Synopsis
of the thesis entitled

"Development of Nanocarriers for Simultaneous Delivery of Drug and Gene Therapeutics in Treatment of Drug Resistant Lung Cancer"

By
Denish R. Bardoliwala
M.Pharm (NDDS)

Under the supervision of

Late Prof. Ambikanandan Misra

And

Dr. (Mrs.) Krutika. K. Sawant
Professor and Dean of Pharmacy Department,
Faculty of Pharmacy

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THE MAHARAJA SAYAJIRAO UNIVERSITY OF BARODA, VADODARA

Faculty of Pharmacy,
The M. S. University of Baroda.
Vadodara -390001, Gujarat, India

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1 Introduction

Lung cancer or lung carcinoma in simple terms can be defined as any abnormal/uncontrolled growth of different types of cells in lungs. Lung cancer can be engendering from lung cells or due to metastasis from other organ cells which have extensive replicative potential (1).

Docetaxel is a lipophilic anticancer agent and a semi-synthetic taxane derived from the European tree Taxus baccata (2). Docetaxel has been approved by the Food and Drug Administration (FDA) and is widely used for different types of cancer, such as breast cancer, ovarian cancer, prostate cancer, non-small-cell lung cancer, gastric adenocarcinoma, and others (3). Docetaxel acts by binding reversibly to microtubules, promoting transitory structure stabilization, leading to cell cycle arrest. Therefore, docetaxel is a cytostatic drug for the control of tumor tissue growth (4).

Docetaxel showed some improved survival outcomes regarding metastatic disease when compared with other chemotherapeutic agents (5). However, the clinical administration of intravenous docetaxel has been limited due to its poor aqueous solubility (4.93 µg/mL in purified water), high lipophilicity (log P = 4.1), low bioavailability and high toxicity. To increase the solubility of docetaxel, the pharmaceutical industry developed some formulations containing surfactants, such as Tween-80, and/or alcohol, to combat these pharmaco-technical problems. Nevertheless, as highly reactive components, these formulations cause some adverse reactions in patients, including hypersensitivity, neurotoxicity, musculoskeletal toxicity and fluid retention (6). In order to reduce these side effects nanocarriers have been used to overcome these drawbacks related to docetaxel.

Multidrug resistance (MDR) is a major obstacle in treating cancer in which cancer cells develop resistance towards ongoing chemotherapy. Mechanisms by which cells develop MDR can be broadly divided into cellular factors and physiological factors (7). Cellular factors involve over expressive efflux pump, reduced rate of cell apoptosis, genetic defects (i.e. gene deletion and polymorphism in gene), increased rate of drug metabolism, etc (8). Whereas physiological factors responsible for MDR includes interaction at cellular level, higher pressure of cell interstitial fluid, low pH environment around tumor, presence of hypoxic region at core of tumor, irregular nature of tumor vasculature, presence of cancer cells in areas that are difficult to penetrate (9).

ABC transporters are the most extensively studied mechanism for the treatment of MDR cancers. Therefore, designing an advanced multifunctional delivery system should be a priority to reverse MDR in cancer chemotherapy. Thus, MDR can be treated by gene knock down approach to inhibit expression of these ABC family proteins which are responsible for efflux of oncological therapeutics and reduced therapeutic action. RNA interference, P-gp inhibitors and few peptides are extensively adopted approaches and gene silencing through RNA interference technology is most impactful tool now-a-days amongst all approaches.

RNA interference (RNAi) is a basic conserved mechanism of cell by which a small double stranded RNA (dsRNA) directs the degradation mRNA which is eventually responsible for the inhibition of specific gene expression (10). Soon after its discovery RNAi has been studies extensively for the role in gene function in the normal cellular biological process and protein synthesis (11).

RNA interference (RNAi) is the artificially induced cellular process for the degradation of particular mRNA and it is induced with the use of double stranded RNA which has a specific sequence related to target mRNA. RNAi mechanism has been observed in all eukaryotes, from yeast to mammals (12). The mammalian cell contains specific enzymes similar to dicer enzyme found in Drosophila which identifies the dsRNA (Double stranded RNA) and breaks it into smaller fragments having a base pair length between 21-25. This double stranded RNA can be shRNA which binds to RISC (RNA induced silencing complex). This RISC complex detaches distal chain from the shRNA which then binds to mRNA (that is for the specific protein synthesis). After mRNA binds to the activated RISC complex, it is cleaved and hence production of specific protein in the cells is inhibited.

siRNA and shRNA are the powerful tools that are used to artificially induce RNAi in the mammalian cells. (13). shRNA has been advantageous over siRNA as it has nuclear expression rather than siRNA which is artificially made. Expression of the siRNA has been limited up to 48 hrs or maximum three cell cycle whereas shRNA is expressed over the period of three years. Only 5 copies of shRNA are enough to produce therapeutic concentration. As it is nuclear expression it has low stimulation of immune system and low cytotoxicity (14). Hence shRNA is a new tool for the RNAi which is proved to be better than siRNA in terms of efficiency, stability and duration of therapy

along with safety. It has high potential to become future of biopharmaceutical medicines.

Nanotechnology- based delivery systems grabbed tremendous attention for delivering cancer therapeutics as they provide benefits including controlled drug release, improved biological half-life, reduced toxicity and targeted delivery. Majority of the nanocarriers consists of either a polymer or a lipid component along with other excipients to stabilize the colloidal system (15). Lipid-based systems provide advantages like better entrapment efficiency, scalability and low- cost raw materials, however, suffer from limitations including instability, a burst release of the drug, and limited surface functionalization (16). On the other hand, polymeric systems provide an excellent diversity of chemical modifications, stability, controlled release, however limited drug loading capacities and scale up limit their use (17).

Hybrid nanocarriers consisting of lipid and polymer were able to overcome some of these disadvantages while retaining the advantages of both the systems. The hybrid architecture made up of polymeric core and layers of lipid has been said to provide several advantages like adjustable particle size and drug release (18). Also, the use of charged phospholipids further provides electrostatic interactions leading to the ease of loading multiple agents, better loading efficiency and serum stability (19). Designing a stable lipid-polymer hybrid system requires a thorough understanding of the material properties and their behaviour in in vitro and in vivo environments.

2 Aim:

The present research focuses on the development of hybrid nanocarriers for the simultaneous delivery of docetaxel and shRNA plasmid for the treatment of drug-resistant lung cancer.

3 Objectives

To develop lipid polymeric hybrid nanocarriers with the combination of docetaxel and shRNA for the reversal of drug resistance in lung cancer. Reversal of drug resistance will directly result in increased sensitization of the drug along with a reduction in therapeutic dose and achieve a milestone in prolongation of treatment due to reduced toxicity. This treatment may possibly help in the complete remission of lung cancer and may prevent its metastasis. This treatment will also increase the survival rate of patients.

4 Supplementary objectives

The present research is proposed for evaluating effectiveness of silencing gene ABCB1 (MDR1) that confers resistance to chemotherapeutic agents in treatment of lung cancer using novel nanocarrier; **PLHNCs (Polymeric-lipid hybrid nanocarriers)** comprising of lipids & polymers by administering them through parental (For comparative in-vivo studies) and pulmonary route (by **formulating Dry Powder Inhaler (DPI)**.

- To develop novel nanocarriers (PLHNCs) for the simultaneous delivery of drug and gene therapeutics.
- It was also conjugated with folate targeting ligand to impart selectivity towards cancer cells.
- To mask the drug resistance in the chemotherapy of lung cancer by silencing drug efflux transporter through the delivery of plasmid for shRNA against ABCB1.
- To potentiate drug efficacy and treatment in cases of drug-resistant lung cancer by formulation & development of PLHNCs that can deliver plasmid for shRNA against ABCB1 along with anti-neoplastic agent docetaxel.
- To compare the developed PLHNCs against P-gp inhibitor Verapamil in cell line studies.

5 Analytical method development:

Aliquots ranging from 0.2, 0.4, 0.6, 0.8, 1.0 ml were taken from working standard solution of docetaxel and diluted up to 1 ml with the mobile phase to give a final concentration of 200, 400, 600, 800, 1000ng/ml. 20 µl of each sample was injected in sampler loop of HPLC for each concentration and chromatogram was taken under the condition mentioned in Table 1. The calibration graph was constructed by plotting peak area against concentration of docetaxel and the regression equation was calculated.

Table 1 HPLC system parameters for docetaxel estimation

System Parameter	Value
HPLC	Agilent Technologies 1260 infinity II
Column	250 mm *4.6 mm* 5 µm, Thermo scientific
Wavelength	231 nm
Flow-rate	1 ml/min.
Run-time	10 min

Injection volume	Rhenodyne 7725 injector valve with a fixed loop at 20 µl
Mobile Phase	ACN: Water (60:40)
Retention time	7.4 mins.

A Docetaxel calibration plot in the range of 200-1000 ng/ml was obtained. The regular curve regression equation was found to be y = 545.06x - 9156.9. It was observed that the correlation coefficient for the system was 0.999, suggesting the presence of a linear relationship between peak area and docetaxel concentration. The retention period was 7.55 minutes.

6 Preformulation study

Pre formulation studies are designed to recognize the physiochemical properties of drugs as well as excipients that may affect the method of manufacture, formulation design, pharmacokinetic properties of the resulting product. The objective of the preformulation study is to develop an elegant, stable, effective and safe dosage form by establishing kinetic rate profile, compatibility with the other ingredients and establish the physico-chemical parameter of new drug substances.

6.1 Organoleptic properties:

The received sample of docetaxel was checked visually for organoleptic characteristic like color and the odor of drug.

Color: White to off-white

Odor: odorless

State: powder

6.2 Melting point determination

The melting point was determined by capillary tube method using melting point apparatus (VEEGO, Mumbai) in which temperature was gradually increased and the temperature at which the drug melt was recorded. The melting point of Docetaxel determined by Capillary Method was found to be in the range of 179°C-182°C which was in the range of reported value (178-183°C).

6.3 Authentication of Docetaxel with FT-IR

The IR spectra of Docetaxel practically obtained using FT-IR spectrophotometer (Bruker, Japan) was compared with standard IR spectra of a drug, which was found to show all characteristic peaks of functional groups. So, it was confirmed that the drug was authentic.

7 Drug-excipients compatibility study

7.1 Compatibility study using Infrared spectroscopy

The IR Spectrum of Docetaxel and characteristic bands were identified. For the compatibility of drug and excipients, IR spectra of Docetaxel and physical mixture (Docetaxel +PEG-PCL+ DPPC: DSPE-PEG_{2000}: DOTAP) was studied as shown in **Error! Reference source not found.**. It exhibited characteristic peaks at 3377.50 cm^{-1} f or OH stretching, 3487.00 cm^{-1} for NH stretching, 1452.57 cm^{-1} for C=C stretching, 1711.85 cm^{-1} for C=O stretching, and 2982.00 cm^{-1} for CH stretching vibrations. All these peaks are considered characteristic to Docetaxel and were prominently observed in IR spectra of Physical mixture as well. No additional peak was observed in physical mixture of drug and excipients. From these results, it was confirmed that there was no interaction between Docetaxel and excipients.

7.2 Compatibility study using DSC

In thermal analysis, Differential Scanning Calorimetry (DSC) is generally used to track endothermic (melting, solid-solid phase transformations and chemical degradation) and exothermic processes (Crystallization and oxidative decomposition) (20). In pre-formulation tests, it is highly useful as it suggests the presence of potential association between drug-excipient or excipient-excipient in formulation.

DSC thermogram of Docetaxel displayed a high endothermic plateau corresponding to Docetaxel melting point at 179.62 °C. Whereas a sharp endothermic peak at 60.25 °C shows the melting point equivalent to crystalline PEG: PCL diblock co-polymer. Even DSC thermogram of the DPPC: DSPE-PEG_{2000}: DOTAP (lipid admixture) doesn’t show any peak due to negative glass transition temperature of resultant lipid mixture. DSC thermogram of physical mixture of Docetaxel, PEG-PCL and lipid admixture demonstrates no change in the endothermic nature of Docetaxel

hence it can be said that Docetaxel and excipients used in the formulation development is compatible with one another.

8 Formulation development and optimization

Developed lipid polymeric nanoparticles have merits of polymeric nanoparticles along with biocompatibility of the lipids. At nano-size range, developed nanocarriers provide a favourable advantage in tumor accumulation and intracellular uptake through endocytosis.

There are two approaches available in the literature for the preparation of PLHNCs. One approach involves that the polymeric core and lipid shell are prepared separately using two independent processes; then the two components are combined by direct hydration, sonication, or extrusion to obtain the desired lipid shell–polymer core structure; the other approach involves a single-step process, in which the hybrid nanoparticles are prepared through a one-pot nanoprecipitation and self-assembly method. All the methods were checked for their feasibility and the best method suited to prepare Docetaxel PLHNCs with favourable characteristics was further optimized.

A systematic screening study was employed to screen the parameters that greatly affect characteristics of PLHNCs. The above-mentioned poor solubility and hypersensitivity of the marketed formulation can be overcome by the targeted profile of the intended PLHNCs: i.e.,1) Higher entrapment efficiency compared to other formulations. 2) Predictable variation range in encapsulation efficiency between the range of different factors. 3) Particle size < 200 nm (To prevent macrophage mediated cell clearance) (21). 4) Higher stability. For this purpose, we have introduced two designs. i.e., Placket-Burman design and Box- Behnken design. Plackett Burman's design was used to evaluate the most prominent parameters influencing Docetaxel encapsulation in PLHNCs as well as its particle size. (22), whereas the Box- Behnken design was used to produce a design matrix and to provide a surface plot for number of parameters influencing the encapsulation and the size of the PLHNCs (23). Currently, researches have employed the principle of QbD to reinforce the formulation and process layout for a better understanding of a variation source to enhance the product quality.

8.1 Single step method

To circumvent the problems of time-consuming preparation steps, a relatively simple approach that combines the dual steps of the two-step method into a single step was evaluated.

8.1.1 Modified solvent extraction/evaporation method

Formulations of PLHNCs were performed using (self-assembly of polymers and various ratios of lipids) single-step nano-precipitation method with certain modifications (24). Two phases (i.e., organic and aqueous) were prepared separately and then, mixed together to form PLHNCs. A fixed quantity of PEG: PCL (1-10 mg/mL) and Docetaxel were dissolved in acetonitrile to prepare the organic phase. DPPC: DOTAP: DSPE-PEG_{2000} was dissolved in 4% ethanolic solution form aqueous phase. The resultant solution was heated at 65ºC to ensure the phase transition of the lipid-bi-layer, when mixed further. After that, Docetaxel containing PEG: PCL solution was added to a preheated lipid solution via a dropwise method at 1 mL/min flow rate with vigorous mixing for at least 0.5 to 2 h to ensure a complete evaporation of the organic solvent and maximum encapsulation of Docetaxel in PLHNCs. Formulated PLHNCs were subjected further for the determination of encapsulation efficiency. Furthermore, PLHNCs suspension was introduced to the series of extrusion cycles and bath sonication to improvise the encapsulation efficiency as well as the size distribution.

The developed formulation of docetaxel hybrid nanocarriers were subjected to the ABCB1 shRNA complexation to obtain the dual drug-gene-loaded hybrid nanocarriers. The positively charged lipid of the docetaxel liposomes was able to make complexation with negatively charged shRNA plasmid when incubated for 45 min. Incubation was carried out at 37 °C with various molar ratios of cationic lipid in hybrid nanocarriers to achieve the highest complexation efficiency. Similarly, ABCB1 shRNA-complexed hybrid nanocarriers without Docetaxel were also prepared using the same lipid composition for in vitro tests. Folate targeted D-sh-PLHNCs (FA-D-sh-PLHNCs) have been formulated using folic acid conjugated DSPE-PEG_{2000} in the formulation.

8.1.2 Formulation optimization:

Table 2 Optimization parameters

Independent variable			Dependent variable
Selection of Material	**Process variables**	**Formulation variables**	

Polymer selection	Speed of stirring	Drug to Polymer ratio	Entrapment efficiency
Lipid selection	Flow of addition (Organic phase to aqueous phase)	Polymer to lipid ratio	Particle size
Solvent selection	Stirring time	Internal lipid ratio (N/P ratio)	Zeta potential
	Sonication time		
	Extrusion cycle		

9 Characterization

9.1 Determination of encapsulation efficiency of Docetaxel and complexation efficiency of shRNA

The exact amount of Docetaxel incorporated in PLHNCs was identified by the RP-HPLC method. Extraction of Docetaxel from PLHNCs was successfully performed before injection using given method. Each mL of PLHNCs suspension was diluted with tertiary butyl methyl ether to make the final volume of 5 mL. Above mixture was then vortexed for 30 sec to achieve homogenity. Subsequently, the vortexed mixture was subjected to centrifugation at 10,000 rpm for10 mins to separate organic layer from the aqueous one. The organic layer was then transferred to a separate vial and subjected to nitrogen drying. Residues of the drying were then reconstituted with 100 µL of the mobile phase (acetonitrile:water). From that, 20 µL of sample was injected into the RP-HPLC system. Estimation of docetaxel content in PLHNCs was performed by reverse-phase HPLC method/RP-HPLC (Agilent Technologies 1260 infinity II) using C18 ODS (octadecyl silane) column (250 mm *4.6 mm* 5 µm, Thermo scientific) at ambient temperature. The mobile phase acetonitrile: water (60:40) was allowed to run at a flow rate of 1 mL/min. Estimation of Docetaxel was examined using a UV-visible detector at a wavelength of 231 nm.

$$\%\ \text{encapsulation efficiency} = \frac{\text{Amount of drug entrapped}}{\text{Total amount of drug}}\ \text{X}\ 100$$

Complexation of ABCB1 shRNA plasmid with the PLHNCs was evaluated by centrifugation and UV analysis using NanoDrop 1000 (ThermoScientific, USA). ABCB1 shRNA plasmid and Docetaxel loaded PLHNCs were centrifuged at 18000 rpm for 2 h at 4°C (Remi centrifuge, Remi, USA) to settle down the PLHNCs. Then, the supernatant was analyzed on the NanoDrop 1000 and the content of pDNA was calculated using the standard expression OD1 = 50 µg/mL of double-stranded pDNA.

Readings from formulations were compared to those from standard dilution of naked ABCB1 shRNA plasmid. Complexation efficiency was calculated using the following equation;

$$\%\ Complexation\ efficiency = \frac{(Absorbance_{naked\ ABCB1\ shRNA\ standard} - Absorbance_{supernent}}{Absorbance_{naked\ ABCB1\ shRNA\ standard}}\ X\ 100$$

9.2 Particle size and zeta potential determination

The particle size of PLHNCs was determined under the principle of dynamic light scattering using Malvern Zetasizer Nano (Nano ZS, Malvern Instruments, UK). The light source was 633 nm He-Ne laser and the scattering angle was 175°. Analyses were carried out at 25 °C temperature after diluting 0.2 mL of formulation to 2 mL using filtered double distilled water. The total number of sub-runs for the size measurement were 15 and each run was for a duration of 10 seconds. The results were reported as Z-average. Zeta potential of the developed PLHNCs was determined using the same instrument as per Smoluchowski's equation from the electrophoretic mobility of the sample at 25 °C.

9.3 Transmission Electron Microscopy (TEM)

TEM analyses were carried at an acceleration voltage of 200 kV on a Technai, Pillips Holland, at the Sophisticated Instrumentation Center for Applied Research and Testing (SICART) Vallabh Vidyanagar instrument. By administering the PLHNCs onto a 300-mesh Formvar-coated copper grid (previously hydrophilized under UV light), the TEM sample was prepared (Electron Microscopy Sciences, Hatfield, PA). After 30 min of incubation, samples were blotted away and grids were negatively stained at room temperature for 10 min with 2 % (w/v) uranyl acetate aqueous solution freshly formulated and sterile-filtered. The grids were double washed with purified water and air dried until imaging.

9.4 In-vitro drug release study and drug release kinetic

To simulate the physiological environment of tumor cells, interstitium of tumors ,blood and normal cell, phosphate buffer with pH 5.5, 6.6, and 7.4 were investigated for in-vitro drug release study (25). A drug release study was performed using a dialysis bag with molecular mass cut-off of 3000 Da for 72 h. 2 mL of the formulation was

filled in a dialysis bag and dipped in receptor media comprising 20 mL phosphate buffer at 37 °C. At 1, 2, 4, 8, 12, 24, 48, 72 h, 1 mL of sample was withdrawn periodically and fresh media was added to maintain sink condition. These samples were analysed using HPLC and the % drug released was calculated and plotted against the time to obtain the release curve.

PLHNCs containing shRNA pDNA were suspended in TE buffer pH 7.4 (1 ml) and incubated at 37°C on a shaker at 100 rpm. At different time-points, the buffer was separated from the PLHNCs by centrifugation at 20,000 r.p.m. for 10 min and analysed for the amount and integrity of released pDNA (26). Then, the supernatant was analysed on the NanoDrop 1000 and the content of pDNA was calculated using the standard expression OD1 = 50 µg/mL of double-stranded pDNA.

9.5 In-vitro cytotoxicity

In-vitro cytotoxicity of the PLHNCs was estimated with the help of MTT assay (27). Drug resistant (DR-A549) cells were seeded into 96 well-plates with a calculated cell density of 5000 cells per well. After the growing phase of 24 h, cells were exposed to various ranges of formulation (Docetaxel equivalent to 0.025, 0.05, 0.1, 0.2, 0.4 and 1.0 µM) in complete media. The cells were incubated as per the period of 24 h, 48 h, and 72 h. After incubation, 100 µL of MTT solution (1 mg/mL) was added to each well and incubated for 4 h. Once the MTT incubation was conducted, the culture medium was removed and 200 µL of DMSO was added to each well. Live cells were able to reduce the MTT dye (yellow colored) to formazan crystals (purple colored) with mitochondrial enzymes. The absorbance of the plate was recorded at 570 nm with a reference band at 655 nm on the ELISA microplate reader, BioRad, USA. Micro well with 0.5 % Triton X100 was taken as positive control in 8 wells for each experiment, whereas for the negative control, wells of an untreated cell were considered. Cell viability was calculated and plotted on the basis of the concentration of Docetaxel on the X-axis against % viability on the Y-axis.

9.6 Cellular uptake studies

Cell uptake studies were performed using FITC labeled sh-RNA plasmid PLHNCs. The lyophilized stock of shRNA plasmid was reconstituted with nuclease-free water and it was used for the further investigations. In order to measure the

qualitative cellular uptake, confocal microscopy was performed; while to analyze the quantitative uptake, flowcytometric technique was employed.

9.6.1 Qualitative cellular uptake by confocal microscopy

DR-A549 cells were seeded into 6 well-plates with an initial cell density of 10,000 cells/well on the covered surface of a sterilized glass coverslip. After the span of 24 h, the cells were transfected with a different formulation and naked shRNA plasmid concentration of 100 nM. Subsequently, cells were incubated for 6 h and then, washed with cold phosphate buffer saline (PBS pH 7.4). Finally, washing was followed by live imaging of the cells, which was subjected to a confocal laser scanning microscope (LSM 710, Carl-Zeiss, USA) for the ultimate visualization (25).

9.6.2 Quantitative uptake by flow cytometry

DR-A549 cells were seeded with an initial density of 5000 cells/well and proliferated for 24 h. After proliferation, cells were treated with FITC labeled negative control shRNA plasmid at a concentration of 100 nM. Treated cells were incubated with coumarin-6 D-sh-PLHNCs and incubated for 6 h at 37 °C with a condition of 5 % CO_2 in an incubator. Then, the cells were harvested and further collected in FACS buffer containing 0.5 % bovine serum albumin (BSA) & 0.5 mL FBS. At last, cells were sorted using fluorescence intensity using a fluorescence-activated cell sorter. Naked FITC loaded NC-shRNA plasmid was considered as a negative control, while Lipofectamine (L2K)-shRNA plasmid complex was considered as a positive control. Raw data was analyzed and processed using FlowJo software (28).

9.7 Cell apoptosis detection

The detection of cell apoptosis was accomplished using a flow cytometer. DR-A549 cells were seeded at an initial cell density of 2000 cells per well in 6-well plates. Cells were incubated for 24 h. Cells were treated with Docetaxel solution, Docetaxel PLHNCs, Docetaxel-shRNA-PLHNCs and FA conjugated D-sh-PLHNCs and incubated at 37 °C for 24 h. Untreated cells were selected as control. Once the incubation period was over, cells were trypsinized using 0.0025% trypsin solution and harvested in 200 mL of binding buffer (0.2 µm sterile filtered 0.1M HEPES (pH 7.4), 1.4M NaCl, and 25 mM $CaCl_2$ solution) in the form of a cell suspension. Immediately, propidium iodide (8 mL) and annexin V-FITC (5 mL) were added to the cell suspension and gently vortexed for uniform mixing, and kept aside for 150 min for permeation of

dyes into the cells (29). The proportion of the apoptotic cells and stained cells were measured using BD FACS Aria III, BD Biosciences, USA.

9.8 Cell cycle analysis

DR-A549 cells were seeded at an initial cell density of 2000 cells per well in a 6-well plate. The cells were allowed to attach for 24 h and then, treated with Docetaxel solution, Docetaxel PLHNCs, Docetaxel-shRNA-PLHNCs and FA conjugated D-sh-PLHNCs. Cells were allowed to incubate at 37 °C for 24 h. Then cells were trypsinized using trypsin and centrifuged at 1500 rpm for 4 min in sterilized cell centrifuge tubes. Pellets of the cells were washed twice with PBS and cells were fixed using 75 % ethanol at 4 °C. After that, the cells were centrifuged again and washed with PBS twice and resuspended in PBS containing propidium iodide at a concentration of 5 mg/mL and ribonuclease A (deoxyribonuclease-free) at a concentration of 50 mg/mL (29). The resulting cell suspension was incubated in dark place for 25 min and patterns of the cell cycle were analyzed using BD FACS Aria III, BD Biosciences, USA.

10 In-vivo studies

In-vivo studies were conducted according to the institutional (IAEC) as well as national guidelines (CPCSEA) of the animal use and care. Surgical procedures and protocol performed in the experiment were approved by the Institutional Animal Ethical Committee (IAEC), Faculty of Pharmacy, The M.S. University of Baroda with protocol Id MSU/IAEC/2018-19/1827 and permission from Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India.

10.1 Acute toxicity study and maximum tolerated dose estimation

Acute toxicity studies were performed in two groups of SD rats; one with D-sh-PLHNCs (dose of 10 mg/kg Docetaxel & 40 ng shRNA plasmid equivalent DPI formulation) and another with Docetaxel solution (30). 6 rats of either sex were fasted overnight before dosing. Docetaxel solution and D-PLHNCs were administered to animal through intra-tracheal route at an initial dose of 50 mg/kg as per the weight of the animal, which was 5 times higher than the therapeutic dose of Docetaxel solution. If the animal survived, then the high dose was injected until 2 or more rats have been found dead in the same group. The dose at which an animal has been found dead was

considered as LD_{50} for respective formulation. The dose intensity was planned as 50, 60, 70 mg/kg and so on.

Sprague Dawley rats were divided into five groups for MTD estimation, each for D-PLHNCs, control saline solution, and Docetaxel solution. 3 Rats in each group were anesthetized and given drug solution and PLHNC dispersion at 10, 20, and 30 mg/kg via an intra-tracheal administration. After that rats were kept for 21 days under observation for estimation of MTD (Maximum tolerated dose). After the span of 21 days, animals were sacrificed and the left lung of the animal was stored in a 10 % solution of formalin and analyzed for histological analysis. Very thin sections (4 mm) were observed under inverted microscope for any sign of inflammation and congestion in muscle with help of eosin and hematoxylin staining. The separated right lung was weighted before drying and subjected for drying at 60°C for 10 mins. The former weight was considered as wet weight (WW) and after drying, weight was considered as dry weight (DW). The edema index was calculated by considering the ratio of the wet weight by dry weight using the following equation (31).

$$Oedema\ index = \frac{Weight\ of\ wet\ lung\ (WW)}{Weight\ of\ dry\ lung\ (DW)}$$

10.2 Pulmonary pharmacokinetics

Animals were divided into two experimental groups (3 rats per group) and intra-tracheal doses were given (1) Docetaxel solution (2) D-PLHNCs (dose of 10 mg/kg Docetaxel). Bronchoalveolar lavage was performed on an anesthetized as well as re-cannulated rat using 12 mL of PBS, which was prewarmed at 37 °C. After that, PBS with pH 7.4 was slowly injected into the trachea with one syringe and BAL (Broncho alveolar lavage) was ejaculated with another syringe. The yielded BAL fluid was centrifuged at 4000 rpm for 5 min. Supernatant was mixed with a 10 % solution of Triton X to burst the PLHNCs for Docetaxel release. Then, Docetaxel was extracted and analyzed by HPLC. After euthenization lungs were excised along with the tracheal portion below the site of instillation and homogenized in 10 mL PBS having 1% Triton-X-100 to analyze diffused drug. Total concentration of drug in the lung was calculated and pulmonary and IV pharmacokinetic (By single IV bolus of lyophilized D-sh-PLHNCs suspension in rat tail vain) of parameters were calculated (32).

The drug concentration in the lung is the drug estimated in lung homogenate (LH), BAL fluid and pulmonary and IV pharmacokinetic parameters as follows:

- **Tmax:** The time point at which maximum drug concentration is attained in lung (i.e. the time interval of Cmax).
- **AUC_{total}**: The area under the curve of drug concentration in lung vs. time, over the period of study (12hrs).
- **$t_{1/2}$** : Pulmonary half-life of drug is calculated by:
    a. Calculating the sum of the values of drug concentration in BAL and LH at individual sampling points.
    b. Regressing the calculated sum over the entire duration of study.
    c. Deriving the time point at which the sum of drug level is 50%compared to instilled quantity (i.e. deriving the median of the regression line).
- **C_{max}**: is the highest concentration of a drug in the blood and target organ after a dose is given.

MRT (Mean residence time): is the average time that molecules of a dosed drug spends in the body.

11 Stability Studies

Stability of Docetaxel loaded PLHNCs in terms of drug content and particle size distribution was monitored for 3 months at 2-8 ^{0}C and RT (25-30 ^{0}C). Periodically, samples were withdrawn and the particle size as well as drug content was determined.

12 Statistical Analysis

Statistical analysis of data was performed by ANOVA using Graphpad Prism software (version 6.0). p value < 0.05 was considered significant.

13 Results and discussion

13.1 Formulation and optimization

From the Placket-Burman and Box-Behnken design, most desirable batch were selected for the further optimization of lyophilization and dry powder formulation on the applied constraint. Confirmation of the responses was done by carrying out the experiment using the selected factor values in triplicate.

13.2 Determination of encapsulation efficiency of Docetaxel and complexation efficiency of shRNA

Docetaxel entrapment also play a critical role in targeted drug delivery. The PDE (Percentage drug entrapment) of the optimized PLHNCs was found to be 91.5 ± 1.4 %. The reason behind such high entrapment efficiency can be attributed to the lipophilic nature of drug. Hybrid nanocarriers have also been reported to give high entrapment efficiency for water insoluble drugs. While the optimized batch was reported to possess 96.2 ± 1.4 % of shRNA pDNA complexation.

13.3 Particle size and zeta potential determination

Size of the PLHNCs is important for establishing drug delivery strategies to specific sites of the body. Smaller PLHNCs (~100nm) may be prone to minimize the particle uptake by nontargeted cells, including their premature clearance by the MPS (mononuclear phagocytic system). The resulting PLHNCs were sized at 126.3±6.1 nm (mean ± SD; n=3). Additionally, lower polydispersity index of 0.108 ± 0.11 indicated monodisperse formulation.

Zeta potential is an important factor to determine the stability of the PLHNCs in dispersion and also plays an important role in the interaction between the cell membrane and the PLHNCs. PLHNCs exhibited zeta potential of 22.4 ± 1.4 mV due to presence of cationic lipids on the surface.

13.4 Transmission Electron Microscopy (TEM)

Negative staining with uranyl acetate in TEM was conducted for structural characterization of PLHNCs, which stains the lipid layer that was observed as a dim ring circling the polymeric centre (as shown in Figure 1). The ring diameter is less than 20 nm. It confirms the morphology and architecture of PLHNCs, i.e., the formation inside the lipid bilayer of several co-polymer amorphous unimers area in a frozen phase segregated in stable matrix centre. With embedded matrix forming, the polymeric centre showed separate PEG-PCL particles inside the PLHNC cavity as well as reinforcing the PLHNC architecture relative to liposomes alone.

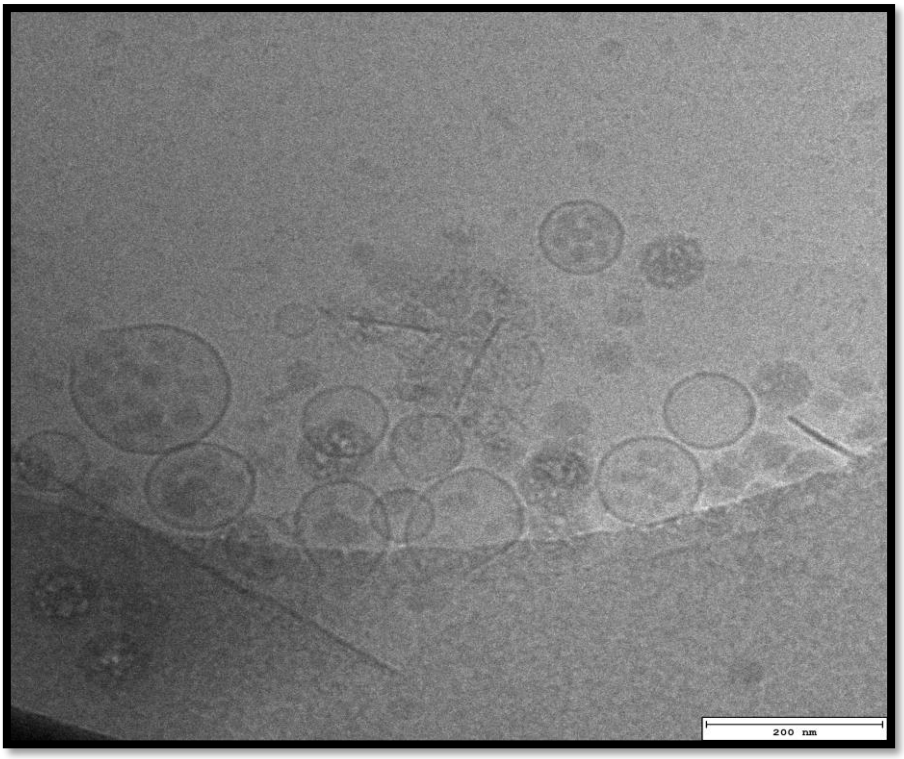

Figure 1 TEM images of optimized batch of PLHNCs

13.5 Release of Docetaxel and shRNA pDNA from PLHNCs

Docetaxel loaded PLHNCs followed the sustained release kinetics. From the three-release (i.e., pH 5.5, pH 6.6, pH 7.4) condition, the highest sustained-release curves were obtained in receptor media pH 5.5, which suggested the maximum sustained release of the drug in the cancer cells (33). Release of the docetaxel from the PLHNCs in the different media has occurred in the decreasing order, pH 5.5 > pH 6.6 > pH 7.4, which supports least docetaxel release in the plasma and blood. The initial pattern of the burst release has been found at pH 5.5 and pH 6.6, which can be due to sudden diffusion of the docetaxel present either on the surface or just beneath the lipid layer in the PLHNCs. The later sustained release pattern was achieved owing to the presence of the drug in the core of the PEG-PCL diblock copolymer.

Release of shRNA pDNA from PLHNCs also followed sustained release profile. Cationic lipid in the formulation is responsible to hold the negatively charged shRNA pDNA molecule for longer period.

13.6 In-vitro cytotoxicity (MTT assay)

Cytotoxicity of the different formulations of PLHNCs were demonstrated with IC_{50} at 24, 48, and 72 h. It was found that all the cells had received the same amount of

Docetaxel. After 24 h, there was a non-significant improvement in IC_{50} of D-PLHNCs and sh-D-PLHNCs due to sustained release of Docetaxel from the PLHNCs. The fold change in the IC_{50} of the Docetaxel solution and D-PLHNCs were found to be 2.00, 1.96 and 1.84 at the period of 24, 48, and 72 h, respectively. The most significant effect on cell cytotoxicity was observed in sh-D-PLHNCs (Redispersed formulation) and Docetaxel solution, where fold change in IC_{50} was 2.68, 2.84, and 2.64 at 24, 48, and 72 h, respectively. The increased cytotoxicity of sh-D-PLHNCs showed the efficiency of ABCB1 shRNA in the inhibition of the P-gp efflux pump, which accounts for the increased Docetaxel concentration in the cells. Thus, the combinatorial approach for simultaneous delivery of the ABCB1 shRNA plasmid along with Docetaxel led to a higher cellular accumulation of Docetaxel. It can be anticipated that the higher cell-cidal effect of the combinatorial approach (sh-D-PLHNCs) possibly mimicked with in-vivo models due to selective extravasation of PLHNCs in tumor vasculature driven by EPR effect (enhanced permeability and retention effect) and passive uptake by tumor cells with a narrow distribution in normal tissues.

13.7 Cell uptake study

The results of cell uptake study confirmed that there was comparatively higher cellular uptake of folate conjugated sh-PLHNCs as compared to non-conjugated sh-PLHNCs and free drug. Higher cellular uptake of folate conjugated sh-PLHNCs was proportional to amount of folate receptor on cell surface and receptor mediated endocytosis pathway. Besides the above mechanism, other reasons may also exist which can explain higher uptake values of folate conjugated sh-PLHNCs. More amounts of the internalized unconjugated nanocarriers underwent exocytosis during the first 2 h as compared to folate-conjugated nanocarriers (34). Hence, greater intracellular uptake of folate conjugated sh-PLHNCs may also be due to lower exocytosis and greater intracellular delivery of drug. Enhanced intracellular uptake of folate conjugated sh-PLHNCs was further confirmed by confocal microscopy where enhanced fluorescence of the folate conjugated sh-PLHNCs showed that they were internalized more in comparison to unconjugated sh-PLHNCs.

13.8 Apoptosis study

To determine apoptosis DR-A549 cell line, apoptosis studies was conducted using FITC-Annexin V/ PI staining procedure. (35). shRNA and Docetaxel loaded

PLHNCs and folate conjugated D-sh-PLHNCs (FA-D-sh-PLHNCs) were able to cause more apoptosis and necrosis in DR-A549 cell lines as compared to D-PLHNCs and Docetaxel solution following reversal of multi drug resistance and site-specific targeting. In our study, Docetaxel diffuses and accumulates directly at site of action at a higher concentration thus resulting in necrosis. However, all three PLHNCs show a slow sustained release phenomenon and at a lower dose apoptosis signals may be activated leading to a higher number of cell deaths. On contrary FA-D-sh-PLHNC showed mixed results, with cells being present both in apoptosis phase as well as in necrosis stage. Targeted delivery and better uptake of FA-D-sh-PLHNCs results in greater accumulation of drug loaded nanoparticles inside tumor tissue. Rapid release of surface adsorbed drug (burst release) from all three PLHNCs formulation was responsible for causing necrosis initially in the A549 cells. However, in later stages slow and sustained release of drug molecules is responsible for eliciting apoptotic signals causing death by apoptosis.

13.9 Cell cycle analysis

Cell cycle analysis demonstrated that the cells treated with docetaxel had a higher proportion of cells in the G1 phase. It was observed that a higher proportion of cells were in the G1 arrest phase following treatment by FA-D-sh-PLHNCs than the cells treated with the native docetaxel drug. There are previous reports confirming higher arrest of higher number of cells in G0/G1 phase when treated with D-PLHNCs as compared to free drug treated cells. However, FA-D-sh-PLHNCs inhibiting more number of cells in G1 phase can be explained on the basis of the intracellular drug levels. It can be said that in case of FA-D-sh-PLHNCs more drug is available at the site of action (following sustained drug release) for a longer period of time than native drug in solution, resulting in greater efficiency of the FA-D-sh-PLHNCs in arresting cell growth.

13.10 Stability studies

The stability study of D-PLHNCs was carried out at 2-8°C / ambient humidity and 30±2 °C/60±5 % humidity for the period of 3 months. There was no significant increase in particle size at 2-8°C / ambient humidity. At 30 ± 2°C/60 ± 5 % humidity condition, particle size was increased. PLHNCs retained more than 95% of drug at both conditions after storage of 3 months.

13.11 Estimation of the maximum tolerated dose, LD_{50}, and Oedema index

The highest dose tolerated by the animal without any unfavourable adverse effect is considered as MTD (36). To estimate MTD, histopathological images and alteration of the oedema index, as well as the severity of damage to pulmonary tissues, were considered. It can be concluded that the oedema index increases proportionally with the concentration of drug solution. For in-vivo studies non-targeted formulation have been used as folate receptors are overexpressing only on tumor cells which are absent in normal rats. At the same concentration, oedema index of the D-sh-PLHNCs was found to be comparatively lower than the same dose of Docetaxel solution. When Docetaxel solution was introduced at 10 mg/kg, there was a mild sign of cell infiltration but bronchial epithelial cells were found undisturbed with no sign of haemorrhage. When the concentration increased to 20 mg/kg, some of the rigidness was seen in bronchial cells and alveolar destruction was noticed. At the dose of 30 mg/kg, complete destruction of the bronchial cell was observed along with oedema development and haemorrhage. A similar study was performed with D-sh-PLHNCs, which showed that the integrity of the broncho-epithelial cells were well maintained with negligible evidence of any cell infiltration and oedema. Hence, it has been confirmed that Docetaxel solution was found safe up to 10mg/kg. At the same time, D-sh-PLHNCs could show the safety till the dose of 30 mg/kg, which revealed a three-fold increase in the safety profile of Docetaxel when administered with the developed PLHNCs formulation.

Estimation of the LD_{50} was accomplished by observing the signs of mortality as well as morbidity on Sprague-Dawley rats after intratracheal administration of the escalated dose of 50, 60, 70, and so on mg/kg. From the results, it was concluded that the Docetaxel solution showed mortality at 100 mg/kg dose with intra-tracheal administration, while at the same concentration, D-sh-PLHNCs did not show any mortality, which suggests the PLHNCs as a safe delivery vector for controlled release of Docetaxel in the lung tissue. The most appropriate reason for mortality of Docetaxel solution is instantaneous exposure of large dose of drug directly to lung whereas in D-sh-PLHNCs, drug is released over prolonged period, which prevents the sudden rise of the Docetaxel in the pulmonary region.

13.12 Pulmonary pharmacokinetics

The t½ of 6.05 h was recorded for D-sh-PLHNCs compared to 1.98 h for Docetaxel solution. A significant improvement was observed in the AUC of the D-sh-PLHNCs compared to the Docetaxel solution in pulmonary pharmacokinetics. Two-three times, higher AUC was achieved with PLHNCs compared to Docetaxel solution in pulmonary pharmacokinetics. The residence time of the D-sh-PLHNCs was also higher compared to the Docetaxel solution. Hence, from the above trend, it can be inferred that Docetaxel was quickly absorbed by the lungs and entered the systemic circulation and further metabolised when administered as a solution form, whereas PLHNCs formulations were better in terms of providing higher residence time of Docetaxel in lungs and improved its anti-cancer activity with a minimal adverse effect on local lung tissues and slows its metabolism.

14 Conclusion

Present investigation shows a promising way to treat lung cancer using targeted delivery of docetaxel encapsulated PLHNCs along with shRNA for reversal of drug resistance. The proposed formulation showed higher targeting and proved by the cell uptake studies. The sustained release effect of D-sh-PLHNCs prevents exposure of high dose initially as compared to native drug solution. Lower IC_{50} value of Docetaxel and shRNA loaded PLHNCs formulation confirms reduced drug efflux from DR-A549 cells and proved improved cytotoxicity. Hence, developed formulation can serve as better treatment for multi-drug resistance lung cancer.

15 Reference

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