

Action of Paclitaxel pH-Sensitive Liposomes on B16F1 Melanoma Cells.

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ABSTRACT

Long circulating pH-sensitive liposomes loaded with Paclitaxel were prepared by thin film hydration method using DOPE and characterized for mean particle size, zeta potential and percentage encapsulation efficiency which were found to be 190nm, -23.0mV and 87% respectively. The transmission electron microscopy (TEM) studies showed that prepared liposomes were of spherical in shape. The *in-vitro* release studies showed the complete destabilization of pH sensitive liposomes in acidic pH 5.0 where as retarded release at physiological pH 7.4. The action of paclitaxel loaded pH-sensitive liposomes on B16F1 melanoma cells (low metastatic) showed better cytotoxicity, increased cytosolic delivery, inhibition of colony formation and reduced cell motility or cell migration.

Keywords: liposomes, pH-sensitive, melanoma, *in-vitro* release, paclitaxel.

1. INTRODUCTION

Paclitaxel is one of the most effective anticancer drugs in the market having significant antitumor activity against a wide variety of tumors, including ovarian carcinoma, breast cancer, head and neck cancers and non-small cell lung cancer. The biggest shortcomings of this drug are its low aqueous solubility and the side effects caused by the vehicles used to formulate it. Neoplastic agents can be targeted through liposomal drug delivery system. However, one of the major drawbacks of the liposomal formulation was its rapid clearance from blood and uptake of the liposomes by the mononuclear phagocytic system (MPS). pH-Sensitive liposomal system attained substantial interest due to their benefits such as enhanced stability and circulation time in blood stream, targeting to specific tissues or cells and facilitation of intracellular delivery. Herein, we report on the development of a PEGylated pH sensitive liposomes of paclitaxel and its evaluation of activity on B16F1 melanoma cells.

2. MATERIALS AND METHODS

2.1 Materials

Di-oleoylphosphatidylethanolamine (DOPE) is the generous gift from Lipoid (Lipoid GmbH, Ludwigshafen, Germany) 1, 2 Dimyristoyl-sn-glycero-3-phosphoglycerol, sodium salt

(DMPG) was the gift from Genzyme Corporation, USA. Hydrogenated soy phosphatidylcholine (HSPC) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-*n*-[methoxy (polyethylene glycol)-2000] (mPEG₂₀₀₀-DSPE) and Cholesterol (CHOL) were purchased from Avanti polar lipids. Cholesteryl hemisuccinate (CHEMS), 3-(4,5-dimethyl thiazole-2yl)-2,5-diphenyl tetrazolium bromide dye (MTT) was purchased from Sigma Chemical (MO, USA). 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES) was purchased from Himedia Labs, Mumbai. Paclitaxel (PCL) was generously gifted by Xechem Pharmaceuticals NJ, USA. All lipids were used without further purification. All other reagents were of analytical grade.

2.2 Cell and culture conditions

B16F1, a low metastatic lung selected subline derived from C57/BL6 murine melanoma was purchased from the National Center for Cell Science (Pune, India) and stock cells were maintained as a continuous culture in Iscove's Modified Dulbecco's Medium (IMDM, Sigma chemicals, USA) supplemented with 10 % inactivated foetal bovine serum (FBS, GIBCO, BRL, MD, USA), penicillin (100 IU/ml) Streptomycin (100 µg/ml) in a humidified atmosphere of 5 % CO₂ at 37 °C until confluent.

2.3 Preparation of liposomes

Paclitaxel loaded pH-sensitive liposomes (PSPT) were prepared by the film hydration method [1,2]. The liposomes were composed of DOPE: HSPC: CHEMS: CHOL at various molar ratios with mPEG₂₀₀₀-DSPE at 5 mol % to phospholipids. The mPEG₂₀₀₀-DSPE is used for preparing sterically stabilized liposomes. Small unilamellar vesicles were prepared as previously described [1]. Briefly, lipids were mixed and dissolved in organic solvents like chloroform-methanol mixture along with drug and the solvent was evaporated using a rotary evaporator; residual solvent was removed under high vacuum. The dried lipid films were hydrated with HEPES buffer pH 8.2 and sequentially extruded through a series of polycarbonate membrane filters (Nucleopore, CA, USA) with pore sizes of 400–100 nm, using an extruder. Paclitaxel containing conventional liposomes (CLPT) were also prepared using the same technique with DMPG, HSPC and Cholesterol in the molar ratio of 6:2:2 and hydrating the dry thin lipid film using 2 ml of PBS, pH 7.4.

2.4 Physicochemical characterization of liposomes

2.4.1 Morphology of liposomes

The morphology of the conventional and the PEGylated liposomes was observed by transmission electron microscopy (TEM) using negative staining technique using 10 µl of 2 % uranyl acetate solution and then observed by TEM (FEI/Philips, Morgagni 268, Netherlands).

2.4.2 Entrapment efficiency

The amount of Paclitaxel encapsulated in liposomes was measured following the method in the literature with slight modification [3,4]. Briefly, aliquots of diluted liposomal dispersion were centrifuged at 1000 rpm for 10 min to remove untrapped paclitaxel from the liposomal suspension. Then, the liposome supernatant was ultracentrifuged for 30 min to separate the liposomal pellet and the pellet was dissolved in organic solvent mixture of methanol and chloroform (9:1, v/v) and the concentration of paclitaxel was determined spectrophotometrically at 227 nm after appropriate dilution. An aliquot of the liposome suspension was also dissolved with the same mixed solvent to determine the total amount of paclitaxel in the liposome suspension, after which the EE was calculated from the following equation:

$$EE(\%) = \frac{\text{amount of paclitaxel in liposome pellet } (\mu\text{g})}{\text{amount of paclitaxel in liposomal dispersion } (\mu\text{g})} \times 100$$

2.4.3 Particle size distribution and zeta-potential

This was performed using Nano-ZS particle size analyzer (Malvern Instruments, UK) which uses New Dynamic Light Scattering Technique to analyze the sample. For determination of mean particle size, size distribution and zeta-potential the samples were filled in respective cuvettes after proper dilution and the readings were taken at 25° C. Uniformity value was also noted down for the sample.

2.4.4 *In-vitro* release of paclitaxel from liposomes

In-vitro release of plain paclitaxel, CLPT and PSPT were studied by dialysis method in phosphate buffered saline, pH 7.4 and pH 5.0 as dissolution media at 37°C using dialysis tube (MWCO 12,000, Sigma Chemicals, USA). The aliquot samples were taken at predetermined time intervals and the samples were analysed for drug content.

2.5 *In-vitro* cell line studies

2.5.1 Cytotoxicity assay

The *in-vitro* cytotoxic activity was assayed on B16F1 cell lines by MTT method [4, 5, 6]. Cells were grown in 96-well tissue culture plates at a concentration of

2×10^4 cells/100 µl/well and treated with serial concentrations of PCL loaded liposomal formulations and plain drug for 48 hours. To evaluate possible effect of formulation components on cell viability, cells were also incubated with blank liposomes (placebos) and vehicle used to prepare drug solution at the highest concentrations used for the study. Post treatment cells were washed with PBS and treated with 20 µl of MTT reagent to each well and incubated for 4 hour at 37° C. Medium was aspirated from the wells and 100 µl of DMSO was added to each to dissolve formazan crystals. The optical density was measured in an ELISA plate reader (Molecular Devices, Spectra Max 190) at 540 nm with a reference wavelength of 690 nm. Cell viability was plotted as a percentage of untreated control from which the IC₅₀ values were noted down. (Table 1)

2.5.2 Leighton tube assay

Subconfluent culture of B16F1 melanoma grown on cover slips was treated with paclitaxel and their liposomal formulations for 48 hours separately. Cover slips were washed in PBS and cells were fixed with methanol, stained with haematoxylin and eosin (H and E) and mounted using DPX mountant and the images were captured under phase contrast microscope.

2.5.3 Confocal microscopy

B16F1 cells were grown to 60 % confluency in petri plates containing square cover slips. Cells were incubated with pH sensitive liposome encapsulated with FITC. After incubation period, cells were washed with PBS and fixed with 4 % paraformaldehyde solution. Treated cell monolayer on cover slip was mounted on slides using DABCO-Glycerol and observed by confocal laser scanning microscope (Zeiss LSM 410) with imaging software (Fluoview FV500) (Figure 2).

2.5.4 Cell proliferation

Colony formation assay was carried out as described below [8]. Briefly, 35 mm petri plates were seeded with 600 viable cells/ml in complete medium and allowed to grow for 24 hours. The cells were then incubated in the presence of PCL and its liposomal formulations for 48 hours. The drug was removed; cells were washed and incubated for 48 hours in complete medium. The colonies obtained were washed with PBS and fixed with methanol for 10 min at room temperature followed by staining with 0.5% crystal violet solution. Colonies with 50 or more number of cells were counted on a Zeiss inverted light microscope.

2.5.5 Cell Motility

Subconfluent culture of B16F1 melanoma was treated with different concentrations of PCL and its liposomal formulations for 48 hours. The cells were rinsed with PBS. Using a sterile 200 µl plastic pipette tip, three separate uniform, cell-free wounds were made through the cells of the petri culture dish. Cells were maintained on low serum

media (1.5 ml; 0.5% serum in IMDM) for another 48 hours. Cells were fixed with methanol and stained with 0.5 % crystal violet solution. Photographs of each wound were taken (Figure 3) and the distance between the opposing edges was measured at 25 points on each wound. The distance migrated in micrometers was calculated as the difference of the scratch width at the beginning of the assay (0 hour) and that at the indicated time point.

2.5.6 Flow cytometry analysis

Subconfluent B16F1 cells were treated with various concentrations of PCL and its liposomal formulations for 48 hours. Control received only complete medium. Cells were harvested, washed twice with PBS and fixed in chilled 70% ethanol. After centrifugation, the fixed cell pellet was treated with RNase-A at a concentration of 0.5 mg/ml (Sigma) and finally stained with propidium iodide (1 mg/ml) (Sigma Aldrich) for 10 min at room temperature and later put on ice. Ten thousand events were acquired on Becton-Dickinson FACS SCAN and analyzed using Modfit (DNA Modeling System) software [8,9].

3. RESULTS AND DISCUSSION

3.1 Formulation development and characterization of paclitaxel liposomes

Among the different drug delivery systems, the liposomal formulation is considered to be a relatively non-toxic technology with considerable potential for encapsulating both hydrophilic and lipophilic drugs. The paclitaxel pH sensitive liposomes were prepared by thin film hydration method using combination of lipids with increased encapsulation efficiency. The untrapped paclitaxel was separated from the liposomal suspension by ultracentrifugation.

3.1.1 Morphology

The image from negative staining TEM (Figure 1) showed that PEGylated pH sensitive liposomes were of discrete and round structure ranging size from 100 to 200 nm which were consistent with the results obtained from particle size measurement.

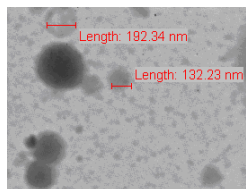


Figure 1. TEM micrographs of paclitaxel pH sensitive liposomes.

3.1.2 Paclitaxel content and entrapment efficiency

The combination of different lipids used in the formulation increased the paclitaxel entrapment efficiency. The maximum drug entrapment was found to be approximately 87 % for both conventional and pH sensitive liposomes.

3.1.3 Zeta potential and particle size

The zeta potential of PEGylated pH sensitive liposomes was more negative (-23.0 ± 2 mV) than that of conventional liposome (-10.3 mV) due to the negatively charged mPEG₂₀₀₀-DSPE. The particle size distribution of the liposomes prepared showed a mono-modal distribution with the mean particle size of 190 nm.

3.1.4 In-vitro release studies

In-vitro release of Paclitaxel loaded pH-sensitive liposomes and conventional Paclitaxel liposomes were carried up to 72 hours in phosphate buffered saline, pH 7.4 and for 1 hour in pH 5.0 as dissolution media. The *in-vitro* release studies revealed the long circulatory property of Paclitaxel loaded pH-sensitive liposomes in pH 7.4 with 29.35 ± 0.35 % release after 72 h whereas, complete destabilization at cytosolic acidic pH of 5.0 within 1h compared to conventional liposomes.

3.2 In-vitro cell line studies

3.2.1 Cytotoxicity assay

In-vitro cytotoxicity assay revealed that the PCL activity did not reduce in the presence of a lipid carrier as reported earlier [10,11]. As anticipated, the blank liposomal formulations did not produce any significant toxicity which proves that the cytotoxicity caused by the liposomal PCL is due to the released drug and not due to the any component of the liposomal bilayer. The cytotoxic effect produced by PCL and its liposomal formulations after 48 h of incubation at 37 °C and 5 % CO₂ is shown in Table 1. As the concentration of the drug (in free form as well as in liposomal formulation) increases there was a gradual reduction in the % cell viability, which explains the concentration dependent cytotoxic effect of PCL and its formulations.

Formulations	IC ₅₀ values (µg/ml)
	B16 F1
PCL	0.50
CLPT	6.00
PSPT	2.20

Table 1. IC₅₀ values of PCL and its liposomal formulations in B16 F1 cell lines by MTT assay after 48 h of incubation.

3.2.2 Leighton tube studies

Haematoxyline and Eosin stained cover slips containing B16F1 cells showed significant changes in the morphology of cells. The untreated samples have the cell membrane and the cellular contents intact whereas, the cells treated with the sub-toxic concentration of PCL solution, CLPT and PSPT showed destruction of the cell wall, nucleus and cell protrusions which resulted in the formation of a large number of dendrites. The marked change in morphology of the cells treated with PSPT showed that activity of the drug was retained even at sub-toxic concentrations.

3.2.3 Confocal microscopy

In-vitro cellular uptake and intracellular distribution of pH sensitive liposome encapsulating FITC-Dextran were evaluated qualitatively by confocal microscopy which revealed the cytosolic delivery of the pH sensitive liposomes (Figure 2). The rapid internalization and increased accumulation of fluorescent material inside the cell was observed which could be related to their narrow size distribution and bioacceptable lipid composition [12].

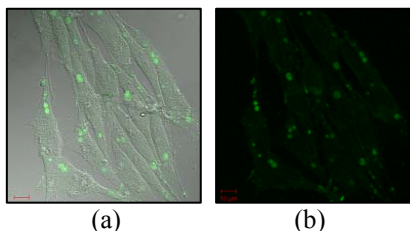


Figure 2. Phase contrast (a) and fluorescent (b) micrographs of paclitaxel pH sensitive liposomes

3.2.4 Cell proliferation (Colony formation)

The colony inhibition effect of PCL solution, CLPT as well as PSPT and placebos for liposomal formulations at the end of 48 hours was observed for B16F1 melanoma cells [8]. The colonies were counted depending upon the number of cells clustered to form it i.e. >50 cells or < 50 cells / colony. The PSPT has shown better inhibition effect compared to PCL and CLPT in the B16F1 cell line after 48 h of treatment in which the colonies were less in number and small colonies are observed compared to other treatments. The percentage colony inhibition for the treatments were in the order of PSPT > PCL > CLPT > Placebos for liposomes.

3.2.5 Wound assay

Wound assay is an easy method for the analysis of migration of cells [7]. Percent migration and relative wound width for wounds treated with PCL, CLPT, and PSPT liposomal preparations after 48 h of treatment was calculated. PCL, CLPT and PSPT provide a dose dependent anti-migration activity i.e. higher concentrations had larger wound width. The percentage migration was found to be in the order - Placebos for liposomes > CLPT > PCL > PSPT where as relative wound width was in the order PSPT > PCL > CLPT > Placebos for liposomes when compared to the 0 h wound reading.

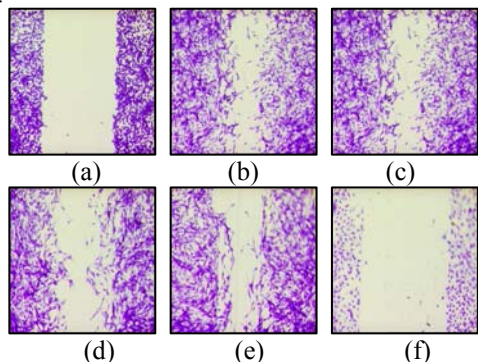


Figure 3. Wound assay micrographs for (a) 0 h (b) untreated control (c) placebo liposome (d) PCL (e) CLPT (f) PSPT.

3.2.6 Cell Cycle analysis

The PSPT arrested B16F1 melanoma cells in the G₂-M phase of cell cycle similarly as that of plain PCL. Analysis of DNA content of Propidium Iodide (PI) stained B16F1 melanoma cells showed significant dose-dependent inhibition in the G₂-M phase transition on Paclitaxel and its liposomal formulations treatment, which resulted in a clear increase in the percentage of cells in G₂-M phase as compared to the control cells and placebos which are in G₀-G₁ phase.

4. CONCLUSION

Paclitaxel loaded pH-sensitive liposomes were successfully prepared and these pH sensitive liposomes exhibited destabilization at cytosolic acidic conditions, with the long circulating potentiality and targetability of the formulation. *In-vitro* B16F1 melanoma studies showed the potential benefits of pH sensitive formulations over the conventional ones. The pH sensitive liposomes encapsulating paclitaxel showed better cytotoxicity, inhibition of colony formation and reduced cell motility or migration compared to plain drugs and conventional liposomes. Based on these results, it can be concluded that the PCL loaded pH sensitive liposomes can be considered as promising system for *in-vivo* anti-cancer delivery.

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