9.1. Introduction

Aim of the QbD based drug development is to design safe and effective drugs with desired characteristics justifying its QTPP. Since in vivo absorption studies in animals are complex, time-consuming, ethically challenging and expensive, there has been a recognized need to develop alternative in vitro methods [1]. Cell culture models are used as intermediately complex systems between whole animal studies and isolated enzymes, membrane fractions or artificial lipid bilayers [2].

The purpose of present study was to evaluate cell viability and quantitative cellular uptake of the developed formulations. Hence, cytotoxicity studies by MTT Assay and cellular uptake studies by FACS analysis were performed prior to in- vivo studies.

9.1.1. Cytotoxicity study by MTT Assay

Tetrazolium reduction assays are characterized by reduction of various tetrazolium analogues (MTT, MTS, XTT, WST-1, and INT) and quantification of the subsequent colour change. The MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay was originally developed as an alternative to the radioactive thymidine incorporation assay. It was one of the first assays to be developed for the 96-well plate format intended for high throughput screening in 1983 by Tim Mossman [3]. The MTT assay measures the cell viability of a solution as opposed to cell proliferation. This assay is therefore is an incredibly useful tool in determining drug cytotoxicity or differentiating between multiple cell lines. Unlike other tetrazolium compounds in other assays MTT can readily pass through the plasma membrane of eukaryotic cells. This allows MTT to be reduced in the cytoplasm whereas other tetrazolium compounds need to be reduced by electrons transported out of the cell by intermediary electron acceptors. This reduction occurs because of dehydrogenase enzymes only present in viable cells which reduce the yellow MTT to purple insoluble formazan crystals which accumulate inside the cell [4]. These formazan crystals must be solubilized before an absorbance reading at 570 nm can be taken and there are many ways to do this such

as adding acidified isopropanol, DMSO, DMF, or SDS. The assay relies on the principle that the darker the purple colour, the more metabolically active cells present [3, 5].

9.1.2. In vitro cellular uptake study

The quantitative cellular uptake of the formulation was performed by FACS-Flow cytometry. FACS is an abbreviation for fluorescence-activated cell sorting, which is a flow cytometry technique that further adds a degree of functionality. FACS analysis allows to concurrently collect data on, and sort a biological sample by a nearly infinite number of different constraints [6]. Just like in conventional flow cytometry, forward-scatter, side-scatter, and fluorescent signal data are collected. The user defines the parameters on how cells should be sorted and then the machine imposes an electrical charge on each cell so that cells will be sorted by charge (using electromagnets) into separate vessels upon exiting the flow chamber [7]. The technology to physically sort a heterogeneous mixture of cells into different populations is useful for a wide range of scientific fields from research to clinical. Nowadays the terms "flow cytometry" and "FACS" are often used interchangeably to describe this laser-based biophysical technique. A population of mixed cells is sorted into a negative sample and a positive sample containing cells of interest by the flow cytometer [8].

9.2. Material and Equipment

9.2.1. Cell Line

The cytotoxicity study was carried out on in vitro model of MCF-7 which was procured from the National Centre for Cell Science (NCCS, Pune, India). MCF-7 over expresses oestrogen and progesterone receptors on it, appropriate to use for studying the efficacy of drug and/or drug loaded formulation acting on endometrial carcinoma. The cells were cultured in T-25 cm^2 tissue culture flask supplemented with Dulbecco's modified eagle medium (DMEM) media with 0.1 % penicillin and streptomycin solution and 10 % v/v foetal bovine serum (FBS) incubated at 37 ± 1 °C, 5 % CO2 in an incubator. The culture medium was replaced every alternative day. Cell culture grade dimethyl sulphoxide (DMSO ≥99%) and other culture solutions mentioned before were procured from Himedia laboratories. The 3– (4, 5-Dimethylthiazol-2-yl)-2, 5-

diphenyltetrazolium bromide (MTT) dye was purchased from Sigma Aldrich (USA). Fluorescein isothiocyanate (FITC) was purchased from SRL chemicals (Mumbai). Various well plates and other materials used in the cell line study were purchased from HI media Laboratories.

Equipment:

- ELISA plate reader (FLUO star, Germany)
- Jouan IGO150 5% CO2 incubator (Thermo-Fischer, Germany)
- Weiber vertical Laminar Air Flow (Weiber, India)
- Nikon H600L Microscope (Nikon, Japan)
- Fluorescence activated cell sorter (BDFACS Calibur, BD Biosciences, USA)

9.3. Cell Cytotoxicity study by MTT Assay

MTT solution: 2.5mg/ml MTT solution was prepared in phosphate buffer saline (PBS) pH 7.4. The solution was filtered through a 0.2 µm filter to eliminate bioburden of the solution and stored at 2-8°C.

PTX-UDNVs and Marketed formulation solutions:

Marketed formulation of PTX (GROS^{TM}-100) and PTX-UDNVs formulation were diluted using sterile PBS pH 7.4 to obtain stock solutions of final concentration of 1000 µg/ml of PTX.

Suitable aliquots of the stock solution of marketed formulation and PTX-UDNVs formulation were added into the wells containing DMEM medium to give a final concentration 0.001 µg/ml, 0.01 µg/ml, 0.1 µg/ml, 1 µg/ml and 10 µg/ml of PTX.

CBP-UDNVs and Marketed formulation solutions:

Marketed formulation of CBP (CARBOKEM NOVA 150) and CBP-UDNVs were diluted using sterile PBS pH 7.4 to obtain stock solutions of final concentration of 1000 µg/ml of CBP.

Suitable aliquots of the stock solution of marketed formulation and CBP-UDNVs formulation were added into the wells containing DMEM medium to give a final concentration 0.001 µg/ml, 0.01 µg/ml, 0.1 µg/ml, 1 µg/ml and 10 µg/ml of CBP.

Sample Coding:

- S1: Marketed formulation of PTX
- S2: PTX-UDNVs formulation
- S3: Marketed formulation of CBP
- S4: CBP-UDNVs formulation

Protocol:

MCF-7 cell line was propagated in Dulbecco's modified eagle medium (DMEM) media with 0.1 % penicillin and streptomycin solution and 10 % v/v foetal bovine serum (FBS) incubated at 37 ± 1 °C, 5 % CO2 in an incubator. Cells were seeded in 96 well plates (5 x 10^3 cells/well) and allowed to attach and grow. After 24 hours cells were treated with different concentrations of the test samples (S1, S2, S3 and S4) individually and incubated at 37 ± 1 °C, 5 % CO2 for 12, 24, 48 hours. After incubation, treatment media was removed and cells were treated with 20 µL (2.5 mg/ml) of MTT dye solution and incubated for 4 hours. The dark blue formazan product formed by the cells was dissolved in 200 µL DMSO under a safety cabinet and read at 570 nm on a plate reader. Furthermore, the blank and negative control were also used in order to avoid false negative errors. The cells without treatment were taken as a negative control and DMSO was taken as blank. The values of percentage viability were plotted with the concentrations and IC50 values were calculated.

9.4. Quantitative cellular uptake analysis by FACS

Preparation of Dye loaded formulations:

Fluorescein isothiocyanate (FITC), a fluorescent model dye, was used for the preparation of dye loaded formulations by similar procedures as explained in formulation development chapters of the respective molecules where the drug was replaced with FITC dye, for quantitative cell uptake studies.

Protocol:

The experiment was initiated following seeding step. The cells (5 x 10^5 cells/well) were seeded in 6-well plate and incubated at 37 ± 1 °C for 24 h. Once the sufficient confluency is reached, the wells were treated with PTX-UDNVs and CBP-UDNVs dye loaded formulations and incubated at 37 ± 1 °C for 24 h. At the end incubation, the culture medium was washed twice with PBS (pH 7.4). Further, the cells were detached by trypsinization with Trypsin-EDTA solution and centrifuged at 6000 rpm for 1 min to get cell pellet. The cells were then re-suspended in FACS buffer (9.8 mL PBS+ 0.1 mL FBS + 100 mg BSA), passed through strainer and were analysed using FACS (FACS Canto-II,BD Biosciences, San Jose, USA) by BD FACS Diva 6.1.3 software, BD Biosciences, USA. Here the untreated cells were taken as control.

9.5. Results and Discussion

9.5.1. Cell Cytotoxicity study

The cytotoxicity studies were performed on MCF-& cell line. The marketed formulations and developed formulations were compared for effectiveness of inhibition of the cancer cells. The results demonstrated that the UDNVs formulation of PTX was more effective in inhibition of cellular growth than the marketed formulation as depicted in the **Fig. 9-1**.

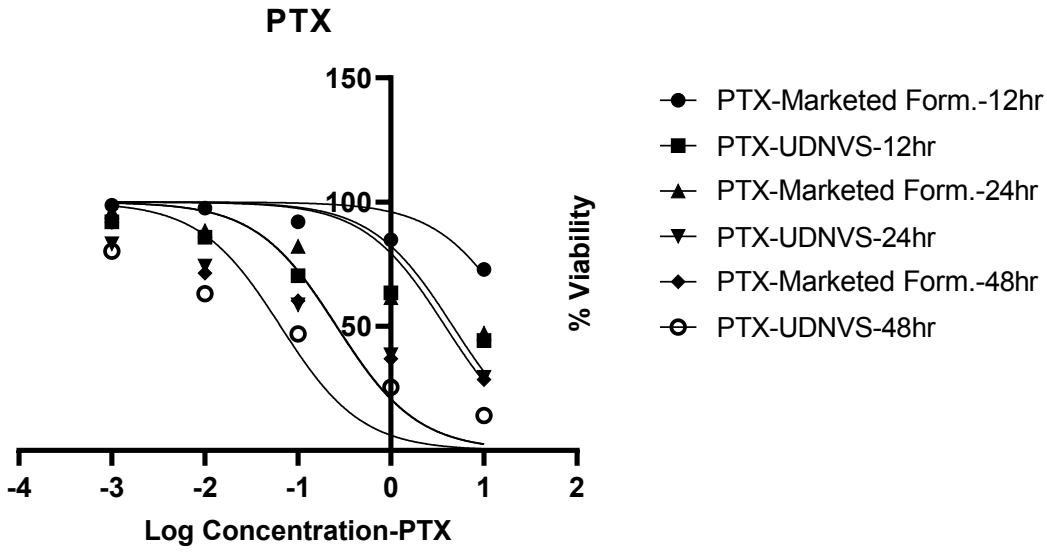

Fig. 9-1: Cytotoxicity of PTX marketed formulation and PTX-UDNVs

It is evidenced from the graphs of 12 h, 24 h and 48 h exposure time that the cytotoxicity increased as exposure time increases. The values of % viability for marketed formulation of PTX was considerably higher than PTX-UDNVs. This indicates that the PTX-UDNVs formulation can produce more cytotoxic effect at the same concentrations to that of PTX marketed formulation. This might be due to higher uptake of the vesicular formulation loaded with PTX than that of PTX present in the solution form in marketed formulation. Moreover, concentration dependent cytotoxicity was observed where cell viability reduced at high concentrations of drug. It is because the cell count in each well remained constant whereas, the PTX concentration was increased which resulted into reduced cell viability.

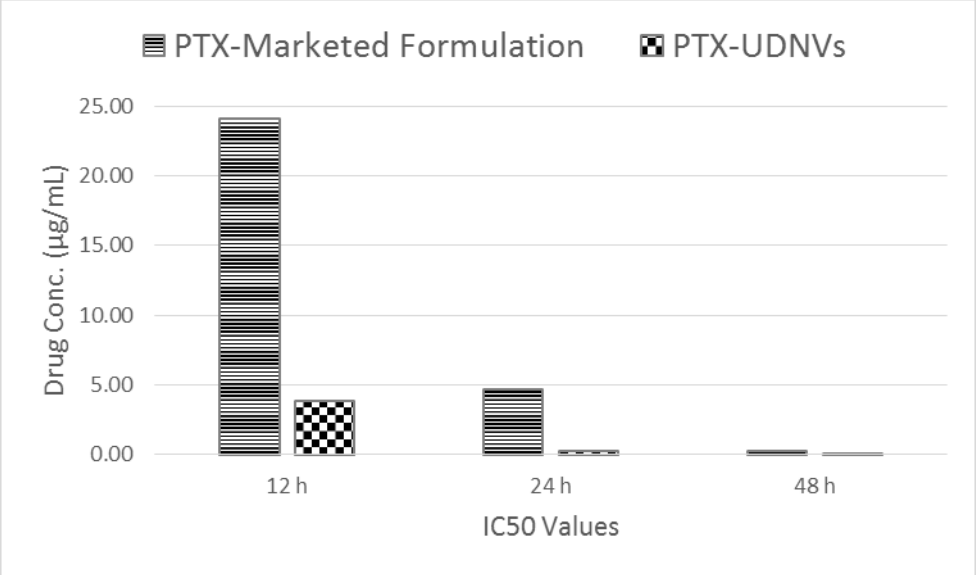

Fig. 9-2: IC50 values of PTX-marketed formulation and PTX-UDNVs

The IC50 values were determined for marketed formulation of PTX and UDNVs formulation loaded with PTX and presented in **Table 9-1** & **Fig. 9-2**. The values of IC50 showed that the inhibitory effect of the vesicular formulation is higher than marketed formulation at relatively lower concentrations also.

Table 9-1: IC50 values of PTX-marketed formulation and PTX-UDNVs

Test Sample	IC50 Values (µg/mL)		
	12 h	24 h	48 h
PTX-Marketed Formulation	24.1	4.64	0.26
PTX-UDNVs	3.38	0.26	0.07

As represented in the graph, it is evidence that the IC50 values for PTX-UDNVs formulation was lower at all the exposure times than that of the marketed formulation of the PTX. This indicated that lower dose of the vesicular formulation is required as compared to the marketed formulation in order to produce 50% inhibition of the cell population. The enhanced antiproliferative activity might be due to the fact that the internalization of the drug via endocytosis is prominent in the case of vesicular drug loaded formulation than the solution of the drug [9].

The cell cytotoxicity efficiency of CBP-UDNVs and CBP-Marketed Formulation was evaluated using MCF-7 cell line and the results are depicted in **Fig. 9-3**

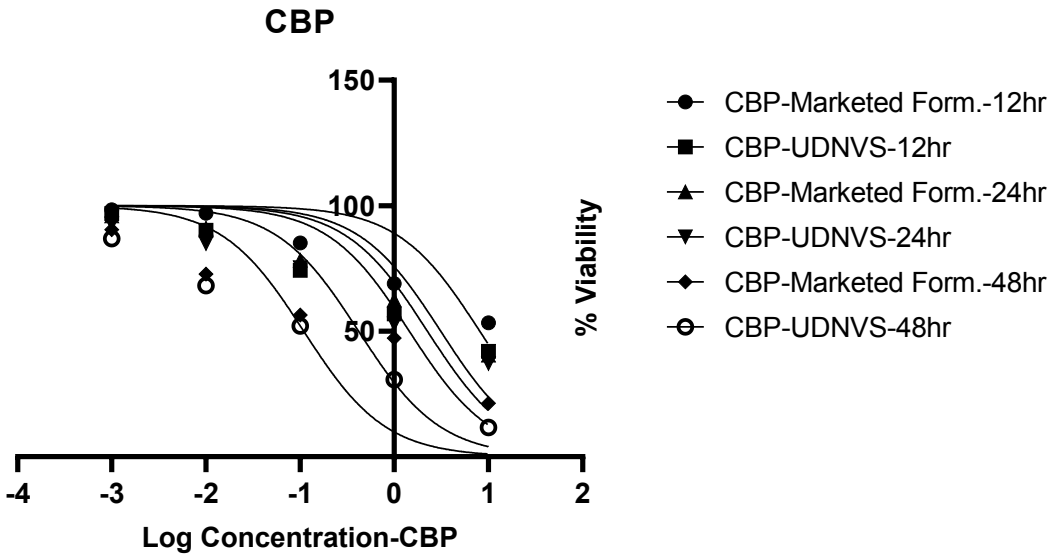

Fig. 9-3: Cytotoxicity of CBP marketed formulation and CBP-UDNVs

It is evidenced from the graphs of 12 h, 24 h and 48 h exposure time that the cytotoxicity increased as exposure time increases. The values of % viability for marketed formulation of CBP was considerably higher than CBP-UDNVs. This indicates that the CBP-UDNVs formulation can produce more cytotoxic effect at the same concentrations to that of CBP marketed formulation. This might be due to higher uptake of the vesicular formulation loaded with CBP than that of CBP present in the solution form in marketed formulation. Moreover, concentration dependent cytotoxicity was observed where cell viability reduced at high concentrations of drug. It is because the cell count in each well remained constant whereas, the CBP concentration was increased which resulted into reduced cell viability.

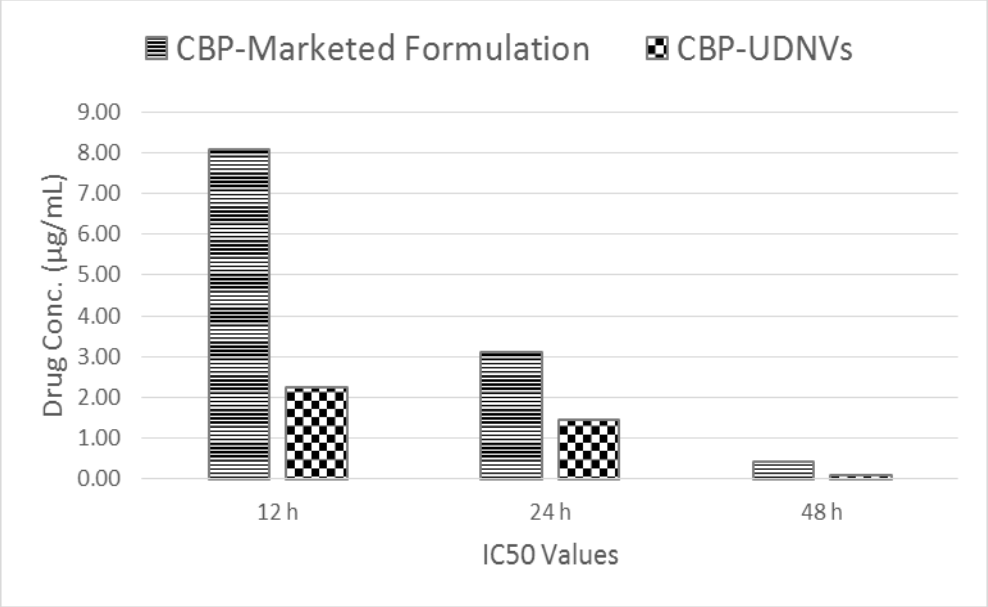

Fig. 9-4: IC50 values of CBP-marketed formulation and CBP-UDNVs

The IC50 values were determined for marketed formulation of CBP and UDNVs formulation loaded with CBP and presented in **Fig. 9-4** & **Table 9-2**. The values of IC50 showed that the inhibitory effect of the vesicular formulation is higher than marketed formulation at relatively lower concentrations also.

Table 9-2: IC50 values of CBP-marketed formulation and CBP-UDNVs

Test Sample	IC50 Values (µg/mL)		
	12 h	24 h	48 h
CBP-Marketed Formulation	8.08	3.11	0.42
CBP-UDNVs	2.25	1.45	0.11

As represented in the graph, it is evidence that the IC50 values for CBP-UDNVs formulation was lower at all the exposure times than that of the marketed formulation of the CBP. This indicated that lower dose of the vesicular formulation is required as compared to the marketed formulation in order to produce 50% inhibition of the cell population. The vesicular formulation loaded with antitumor agent is able to enter the cell more efficiently than the plain drug solutions which could be the reason for enhanced cell growth inhibition for CBP-UDNVs formulation than CBP-marketed formulation.

9.5.2. Quantitative cellular uptake study

Relative extent of uptake of the FITC loaded vesicular formulations of PTX and CBP in comparison with the plain dye solution was analysed by FACS. The results of FACS study showed that the fluorescence intensities inside the cells increased significantly with vesicular formulations when compared to the plain dye solution. The enhanced fluorescence intensities represent higher uptake and internalization for PTX and CBP UDNVs formulations [10, 11]. The enhancement of the fluorescence intensity in the case of PTX and CBP vesicular formulation was more than double which supported the superiority of the developed formulations over plain drug solution in cell uptake.

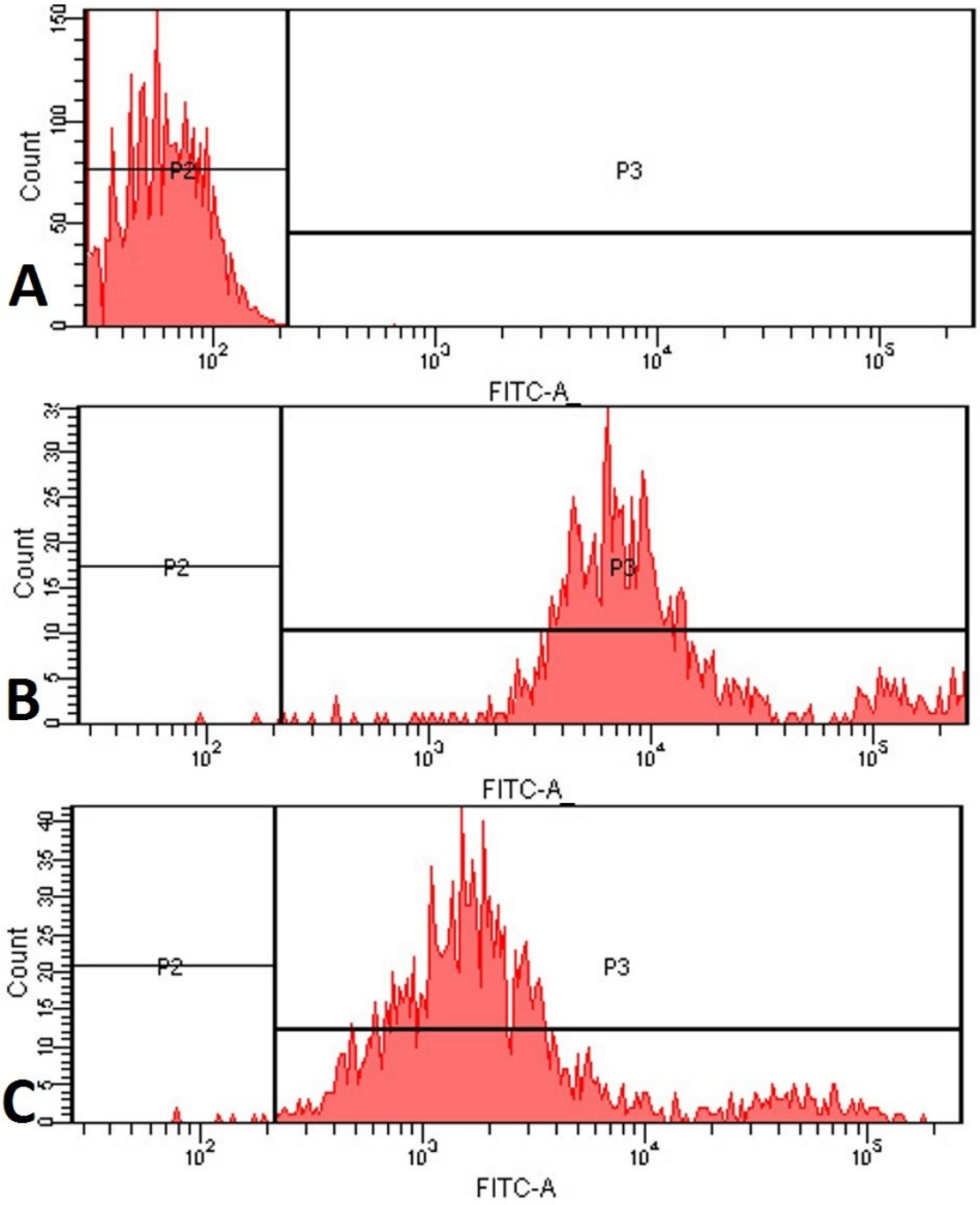

Fig. 9-5: Quantitative intracellular uptake histograms for A) FITC dye solution treated sample B) FITC loaded PTX-UDNVs treated sample C) FITC loaded CBP-UDNVs treated sample

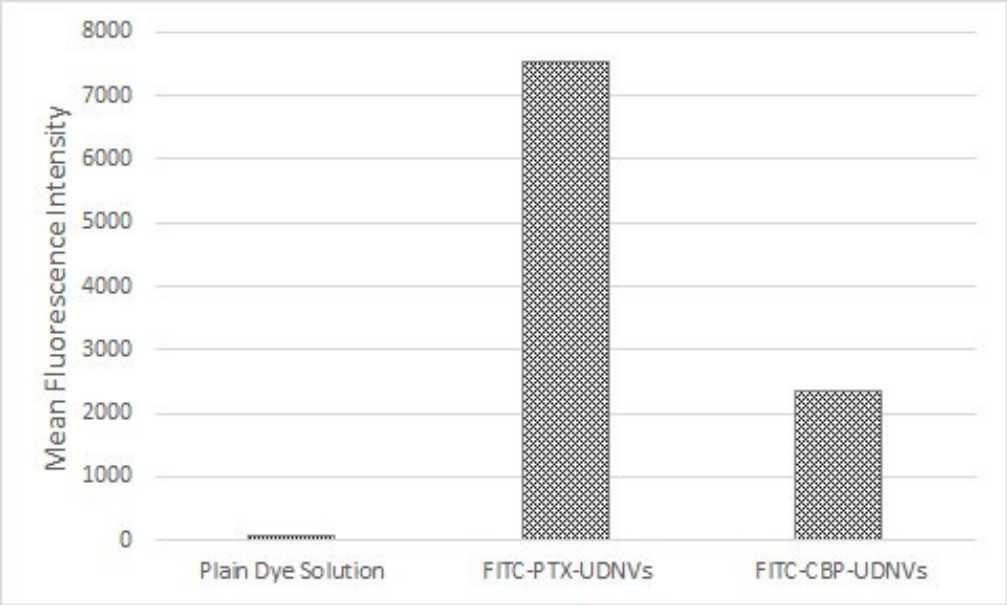

Fig. 9-6: Mean fluorescence intensity plot of plain dye solution, FITC-PTX-UDNVs and FITC-CBP-UDNVs

The augmented intracellular uptake for UDNVs formulation as compared to the plain dye solution was further supported by the mean fluorescence intensities (MFI) values for all three samples. As evidenced from **Fig. 9-6**, mean fluorescence intensities were significantly larger than the plain dye solution representing intensified intracellular uptake of the developed formulations.

The results are in agreement with the findings of different researchers that due to the lipophilic nature, liposomes facilitate diffusion of entrapped drugs through various biological membranes [12-16]. It can also potentiate the efficacy of chemotherapy and other intracellular active drugs by bypassing multi-drug resistance pumps, thus achieving intracellular delivery to targeted cells [14, 17, 18].

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