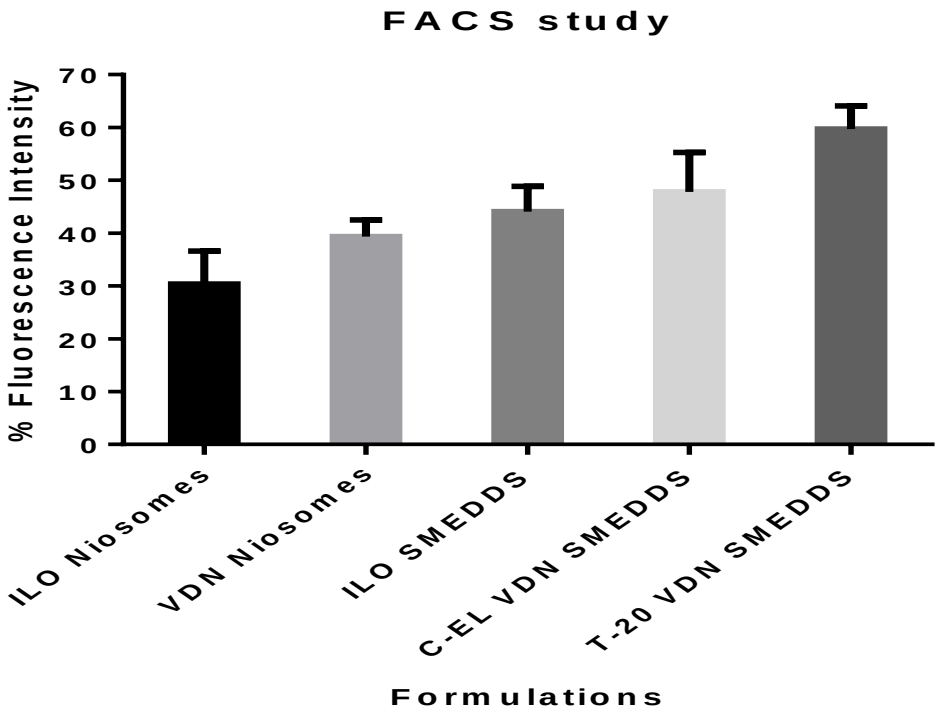



Figure 7.6 % Fluorescence intensity after treatment with formulation in Caco2 cells

For excitation of coumarin 6 flurophore, blue light laser source which emits fluorescence in the green spectrum range was used [18]. Hence, filter optics were set to measure green fluorescence of the excited coumarin 6. Those Caco2 cells in which endocytosis of the formulation has occurred, only that will emit green fluorescence. As the color of exciting light (blue) is different from emitted light (green), they were easily separated by optical filters and the cells which had uptaken the coumarin 6 dye loaded formulations were counted [19].

Generalized observation of the rightward shift of dot plot towards P3 population indicates increased cellular uptake of formulations [20]. The rightward shift after treatment of lipid-based formulations depicts that the lipid formulations were easily uptaken by the cells through endocytosis due to their nano size. FACS uptake studies showed that the fluorescence intensity inside the cells got increased by using SMEDDS as compared to Niosomes. Thus, higher endocytosis was seen with SMEDDS formulations compared to its counterpart Niosomes formulation.

With respect to control (A), Niosomes showed uptake of 30.37% (B – ILO Niosomes) and 39.32% (C-VDN Niosomes). Whereas, the uptake was increased further for SMEDDS formulations. For, ILO SMEDDS uptake was 44.08% (D), whereas for C-EL VDN SMEDDS it was 47.82% (E). It increased drastically with T-20 VDN SMEDDS with the uptake of 59.70% (F). This indicates superiority of SMEDDS formulation as compared to niosomes (p-value<0.001). This might be due to swollen micellar structure of SMEDDS which is smaller than niosomes. Additionally, the surfactants C-EL and T-20 used for SMEDDS formulations have ability of intracellular membrane solubilization due to their high HLB values as compared to Span 60 used for Niosomes. This might have contributed to increased influx of formulations [21].

**7.9 In vitro permeability study**

A single layer of epithelial cells covers the inner intestinal wall which is a rate-limiting barrier for the absorption of dissolved drugs. Therefore, *in vitro* cultivation of Caco2 epithelial cells was carried out on transwell which could imitate intestinal barrier. When cultured for 21 – 23 days, Caco2 cells slowly differentiated into a phenotype having small intestinal villus epithelium. The integrity of cell monolayers cultured for 21–23 days was regularly monitored by measuring the TEER value. Monolayers with TEER of more than 300 $\Omega cm^2$ were used in the transport experiments.

Trans-epithelial permeation of ILO, VDN and their SMEDDS and Niosomes formulations through the Caco2 monolayer were determined. Practically, substances with an apparent permeability coefficient (*P*app) of less than 1×10−6 cm/s are considered as low permeability substances. Medium permeability substances have *P*app values below 1×10−5 cm/s and high permeability substances exhibit apparent permeability coefficients of >1×10−5 cm/s [22,23]. Permeability of drugs and formulations are shown in figure 7.7.

The ILO active material showed *P*app value (0.65085E-06 cm/s) and was considered to have low-medium permeability. Whereas, its Niosomes and SMEDDS exhibited a *P*app value of 1.0909E-06 cm/s and 1.7962E-06 cm/s respectively. Hence, according to this experiment, the

formulations showed medium to high permeability compared to pure ILO (p value<0.01). Similar observations were obtained for VDN and its formulations. Compared to VDN (0.49080E-06 cm/s), its SMEDDS (2.0205E-06 cm/s for T-20 SMEDDS and 1.6934E-06 cm/s for C-EL SMEDDS) and Niosomes (1.1130E-06 cm/s) were found to be moderately permeable (p value<0.01). This might be because of nano size of SMEDDS and Niosomes formulation, combined with amphiphilic nature of non-ionic surfactants present in the formulation. In addition to surfactants action, lipidic component of formulations help solubilization of lipophilic drugs in formulation which in presence of surfactants form mixed micelles that facilitate diffusion through the aqueous diffusion layer, thus, improving absorption [24].

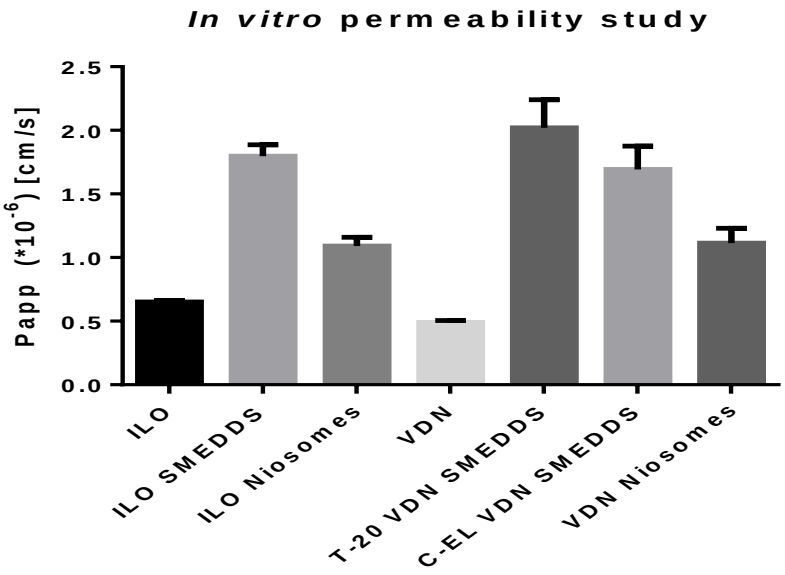



Figure 7.7 Apparent Permeability co-efficient (Papp) for Apical to Basolateral direction for the tested compounds

Moreover, drug permeation rate also depends on the mode of interaction of microemulsions and nanovesicles with cells. This may occur in different modes (1) collision and possible adsorption of the microemulsion and extracellular release of its contents (solubilized active material) and subsequent transport of these contents into the cells; (2) collision and possible adsorption of the microemulsion followed by selective transfer of lipophilic compounds

constructing the microemulsion and the solubilized active material directly to the plasma membrane; (3) endocytotic internalization of the microemulsion followed by intracellular release of its content; (4) fusion of the microemulsion with the plasma membrane or intracellularly with the endosomal membrane, thereby releasing the microemulsion contents into the cytoplasm [25]. The transfer of the drug from the formulation to the cytoplasmic membrane could be rate determining and depends on the comparative interaction of the drug with the formulation structure and the lipid pool of the cell monolayer. In case of SMEDDS, the interaction with the microemulsion is strongly dependent on the type of surfactant and the oil phase, and the ratio between the oil phase components or surfactant to oil phase. Whereas for niosomes, along with surfactants it largely depends on the localization of drug. If the drug, as in the present case, is localized in bilayer formed by cholesterol and surfactant, it will release faster than the drug localized in aqueous cavity.

The results of this study indicated that the permeability of ILO and VDN was clearly enhanced by nano-sized lipid-based formulations. However, other pharmacokinetic parameters such as clearance, volume of distribution and elimination half-life could not be predicted by this experimental approach. Therefore, in order to obtain the pharmacokinetic data of the developed formulations, *in vivo* studies were performed.

**7.10 Actin Visualization**

Actin, an important protein present in adherence junctions between the cells, has an essential role in the functionality of tight junctions. In intact tight junctions, these proteins are strongly associated with the cytoplasmic membrane [26]. Such far, no study has been reported about the effect of SMEDDS and niosomes on tight junctions at a molecular level. It is hypothesized that SMEDDS and niosomes might enhance the absorption of drug not only by increasing the membrane permeability, but also by opening tight junctions.

To test the hypothesis, we designed experiments to examine the effect of SMEDDS and niosomes on actin. The staining of the cells was carried out for cells of control wells and formulation treated wells. Staining by Phalloidin Alexa 488 green dye specifically stains actin

filaments only [5]. Phalloidin reacts stoichiometrically with actin and prevents its depolymerization to stabilize actin polymers [27]. As such phalloidin does not produce fluorescence on its own. Hence, alexa 488 tagged phalloidin which produces green fluorescence upon excitation was used herein.

As shown in figure 7.8 (A), the actin filaments of Caco-2 cell monolayers in the control group were localized at the apical peri-junctional area and appeared as a continuous band encircling the cells at the cellular borders. Whereas, the filaments treated with the formulations appeared discontinuous and less ordered, like "necklace". For formulation treated cells (B-F), actin staining was widespread inside the cell; rather than the peri-junctional areas as observed in control (A). This indicated that after treatment with formulations, the actin filaments were progressively disrupted as evidenced by their breakage and clumping (B-F).

As shown in figure 7.8 (B) and (C), when the cells were pretreated with niosomes formulation, predominant mechanism was endocytosis, which shows arrangement of actin filament somewhat similar to control (A). Whereas for SMEDDS (D, E and F), significant changes in F-actin staining were observed in comparison with the control cells. The change of the actin ring resulted in loss of tight junction integrity [28]. When the formulations bind to the epithelial cell membrane, a higher concentration of surfactants in the surface of cell could cause disrupting of tight junction.

In conclusion, ILO and VDN's formulations: SMEDDS and niosomes were not cytotoxic to the Caco2 cell lines as indicated by MTT assay. The study by confocal microscopy indicated qualitative intracellular uptake of formulation. Qualitatively it was proved by flow cytometry. Compared to niosomes, its counterpart SMEDDS formulations were found to have increased uptake as shown by FACS results. This might be due to smaller globular size of SMEDDS upon dilution as compared to vesicular size of niosomes. SMEDDS and niosomes were found to be able to enhance the paracellular transport of drugs across Caco-2 cell monolayers. Similar to the flow cytometry studies' results, SMEDDS increased more permeation than their counterpart niosomes formulations. The mechanistic study of opening of tight junctions by SMEDDS

demonstrated the involvement of the change of F-actin-related distribution. This mechanism might be the reason of increased uptake of SMEDDS formulation than niosomes.

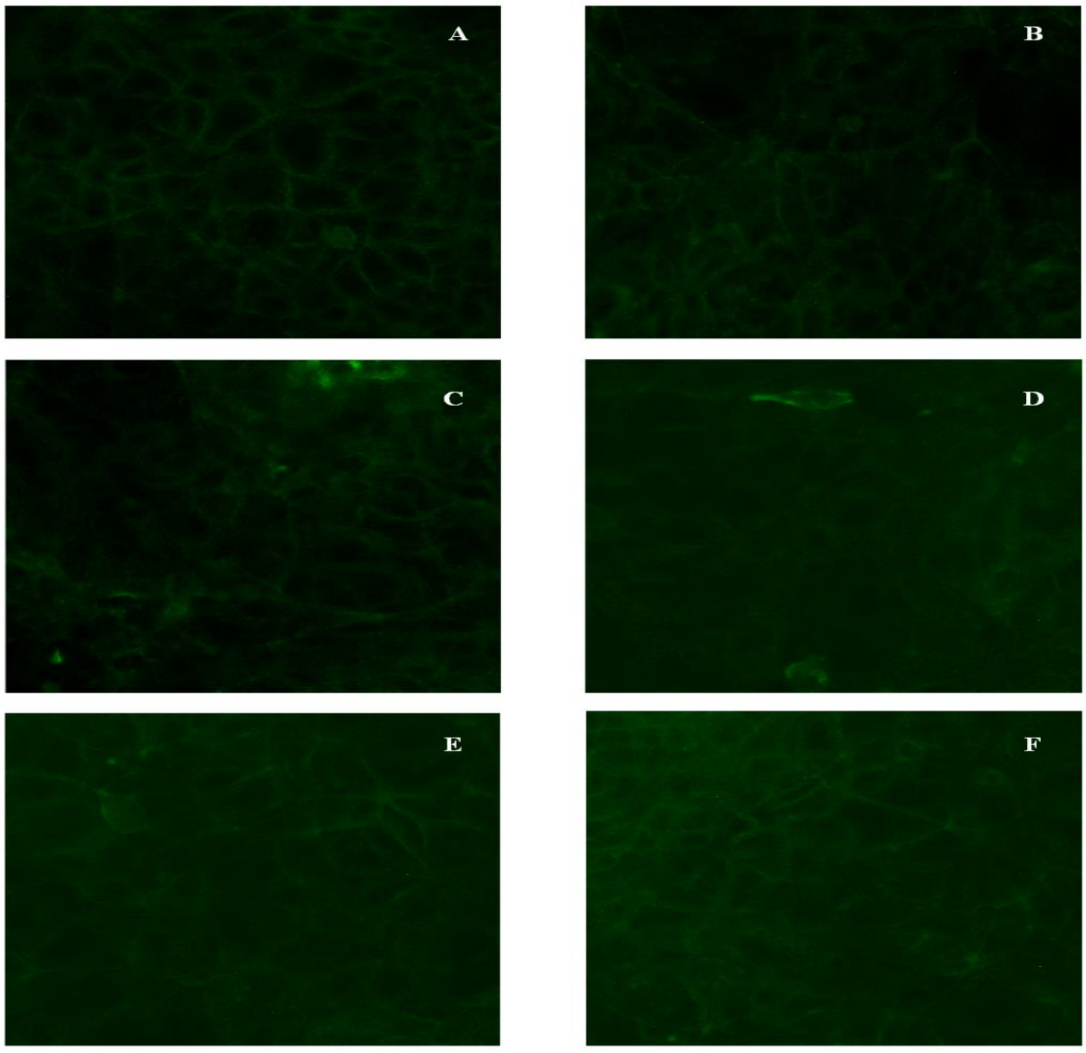



Figure 7.8 Actin visualization (A-control, B-ILO Niosomes, C-VDN Niosomes, D-ILO SMEDDS, E- C-EL VDN SMEDDS, F- T-20 VDN SMEDDS)

**References:**


1. Riss, T. L., & Moravec, R. A. (2006). Cell proliferation assays: improved homogeneous methods used to measure the number of cells in culture. In *Cell Biology (Third Edition)* (pp. 25-31): Elsevier.
2. Joshi, G., Kumar, A., & Sawant, K. (2016). Bioavailability enhancement, Caco-2 cells uptake and intestinal transport of orally administered lopinavir-loaded PLGA nanoparticles. *Drug Delivery, 23*(9), 3492-3504, doi:10.1080/10717544.2016.1199605.
3. Mundada, V. P., & Sawant, K. K. (2018). Enhanced Oral Bioavailability and Anticoagulant Activity of Dabigatran Etexilate by Self-Micro Emulsifying Drug Delivery System: Systematic Development, In vitro, Ex vivo and In vivo Evaluation. *J Nanomed Nanotechnol 9*(1), 1-13, doi:10.4172/2157-7439.1000480.
4. Hubatsch, I., Ragnarsson, E. G. E., & Artursson, P. (2007). Determination of drug permeability and prediction of drug absorption in Caco-2 monolayers. *Nature Protocols, 2*, 2111-2119, doi:10.1038/nprot.2007.303.
5. Tripathi, A., Parikh, Z. S., Vora, P., Frost, E. E., & Pillai, P. P. (2017). pERK1/2 Peripheral Recruitment and Filopodia Protrusion Augment Oligodendrocyte Progenitor Cell Migration: Combined Effects of PDGF-A and Fibronectin. *Cellular and Molecular Neurobiology, 37*(2), 183-194, doi:10.1007/s10571-016-0359-y.
6. De Jong, W. H., & Borm, P. J. A. (2008). Drug delivery and nanoparticles: Applications and hazards. *International Journal of Nanomedicine, 3*(2), 133-149.
7. Araújo, G. L. d., Campos, M. A. A., Valente, M. A. S., Silva, S. C. T., França, F. D., Chaves, M. M., et al. (2014). Alternative methods in toxicity testing: the current approach. *Brazilian Journal of Pharmaceutical Sciences, 50*, 55-62.
8. Judson, R., Richard, A., Dix, D. J., Houck, K., Martin, M., Kavlock, R., et al. (2009). The toxicity data landscape for environmental chemicals. *Environmental health perspectives, 117*(5), 685-695, doi:10.1289/ehp.0800168.
9. van Meerloo, J., Kaspers, G. J. L., & Cloos, J. (2011). Cell Sensitivity Assays: The MTT Assay. In I. A. Cree (Ed.), *Cancer Cell Culture: Methods and Protocols* (pp. 237-245). Totowa, NJ: Humana Press.
10. Mandal, S., Zhou, Y., Shibata, A., & Destache, C. J. (2015). Confocal fluorescence microscopy: An ultra-sensitive tool used to evaluate intracellular antiretroviral nano-drug delivery in HeLa cells. *AIP Advances, 5*(8), 1-9, doi:10.1063/1.4926584.
11. Rivolta, I., Panariti, A., Lettiero, B., Sesana, S., Gasco, P., Gasco, M. R., et al. (2011). Cellular uptake of coumarin-6 as a model drug loaded in solid lipid nanoparticles. *Journal of Physiology and Pharmacology, 62*(1), 45-53.
12. Neves, A. R., Queiroz, J. F., Costa Lima, S. A., Figueiredo, F., Fernandes, R., & Reis, S. (2016). Cellular uptake and transcytosis of lipid-based nanoparticles across the intestinal barrier: Relevance for oral drug delivery. *Journal of Colloid and Interface Science, 463*, 258-265, doi:https://doi.org/10.1016/j.jcis.2015.10.057.
13. Miao, X., Li, Y., Wyman, I., Lee, S. M. Y., Macartney, D. H., Zheng, Y., et al. (2015). Enhanced in vitro and in vivo uptake of a hydrophobic model drug coumarin-6 in the presence of cucurbit[7]uril. [10.1039/C5MD00189G]. *MedChemComm, 6*(7), 1370-1374, doi:10.1039/C5MD00189G.

14. Mui, B., Ahkong, Q. F., Chow, L., & Hope, M. J. (2000). Membrane perturbation and the mechanism of lipid-mediated transfer of DNA into cells. *Biochimica et Biophysica Acta (BBA) - Biomembranes, 1467*(2), 281-292, doi:https://doi.org/10.1016/S0005-2736(00)00226-1.
15. Peetla, C., Stine, A., & Labhasetwar, V. (2009). Biophysical interactions with model lipid membranes: applications in drug discovery and drug delivery. *Molecular pharmaceutics, 6*(5), 1264-1276, doi:10.1021/mp9000662.
16. Pérez-Rosés, R., Risco, E., Vila, R., Peñalver, P., & Cañigueral, S. (2014). Antioxidant activity of Tween-20 and Tween-80 evaluated through different in-vitro tests. *Journal of Pharmacy and Pharmacology, 67*(5), 666-672, doi:10.1111/jphp.12369.
17. Gottstein, C., Wu, G., Wong, B. J., & Zasadzinski, J. A. (2013). Precise Quantification of Nanoparticle Internalization. *ACS nano, 7*(6), 4933-4945, doi:10.1021/nn400243d.
18. Donovalová, J., Cigáň, M., Stankovičová, H., Gašpar, J., Danko, M., Gáplovský, A., et al. (2012). Spectral properties of substituted coumarins in solution and polymer matrices. *Molecules, 17*(3), 3259-3276.
19. Steen, H. B. (1991). Flow Cytometry Instrumentation. In S. Demers (Ed.), *Particle Analysis in Oceanography* (pp. 3-29). Berlin, Heidelberg: Springer Berlin Heidelberg.
20. Negi, L. M., Tariq, M., & Talegaonkar, S. (2013). Nano scale self-emulsifying oil based carrier system for improved oral bioavailability of camptothecin derivative by P-Glycoprotein modulation. *Colloids and Surfaces B: Biointerfaces, 111*, 346-353, doi:https://doi.org/10.1016/j.colsurfb.2013.06.001.
21. Kaur, P., Garg, T., Rath, G., Murthy, R. S. R., & Goyal, A. K. (2016). Development, optimization and evaluation of surfactant-based pulmonary nanolipid carrier system of paclitaxel for the management of drug resistance lung cancer using Box-Behnken design. *Drug Delivery, 23*(6), 1912-1925, doi:10.3109/10717544.2014.993486.
22. Kogan, A., Kesselman, E., Danino, D., Aserin, A., & Garti, N. (2008). Viability and permeability across Caco-2 cells of CBZ solubilized in fully dilutable microemulsions. *Colloids and Surfaces B: Biointerfaces, 66*(1), 1-12, doi:https://doi.org/10.1016/j.colsurfb.2008.05.006.
23. Shah, P., Jogani, V., Mishra, P., Mishra, A. K., Bagchi, T., & Misra, A. (2008). In Vitro Assessment of Acyclovir Permeation Across Cell Monolayers in the Presence of Absorption Enhancers. *Drug Development and Industrial Pharmacy, 34*(3), 279-288, doi:10.1080/03639040701655952.
24. Kalepu, S., Manthina, M., & Padavala, V. (2013). Oral lipid-based drug delivery systems – an overview. *Acta Pharmaceutica Sinica B, 3*(6), 361-372, doi:https://doi.org/10.1016/j.apsb.2013.10.001.
25. Sermkaew, N., Wiwattanawongsa, K., Ketjinda, W., & Wiwattanapatapee, R. (2013). Development, Characterization and Permeability Assessment Based on Caco-2 Monolayers of Self-Microemulsifying Floating Tablets of Tetrahydrocurcumin. *AAPS PharmSciTech, 14*(1), 321-331, doi:10.1208/s12249-012-9912-2.
26. Hartsock, A., & Nelson, W. J. (2008). Adherens and Tight Junctions: Structure, Function and Connections to the Actin Cytoskeleton. *Biochimica et biophysica acta, 1778*(3), 660-669, doi:10.1016/j.bbamem.2007.07.012.
27. Cooper, J. (1987). Effects of cytochalasin and phalloidin on actin. *The Journal of Cell Biology, 105*(4), 1473-1478.

28. Sha, X., Yan, G., Wu, Y., Li, J., & Fang, X. (2005). Effect of self-microemulsifying drug delivery systems containing Labrasol on tight junctions in Caco-2 cells. *European Journal of Pharmaceutical Sciences, 24*(5), 477-486, doi:https://doi.org/10.1016/j.ejps.2005.01.001.