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Research Article

In Vitro Cytotoxic Potential of Paclitaxel-Encapsulated Lipid-Based Drug Delivery Systems

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Abstract

Purpose: This study was carried out to investigate the *in vitro* cytotoxic effects of Conventional Liposomes (PCL-CL) and Stealth Liposomes (PCL-SL) of Paclitaxel (PCL) prepared using thin film hydration method and PCL Lipid Nanoparticles (PCL-GMS SLN and PCL-GPS SLN) prepared using high-pressure homogenization.

Methods: The encapsulated formulations of PCL were compared for their anti-proliferative activity using chemosensitivity assay and colony formation assay, anti-migration activity using wound assay, and cytopathic effects using Leighton tube assay. Based on the chemosensitivity assay, sub-toxic concentrations of PCL-encapsulated formulations (PCL-CL: 0.25 µg/ml and 0.5 µg/ml; PCL-SL: 2.5 µg/ml and 5 µg/ml; PCL-GMS SLN: 0.25 µg/ml and 0.5 µg/ml and PCL-GPS SLN: 2.5 µg/ml and 5 µg/ml) and PCL solution (0.025 µg/ml and 0.05 µg/ml) were selected for the wound assay, colony formation assay and Leighton tube assay.

Results: PCL-GPS SLN and PCL-SL demonstrated better anti-migration activity and colony inhibition in comparison to other encapsulated PCL formulations. These formulations were also found to render maximum cellular damage.

Conclusion: Encapsulation as liposomes or nanoparticles prolonged PCL activity. PCL-GPS SLNs (0.05 µg/ml) and PCL-SL (5 µg/ml) were found to have better cytotoxicity compared to PCL-GSM SLN and PCL-CL.

Keywords: Paclitaxel; B16F10 cell lines; Lipid nanoparticles; Liposomes

Abbreviations

PCL: Paclitaxel; CL: Conventional Liposomes; SL: Stealth Liposomes; SLN: Solid Lipid Nanoparticles; GMS: Glyceryl Monostearate; GPS: Glyceryl Palmitostearate; IMDM: Iscove's Minimum Dulbecco's Medium; PCI: Percent Colony Inhibition

Introduction

Paclitaxel is a potent cytotoxic agent effective against a wide range of refractory and metastatic malignancies [1]. The development of an intravenous dosage form is a challenge due to the poor aqueous solubility of PCL (< 0.01 mg/mL) [2]. As an alternate, particulate systems such as nanoparticles and liposomes have been suggested as effective systems to deliver PCL. PCL-loaded in PLGA nanoparticles has been effective against NCI-H69 cell line [3]. Similarly, PCL-encapsulated in liposomal formulations have demonstrated good cytotoxic effect against various cell lines including B16F10 melanoma cell lines and also against human cell lines [4-6].

B16F10 melanoma cell line is a highly metastatic cell line and has been used to evaluate many delivery systems. The cell line experiments are preferred as an initial study to establish preliminary information to predict the *in vivo* performance of the drug-loaded particulate systems. These experiments provide advantages of better reproducibility and allow screening of a higher number of samples compared to *in vivo* models [7]. Apart from the cytotoxicity aspects, these cell lines help us to assess the various other mechanisms of cytotoxicity of the drug as well as the delivery systems. The *in vitro* cell lines form an essential prerequisite to evaluate drug delivery systems intended for cancer therapy before moving towards *in vivo* studies.

In the present study, *in vitro* anti-metastatic activity of PCL-loaded SLNs and liposomal formulations were assessed using metastatic B16F10 melanoma cell lines. The formulations were subjected to MTT assay, wound assay, colony formation assay and Leighton tube assay.

Materials and Methods

Cell and culture conditions

B16F10 murine melanoma cell line was purchased from National center for cell science, Pune, India. The cell line was maintained as a continuous culture in IMDM (Iscove's Minimum Dulbecco's Medium, GIBCO BRL, MD, USA) supplemented with 10% fetal bovine serum (FBS, Himedia, Mumbai, India), 100 U/ml Penicillin and 100 µg/ml streptomycin. Cells were grown in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. Media was replenished every third day.

Preparation of liposomes

Liposomes were prepared by conventional thin film hydration technique as reported previously [8]. Briefly, the lipid phase comprising of HSPC, cholesterol, and PCL were dissolved in 10mL

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organic solvent mixture (Methanol:Chloroform - 2:1) and evaporated at 620 mm of Hg pressure at 120 rpm in a rotary vacuum evaporator (Superfit, Rotovap, Mumbai). The process was continued until a dry lipid film deposited on the wall of the flask. Residual traces of solvent were removed by applying it to vacuum dryer for 1 h. The film was hydrated using distilled water at 100 rpm and between 55-58°C. The dispersion was allowed to stand for further 2 h at room temperature for annealing.

The size reduction of liposomes was carried out in a high-pressure homogenizer (Avestin E5, Canada). The PEGylated liposomes were prepared optimizing DSPE-PEG 2000 ratio to the optimized conventional liposome formulation using electrolyte flocculation test.

Preparation of solid lipid nanoparticles

The SLNs were prepared by modified Hot homogenization method as described previously [9,10]. Briefly, GMS/GPS and PCL were dissolved in ethanol and added to a hot P407 solution (5° C above the melting point of the lipid) under stirring using an Ultra turrax T 18 (IKA, Germany). The resultant dispersion was then passed through a high-pressure homogenizer (Emulsiflex E5, Canada) at 65°C for 3 cycles.

Chemosensitivity assay

MTT [(3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)] method was used to evaluate the cytotoxicity of PCL solution, PCL-loaded GPS/GMS SLNs, PCL-loaded CL/SL and their corresponding blanks [11, 12]. The experiment was carried out as follows: 100 µl cell culture medium (IMDM supplemented with 10% fetal bovine serum and antibiotics) containing 4×10^4 cells, were added to each well in a 96 - well plate and incubated for 24 hrs (similarly, 2×10^4 cells for 48 hrs). The confluent wells were treated with PCL-loaded GPS/GMS SLNs, PCL-loaded CL/SL and their corresponding blanks.

After 24 and 48 hours of incubation (in moistured atmosphere, enriched by CO_2 5%), the plates were washed with phosphate-buffered saline. 100 µl of culture medium containing 20 µl of MTT was added to the plates and incubated for a further 4 hours at 37° C. After this, the contents of the plates were replaced with 50 µl of dimethyl sulfoxide and Optical density at 570nm was measured after background correction at 690 nm by a Microplate Spectrometer (Spectromax 190, USA). The 96-well plate was divided in such a way that each formulation was repeated 5 times. From the chemosensitive assay, sub-toxic concentrations of the formulations were calculated and these concentrations were used for the other assays.

Wound assay

B16F10 Cells were plated in 35 mm petriplates and were allowed to grow to 60% confluency. The plates were treated with doses of PCL solution, PCL-loaded GPS/GMS SLNs, PCL-loaded CL/SL and their corresponding blanks (for 24 and 48 hours). At the end of incubation, the cells were washed with PBS and wound was prepared on the monolayer. A zero time point wound was kept as reference plate. Remaining plates were incubated for 24 and 48 hours in the presence of Serum free IMDM. The plates were fixed with Methanol and stained with crystal violet. The wound widths were measured using Laser Capture Microdissection Microscope using PalmRobo software after 24 and 48 hours of incubation.

The width of the wound was calculated for each dose and percentage migration was expressed as:

$$\text{Percent Relative wound width} = \frac{\text{Wound with of Untreated or formulations}}{\text{Wound width at Zero hour}}$$

25 wound readings of each formulation were taken. The experiment was performed in duplicate. Percent relative wound widths were calculated and the statistical significance in the case of Relative wound widths was calculated using SPSS package.

Colony formation

B16F10 melanoma cells were incubated for 24 hours (4×10^3cells per plate) and 48 hours (2×10^3 cells per plate). The plates were then treated with respective concentrations of PCL solution, PCL-loaded GPS/GMS SLNs, PCL-loaded CL/SL and their corresponding blanks. After incubation, the plates were washed with PBS and then incubated with complete medium (10% FBS and IMDM) for another 48 hours. The cells were then fixed using 70% alcohol and stained using Crystal violet. Colonies having more than 50 cells were counted and the Percent Colony Inhibition (PCI) capacity of the formulations was calculated as follows:

$$\text{PCI} = \frac{\text{No. of colonies in UC} - \text{No of colonies in formulations}}{\text{No. of colonies in UC}} \text{X100}$$

UC: Untreated Control

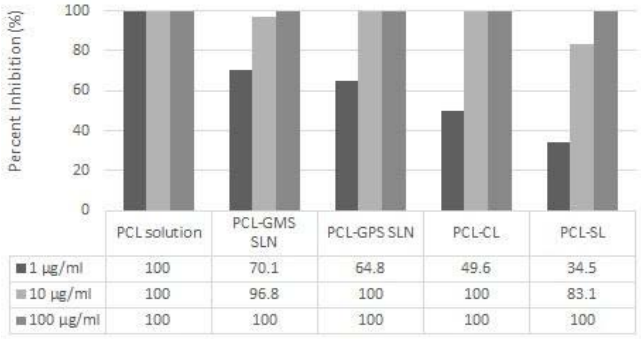
Leighton tube studies

B16F10 melanoma cells were grown on cover slips in 30 mm petriplates in presence of complete medium (10% FBS with IMDM). The plates were treated with PCL solution, PCL-loaded GPS/GMS SLNs, PCL-loaded CL/SL and their corresponding blanks and incubated for 24 and 48 hours. After incubation, the cells are fixed using 70% alcohol and then stained using Hematoxyline and counterstained with Eosin. The cover slips were washed in xylene and finally mounted on the slides. The changes in morphology of B16F10 cells after the treatments of PCL solution, PCL-loaded GPS/GMS SLNs, PCL-loaded CL/SL and their corresponding blanks were assessed under the electron microscope.

Results and Discussion

Chemosensitivity assay of the encapsulated formulations of PCL

The chemosensitivity assay showed that the anti-proliferation effect of PCL and the encapsulated formulation was time- and



	PCL solution	PCL-GMS SLN	PCL-GPS SLN	PCL-CL	PCL-SL
1 µg/ml	100	70.1	64.8	49.6	34.5
10 µg/ml	100	96.8	100	100	83.1
100 µg/ml	100	100	100	100	100

Figure 1: Percentage inhibition of B16F10 cells by PCL and PCL-encapsulated formulations at 48 h at 1µg/ml, 10 µg/ml and 100 µg/ml.

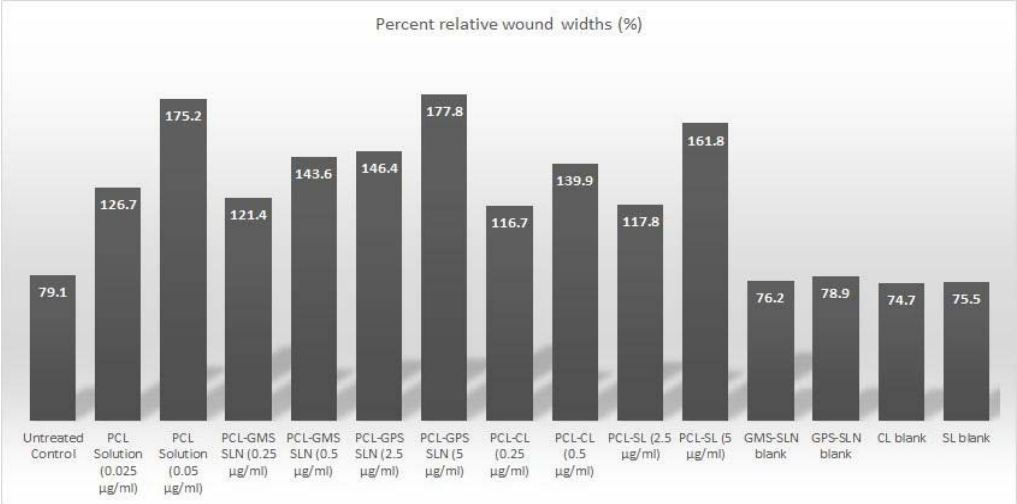


Figure 2a: Percentage relative wound widths of PCL and PCL-encapsulated formulations at 48 h.

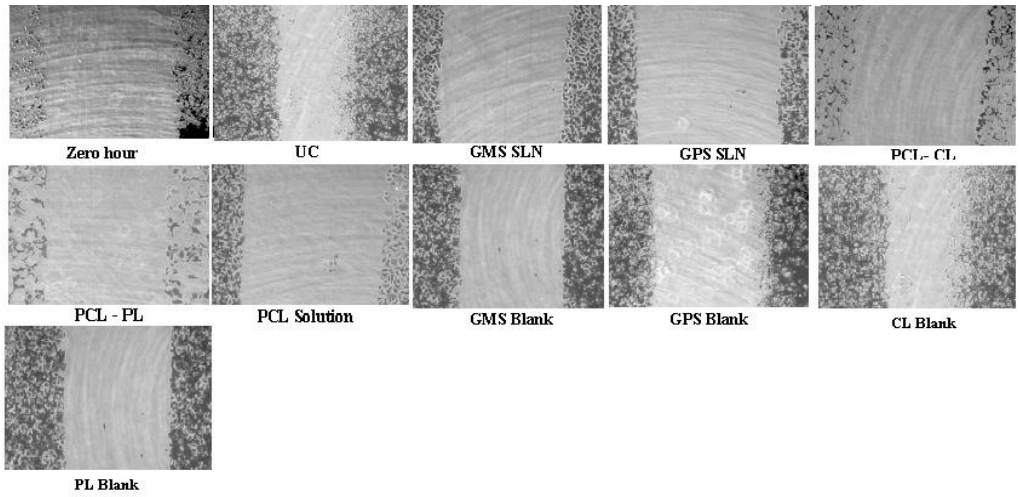


Figure 2b: Pictures of wound widths of different PCL encapsulated formulations at 48 h.

concentration-dependent (Figure 1). At 100 µg/ml, the encapsulated formulations showed comparable cytotoxic activity as that of PCL solution. The blank formulations of each of the encapsulated formulations showed absence of any cytotoxic action of the excipients used in the formulation. Based on the IC50s of the formulations, sub-toxic concentrations of PCL-encapsulated formulations (PCL-CL: 0.25 µg/ml and 0.5 µg/ml; PCL-SL: 2.5 µg/ml and 5 µg/ml; PCL-GMS SLN: 0.25 µg/ml and 0.5 µg/ml and PCL-GPS SLN: 2.5 µg/ml and 5 µg/ml) and PCL solution (0.025 µg/ml and 0.05 µg/ml) were selected for further studies.

Wound assay

Sub-toxic concentrations of PCL solution and encapsulated formulations were found to inhibit the cell motility of B16F10 melanoma cells compared to control and zero hour ($p > 0.5$) (Figure 2a). Overall, PCL-GPS SLN (0.05 µg/ml) was found to have significant anti-migration activity among the encapsulated formulations ($p > 0.05$). There were no significant differences in the anti-migration activity of PCL-GPS SLN (0.05 µg/ml) and PCL solution (0.025 µg/ml). PCL-SL (5 µg/ml) was found to have the best anti-migration activity among the liposomal formulations ($p > 0.05$). As anticipated the blank formulations did not have any anti-migration activity (Figure 2b).

Colony formation assay

Sub-toxic concentrations of PCL solution and encapsulated formulations were found to inhibit the colony of B16F10 melanoma cells compared to control ($p > 0.5$) (Figure 3). However, PCL solution (0.025 µg/ml) had significantly better colony inhibition compared to all the encapsulated formulations ($p > 0.05$). Among the SLNs, PCL-GPS SLN (0.05 µg/ml) had better colony inhibition than PCL-GMS SLN (0.75 µg/ml), while PCL-SL (5 µg/ml) was had better colony inhibition than PCL-CL (0.5 µg/ml). There was no significant difference between had comparable anti-colony forming activity as that of PCL solution. As anticipated the blank formulations did not possess any anti-migration activity.

Leighton tube

The morphological characterization at 48 hours revealed that

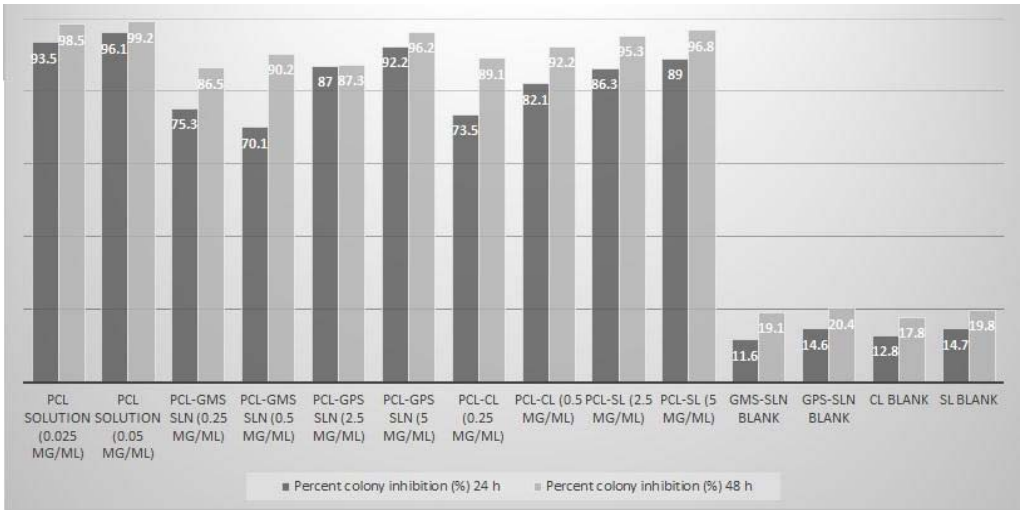


Figure 3: Percent colony inhibition of PCL and PCL-encapsulated formulations at 24 h and 48 h.

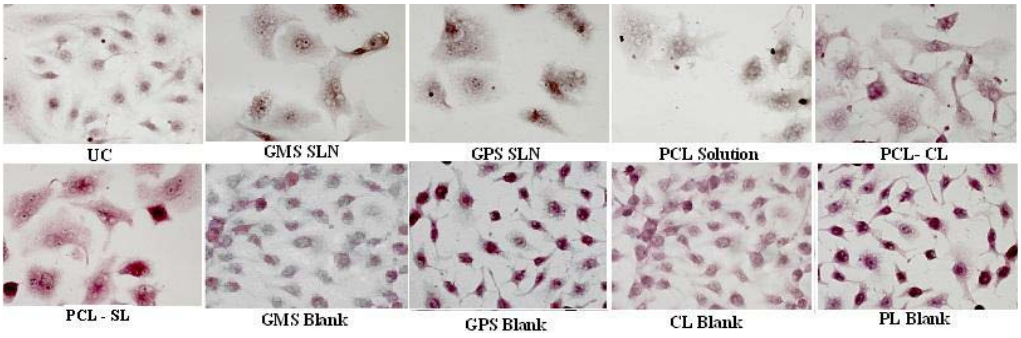


Figure 4: Effect of PCL solution and PCL-encapsulated formulations on the morphology of B16F10 cell lines.

PCL-SL caused maximum damage to the B16F10 melanoma cells (Figure 4). Destruction of the cell wall and release of cellular contents was observed at sub-toxic levels in the PCL-SL formulations. Although cytotoxic activity was also found in cases of other formulations, the extent of damage was minimal. PCL-GPS SLN (0.05 µg/ml) showed drastic morphological changes compared to PCL-GMS SLN (0.75 µg/ml). The cell membrane and the cellular contents were intact in case of blank formulations.

Discussion

PCL is an effective anticancer drug having good activity against a variety of tumors. PCL possesses cytotoxic effects against a wide range of human cell lines such as breast, lung, ovarian, colorectal and melanoma cell lines [13]. In *in vitro* studies, PCL effectively inhibits the growth of B16F10 melanoma cell lines [14]. Particulate systems such as nanoparticles and liposomes have been suggested as alternatives in the effective delivery of PCL. Drug delivery systems such as liposomes and niosomes encapsulating anticancer drugs have been effectively evaluated using B16 F10 melanoma cells for their anti-metastatic activity [15,16]. Previously, we have reported the preparation of PCL lipid nanoparticles [9,10] and liposomal formulations [8]. These formulations showed promising characteristics in terms of *in vitro* release and anti-proliferation activity. To our knowledge, this is the first study that compares the PCL-loaded liposomes and lipid nanoparticles from different lipid carriers.

In the chemosensitivity assay, the IC50 values for SLNs and liposomes were high due to the retarded release from their carrier systems. The lower cytotoxicity of PEGylated than conventional liposomes is probably related to the steric effect of PEG chains, which may delay internalization of the drug in the cells by masking the negative liposomal charge: negatively charged liposomes generally exhibit stronger binding than do neutral ones because of the existence of a membrane receptor recognizing negatively charged particles [17]. The results obtained in the study comply with several previous results carried out on PCL-loaded lipid nanoparticles [18,19], nanoparticles [20] and liposomes [5].

Cell motility is an important component of cell invasion and spread of cancer cells throughout the body. The ability to exploit factors that enable cell motility may endow a tumor cell with a greater ability to metastasize. PCL being a microtubule stabilizing drug inhibits cell migration [21]. It reduces cell migration by perturbing the endothelial cell function and stabilization of the microtubule cytoskeleton [22]. Wound assay is an easy method for the *in vitro* analysis of migration of cells [23]. In the study, the PCL-loaded formulations in its sub-toxic concentrations were able to exert anti-migration effect on B16F10 cell lines. The results with PCL-SL was similar to the migration inhibition observed in a previous study

carried out in HUVEC cell lines [1].

PCL by the virtue of its action on microtubule inhibits colony formation of endothelial cells. It has also been found to inhibit colony formation of B16F10 melanoma cells [14] and even against a wide range of human breast, lung ovarian, colorectal cancer and melanoma cell lines [13]. In the present study, sub-toxic concentrations of PCL formulations were able to exert colony inhibiting activity despite being encapsulated. The colony inhibition effects of PCL-loaded liposomes have been demonstrated in a previous study [24]. A similar reduction in colony formation was observed with PCL liposomal formation, in this study.

Leighton tube study was carried to assess the extent of cellular damage caused by the PCL formulations in comparison to the PCL solution. The cells treated with sub-toxic concentration was found to cause similar cellular damage as that of PCL solution at sub-toxic level.

Conclusion

The prepared PCL-loaded SLNs and liposomal formulations possessed comparable cytotoxicity compared to the PCL solution. All encapsulated formulations showed good anti-migrational activity, colony inhibition activity, and cytopathic effects. Among them, PCL-GPS SLNs and PCL-SL possessed promising characters as delivery systems for PCL.

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