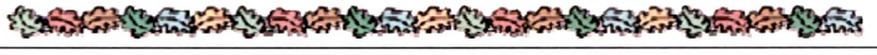
Chapter 6
In-vitro Cell Line Studies



6. *In-vitro* cell line studies:

6.1 Introduction

After all preliminary *in-vitro* testing of the formulations for drug entrapment, release rate and stability studies the formulations should be evaluated for their safety and efficacy by checking for various cellular interactions of the formulations at tissue culture level which then leads to *in-vivo* studies in animal models. Hence the Cytotoxicity studies, cell uptake and antimetastatic activities were taken up prior to *in-vivo* efficacy studies. The cell uptake of liposomal formulations was evaluated using B16F1 (low metastatic) and B16F10 (highly metastatic) melanoma cells as an *in-vitro* model with the aim to apply Paclitaxel (PCL) and Irinotecan Hydrochloride (IH) liposomal formulations for cancer chemotherapy with the view to explore the possible effects of particle size, surface charge and pH sensitivity of the formulation.

Different PCL and IH containing formulations like conventional liposomes, pH sensitive liposomes, placeboes for both the conventional and pH sensitive liposomes and plain drugs were selected for *in-vitro* Cytotoxicity performance evaluation.

In-vitro studies were carried out in B16F1 (low metastatic) & B16F10 (highly metastatic) melanoma cell lines for Cytotoxicity by MTT assay (Yang et. al., 2007: Carvalho et. al., 2007: Gude et. al., 1999), cell proliferation by colony formation (Liao et. al, 2005), cell motility by wound assay (Gude et. al., 2001), cell cycle analysis by Fluorescent Activated Cell Sorting (FACS) (Liao et. al., 2005, Dua et. al., 2007) and Cell uptake by confocal microscopy using fluorescent labeled liposomes (Miglietta et. al., 2000).

Cell culture

B16F1 and B16F10 stock cells were cultured in DMEM (Sigma chemicals, USA) supplemented with 10 % inactivated foetal bovine serum (FBS), penicillin (100 IU/ml) Streptomycin (100 µg/ml) in a humidified atmosphere of 5 % CO_2 at 37 °C until confluent. The cells were dissociated with 0.2 % trypsin, 0.02% EDTA in phosphate Buffer saline solution, the stock culture cells were grown in 25 ml tissue culture flasks and all experiments were carried out in 96-well microtitre plates. 3-(4, 5-dimethyl thiazole-2yl)-2, 5-diphenyl tetrazolium bromide dye (MTT) was prepared in phosphate buffer saline.

6.2 Cytotoxicity Assay (MTT assay):

Cell lines in the exponential growth phase were washed, trypsinized, harvested and resuspended in complete culture medium. Cells were seeded in 96-well flat bottom tissue culture plates at a concentration of $2x10^4$ cells/100 µl/well. The cells were allowed to grow and stabilize for 48 hours during which a partial monolayer forms. Subsequently, the cells were treated with serial concentrations prepared in complete medium of PCL / IH loaded liposomal formulations and Plain drugs 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. Each treatment was performed in six well replicates. Post treatment cells were washed with PBS and the cell viability was determined by MTT colorimetric assay according to the reported method.

Briefly, 20 µl of MTT reagent (Sigma Aldrich) was added to each well to make a final concentration of 1 mg/ml of media and incubated for 4 hour at 37° C. Plates were then centrifuged at 2,000 rpm for 10 min. Medium was aspirated from the wells and 100 µl of dimethyl sulphoxide was added to each to dissolve formazan crystals. The optical density was measured in an ELISA plate reader (Molecular Devices, Spectra Max 190 with Soft max Pro) at 540 nm with a reference wavelength of 690 nm. Cell viability was plotted as a percentage of untreated control. Results are expressed as Mean ± SEM and are representative of three independent experiments. Inhibitory concentration 50 (IC_{50}) of Paclitaxel and its liposomal formulations were determined from the dose effect curve as the drug concentration that decreased the cell viability to 50%, relative to control.

$$\% \text{ Cell viability} = \frac{Abs_{sample}}{Abs_{control}} \times 100$$

Where Abs_{sample} is the absorbance of cells tested with various formulations and $Abs_{control}$ is the absorbance of control cells (incubated with cell culture media only) The toxicity of samples was expressed as the drug concentration required to inhibit growth of the cells by 50 % relative to control (IC_{50}) (Table 6.3 and Table 6.6)

Table 6.1. Cell viability of PCL and its liposomal formulations on B16F1 cells after 48 h incubation period.

Paclitaxel concentration (µg/ml)	%Cell viability (B16 F1)		
	PCL	CLPT	PSPT
25.00	0.00	15.71	0.00
12.50	12.78	38.57	4.29
5.00	45.46	51.43	38.86
0.50	50.00	70.35	64.71
0.10	62.12	80.30	76.86
0.01	66.67	86.36	82.45
0.001	72.72	92.42	86.57
Untreated control (UC)	100.00	100.00	100.00

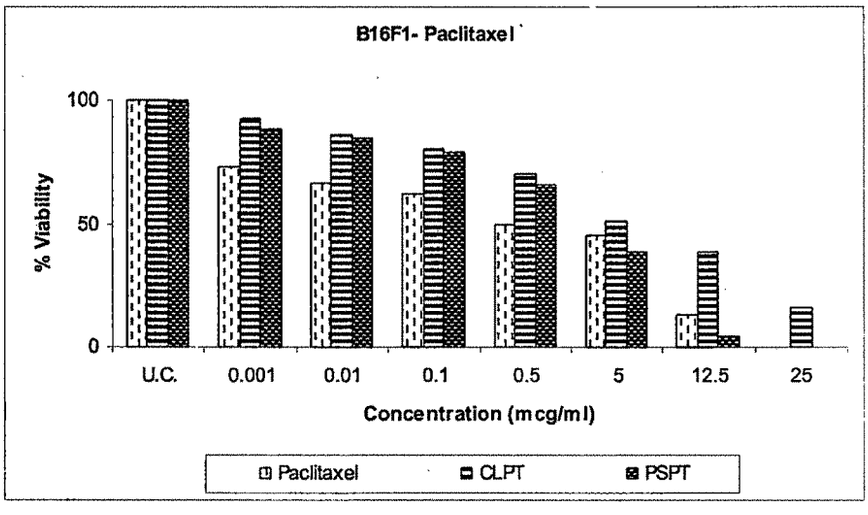


Figure 6.1. Cell viability of Paclitaxel and its liposomal formulations on B16 F1 cells after 48 h incubation period, $p<0.05$. (U.C. = Untreated Control)

Table 6.2. Cell viability of PCL and its liposomal formulations on B16F10 cells after 48 h incubation period.

Paclitaxel concentration (µg/ml)	% Cell viability (B16 F10)		
	PCL	CLPT	PSPT
25.00	0.00	13.57	0.00
12.50	6.47	45.45	0.00
5.00	24.31	48.25	24.48
0.50	35.29	60.84	43.36
0.10	48.96	70.63	50.94
0.01	60.78	80.42	68.04
0.001	83.13	92.90	89.23
Untreated control (UC)	100.00	100.00	100.00

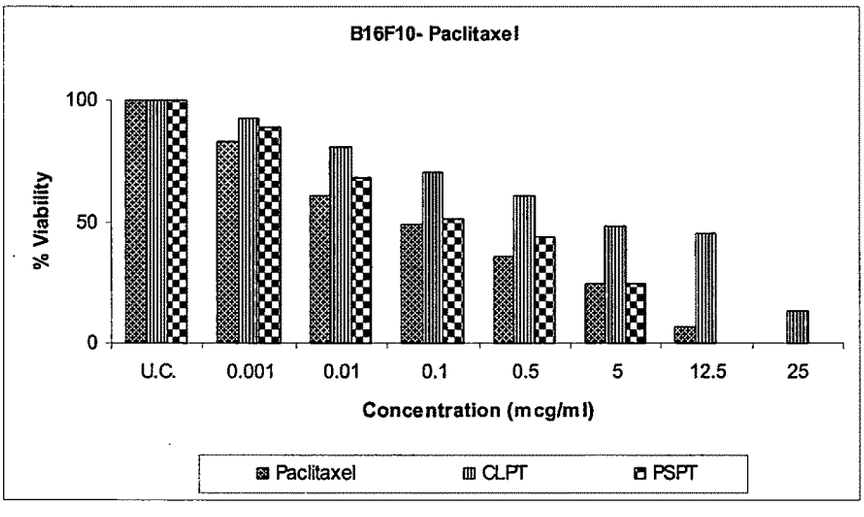


Figure 6.2. Cell viability of PCL and its liposomal formulations on B16 F10 cells after 48 h incubation period, *p<0.05. (U.C.= Untreated Control)

Table 6.3. IC_{50} values of PCL and its liposomal formulations in B16 F1 & B16 F10 cell lines by MTT assay after 48 h incubation period.

Formulations	IC_{50} values (µg/ml)*	
	B16 F1	B16 F10
PCL	0.50	0.09
CLPT	6.00	3.50
PSPT	2.20	0.14

Table 6.4. Cell viability of IH and its liposomal formulations on B16 F1 cells after 48 h incubation period.

Irinotecan concentration (µg/ml)	% Cell viability (B16 F1)		
	IH	CLIH	PSIH
25.00	0.00	0.00	0.00
12.50	0.85	2.45	0.00
5.00	4.39	5.58	0.19
0.50	13.41	40.89	10.97
0.10	50.24	70.67	57.06
0.01	70.98	82.74	76.89
0.001	80.19	89.78	83.02
Untreated control (UC)	100.00	100.00	100.00

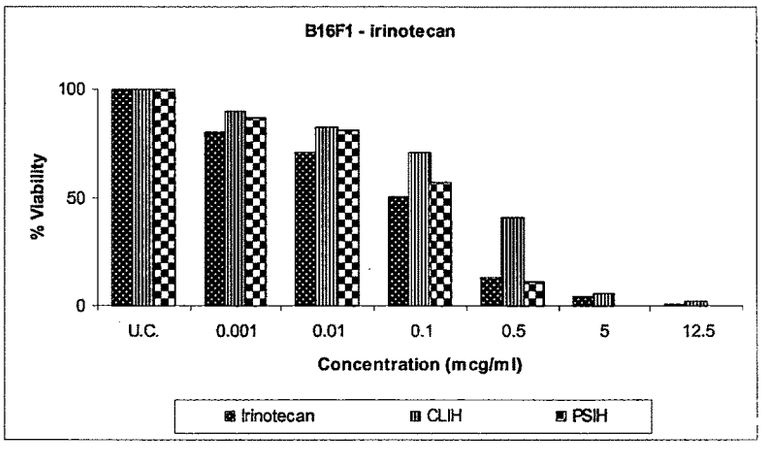


Figure 6.3. Cell viability of IH and its liposomal formulations on B16 F1 cells after 48 h incubation period, *p<0.05. (U.C. = Untreated Control)

Table 6.5. Cell viability of IH and its liposomal formulations on B16 F10 cells after 48 h incubation period.

Irinotecan concentration (µg/ml)	% Cell viability (B16 F10)		
	IH	CLIH	PSIH
12.50	0.00	0.00	0.00
5.00	2.65	7.46	0.00
0.50	26.76	33.09	11.79
0.10	40.79	63.87	56.16
0.01	71.71	86.76	73.94
0.001	77.87	88.52	83.27
Untreated control (UC)	100.00	100.00	100.00

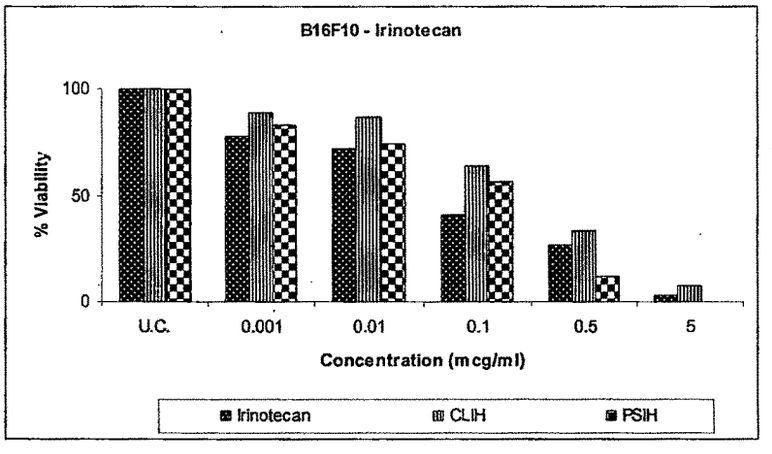


Figure 6.4. Cell viability of IH and its liposomal formulations on B16 F10 cells after 48 h incubation period, *p<0.05. (U.C. = Untreated Control)

Table 6.6. IC_{50} values of IH and its liposomal formulations in B16 F1 & B16 F10 cell lines by MTT assay after 48 h incubation period.

Formulations	IC_{50} values (µg/ml)*	
	B16 F1	B16 F10
IH	0.10	0.05
CLIH	0.31	0.21
PSIH	0.15	0.15

6.3 Leighton Tube Assay

Drug and its formulations-induced changes in cell and nuclear morphology were observed by Haematoxylin - Eosin (H and E) staining. In brief, subconfluent culture of B16F1 and B16F10 melanoma grown on cover slips was treated with different concentrations below their respective IC_{50} values obtained for Paclitaxel, Irinotecan Hydrochloride and their liposomal formulations in complete medium for 48 hours separately. Cells were also incubated with blank liposomes (placebos) at the highest concentrations used for the study to evaluate the possible effects of formulation components. Cover slips were washed in PBS and cells were fixed with methanol. Fixed cells were stained with haematoxylin and eosin, washed and mounted using DPX mountant. The cytoplasmic and nuclear changes were observed under a Phase contrast Microscope and images were captured at 10X & 40X magnification.

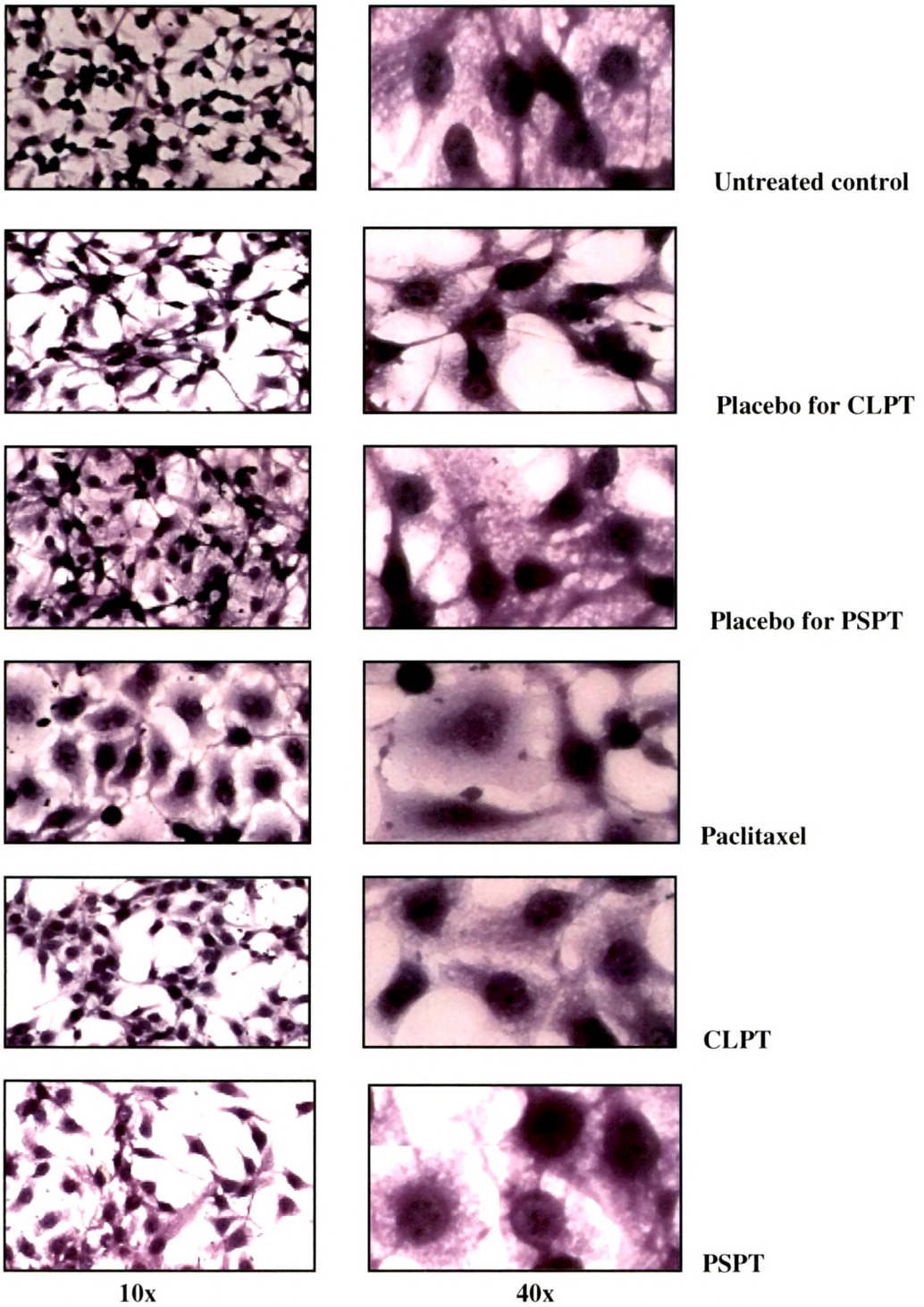


Figure 6.5. Leighton tube experiment for Paclitaxel and its liposomal formulations on B16F1 melanoma cells.

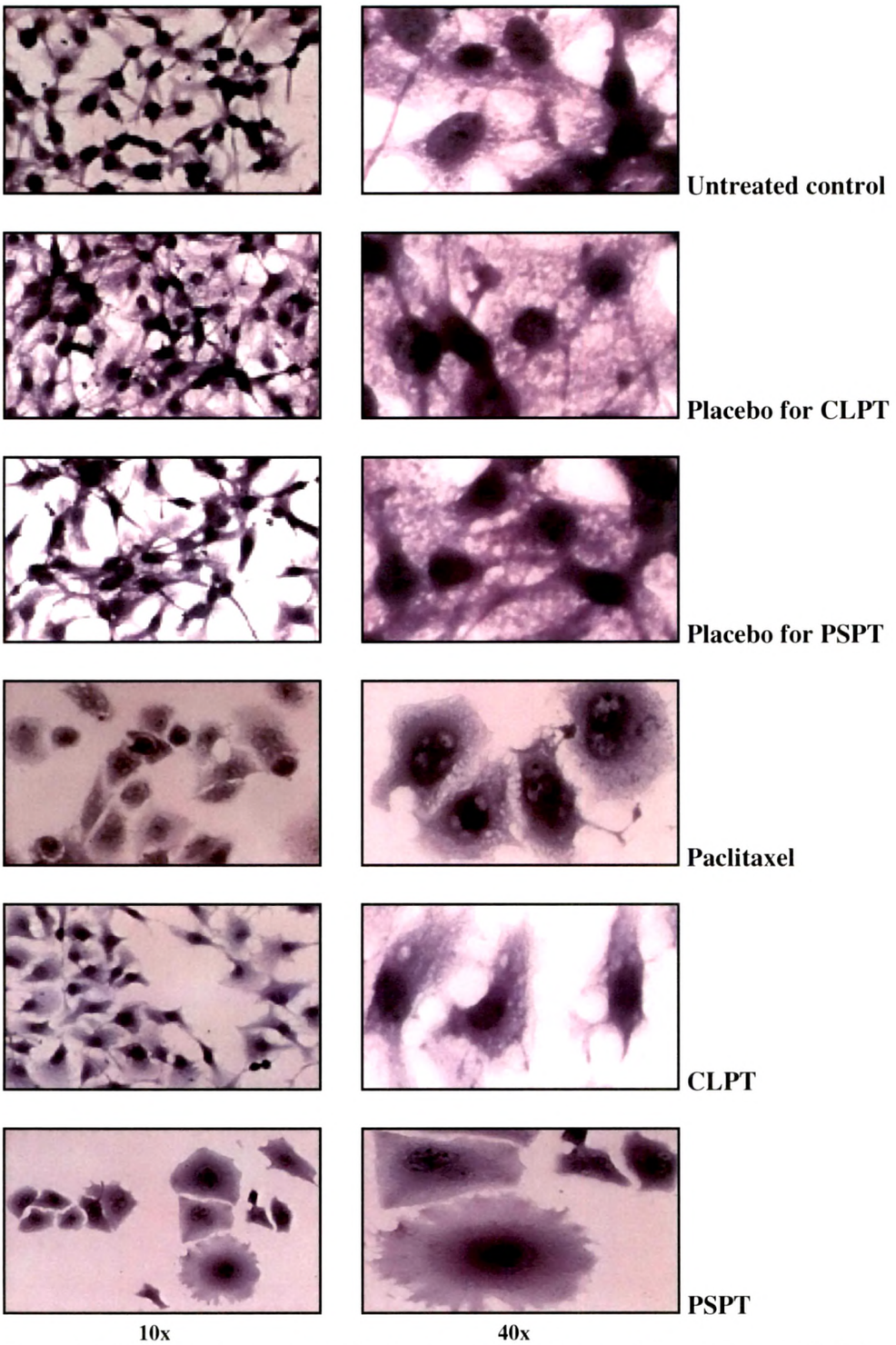


Figure 6.6. Leighton tube experiment for Paclitaxel and its liposomal formulations on B16F10 melanoma cells.

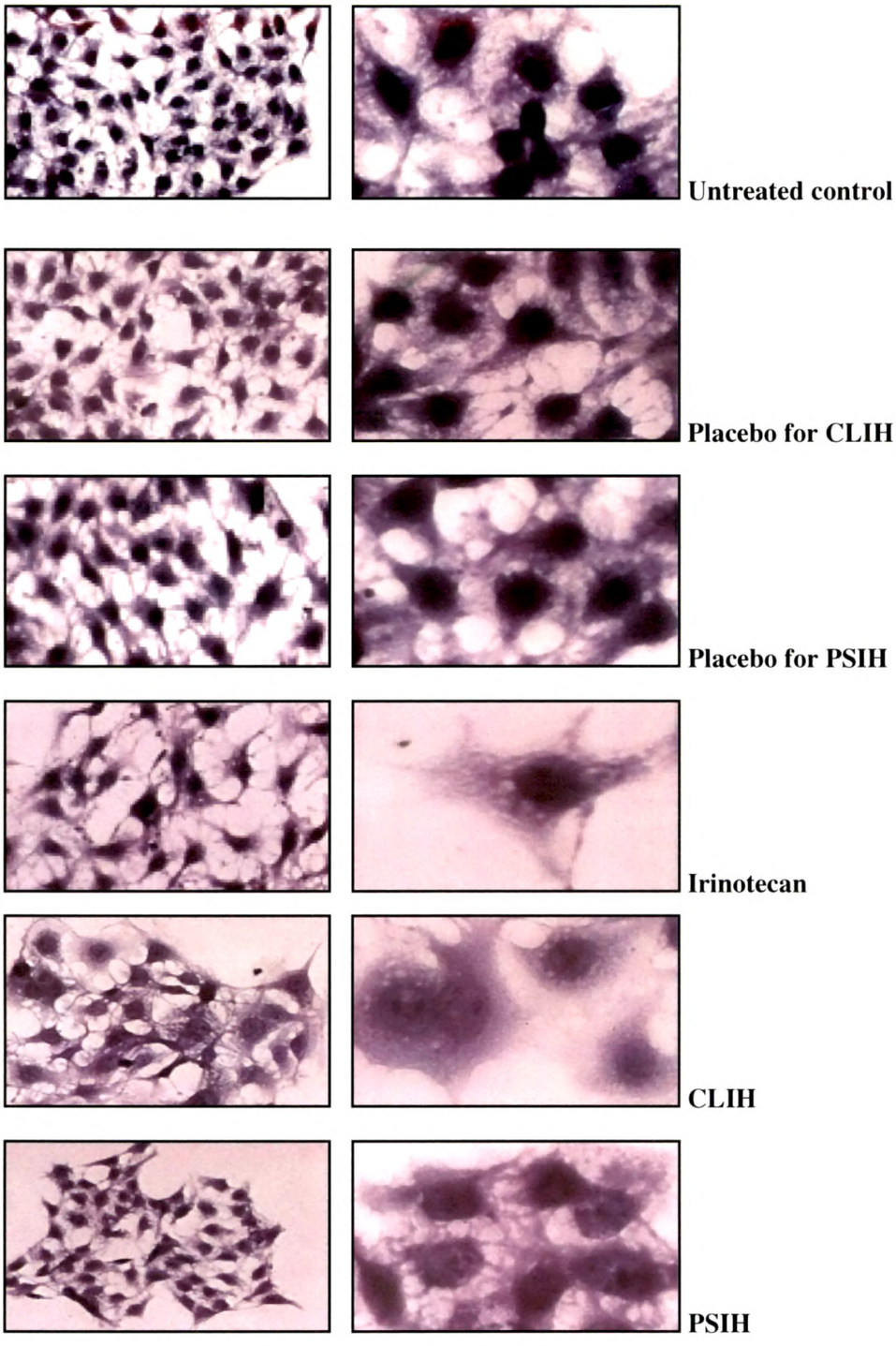


Figure 6.7. Leighton tube experiment for Irinotecan and its liposomal formulations on B16F1 melanoma cells.

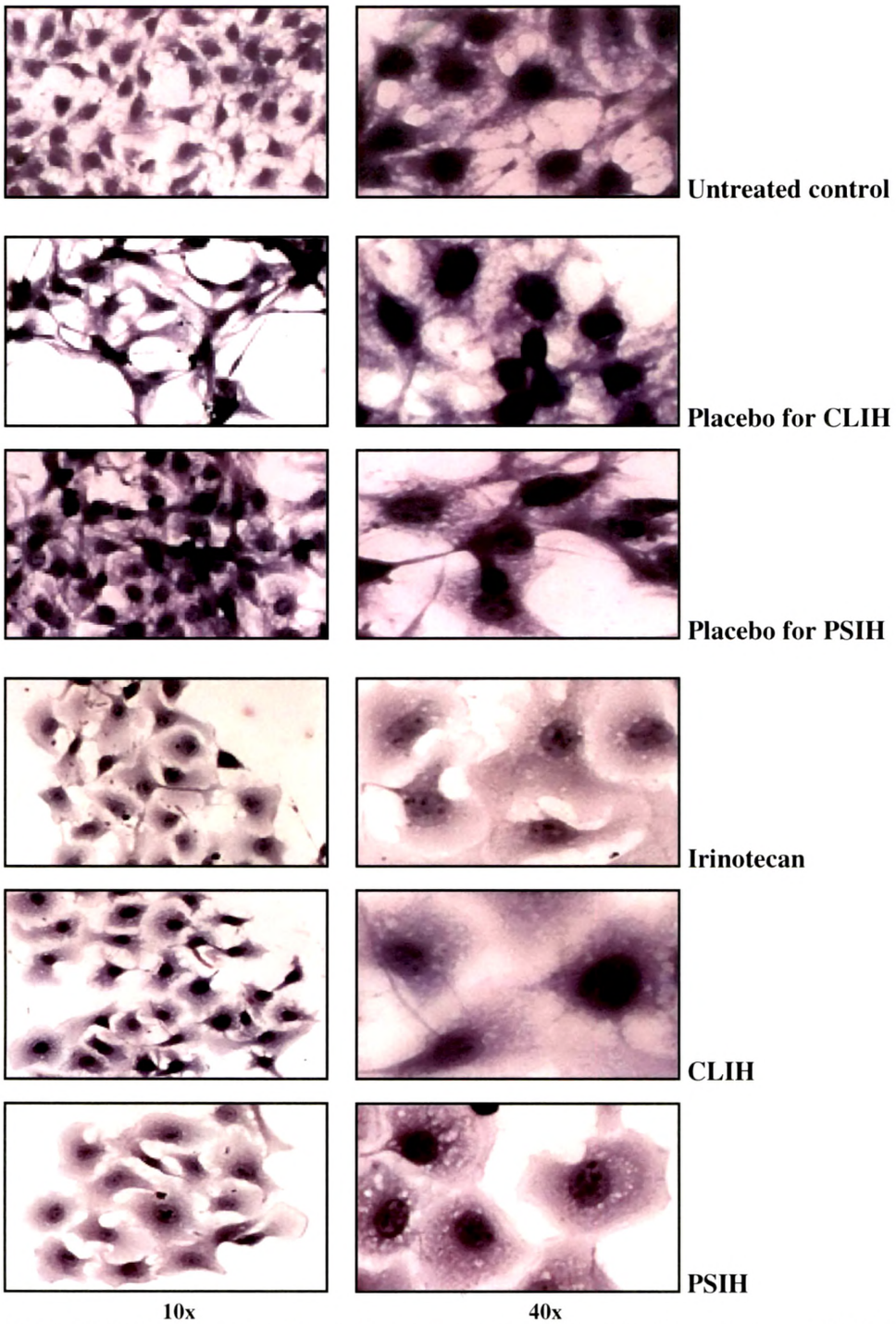


Figure 6.8. Leighton tube experiment for Irinotecan and its liposomal formulations on B16F10 melanoma cells.

6.4 Confocal microscopy

In vitro cellular uptake and intracellular distribution of liposome encapsulating FITC-Dextran were evaluated qualitatively by fluorescence microscopy. B16F1 and B16F10 cells were seeded and grown to 60 % confluency in 35mm petri plates containing square cover slips. Cells were incubated for 1, 24 and 48 hours with pH sensitive liposome encapsulated with FITC. After incubation period, cells were washed four times with PBS and fixed with 4 % Paraformaldehyde solution. Treated cell monolayer on cover slip was mounted on slides using DABCO-Glycerol. The slides were observed by confocal laser scanning microscope (Zeiss LSM 410) with imaging software (Fluoview FV500). (Figure 6.9 and Figure 6.10)

6.5 Cell proliferation

Colony formation assay was carried out as described below. 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 / IH and its liposomal formulations at concentrations below their respective IC_{50} values for 48 hours. Cells were also incubated with blank liposomes (placebos) at the highest concentrations used for the study to evaluate the possible effects of formulation components. The drug was removed; cells were washed in PBS and incubated for 48 hours in complete medium. Each treatment was done in duplicate. 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 (Figure 6.13, 6.14 and Figure 6.17, 6.18).

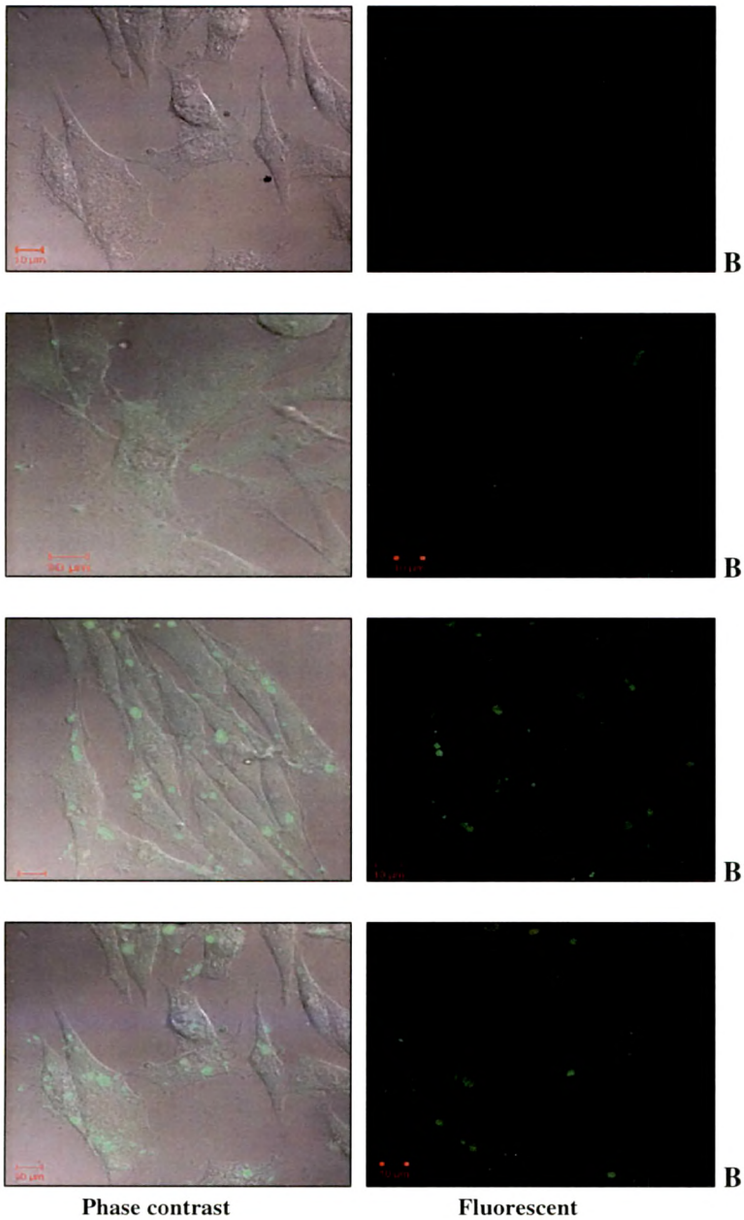


Figure 6.9. Confocal micrographs of pH sensitive liposomal formulations in B16F1 melanoma cells at 1, 24, and 48 hours incubation.

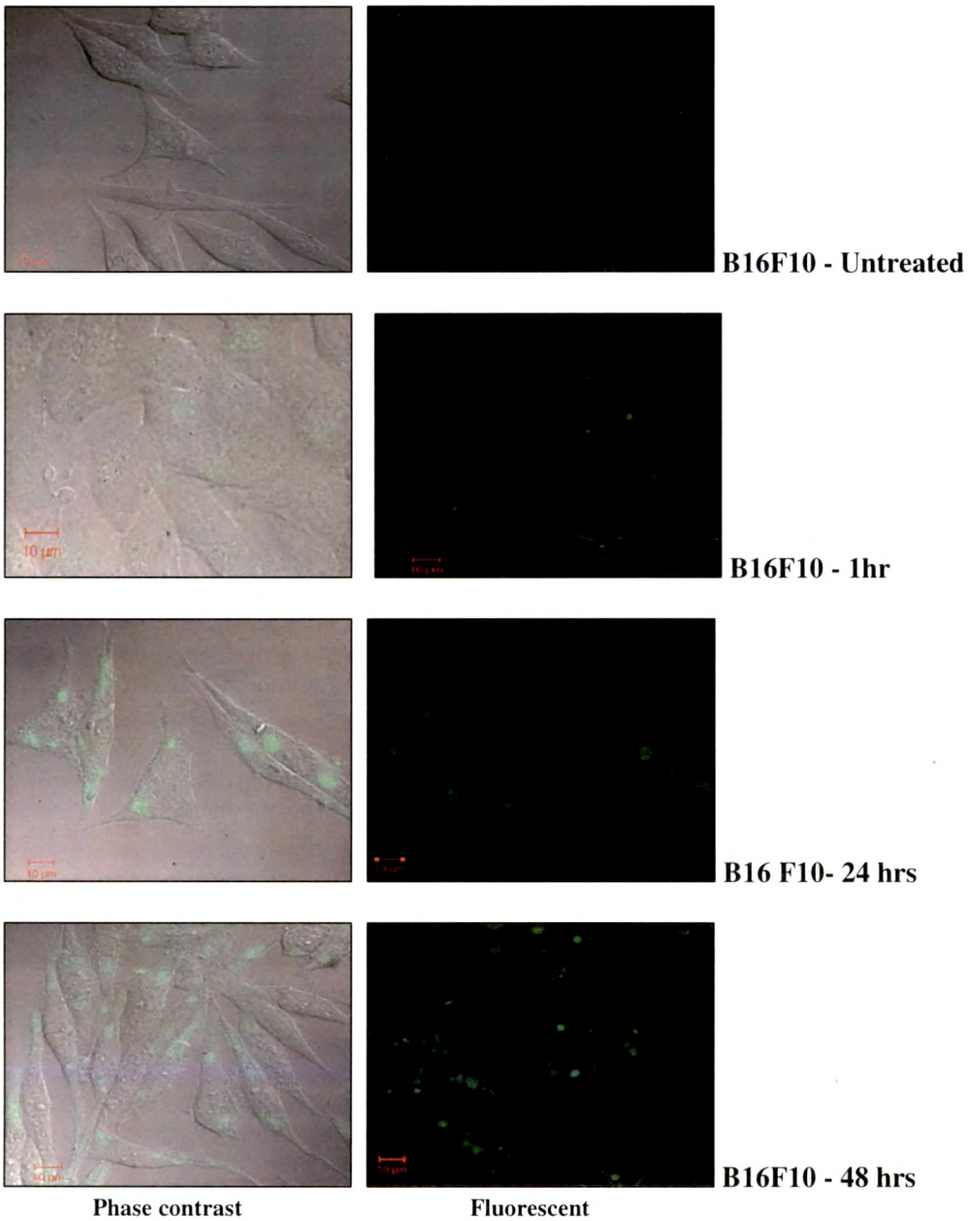


Figure 6.10. Confocal micrographs of pH sensitive liposomal formulations in B16F10 melanoma cells at 1, 24, and 48 hours incubation.

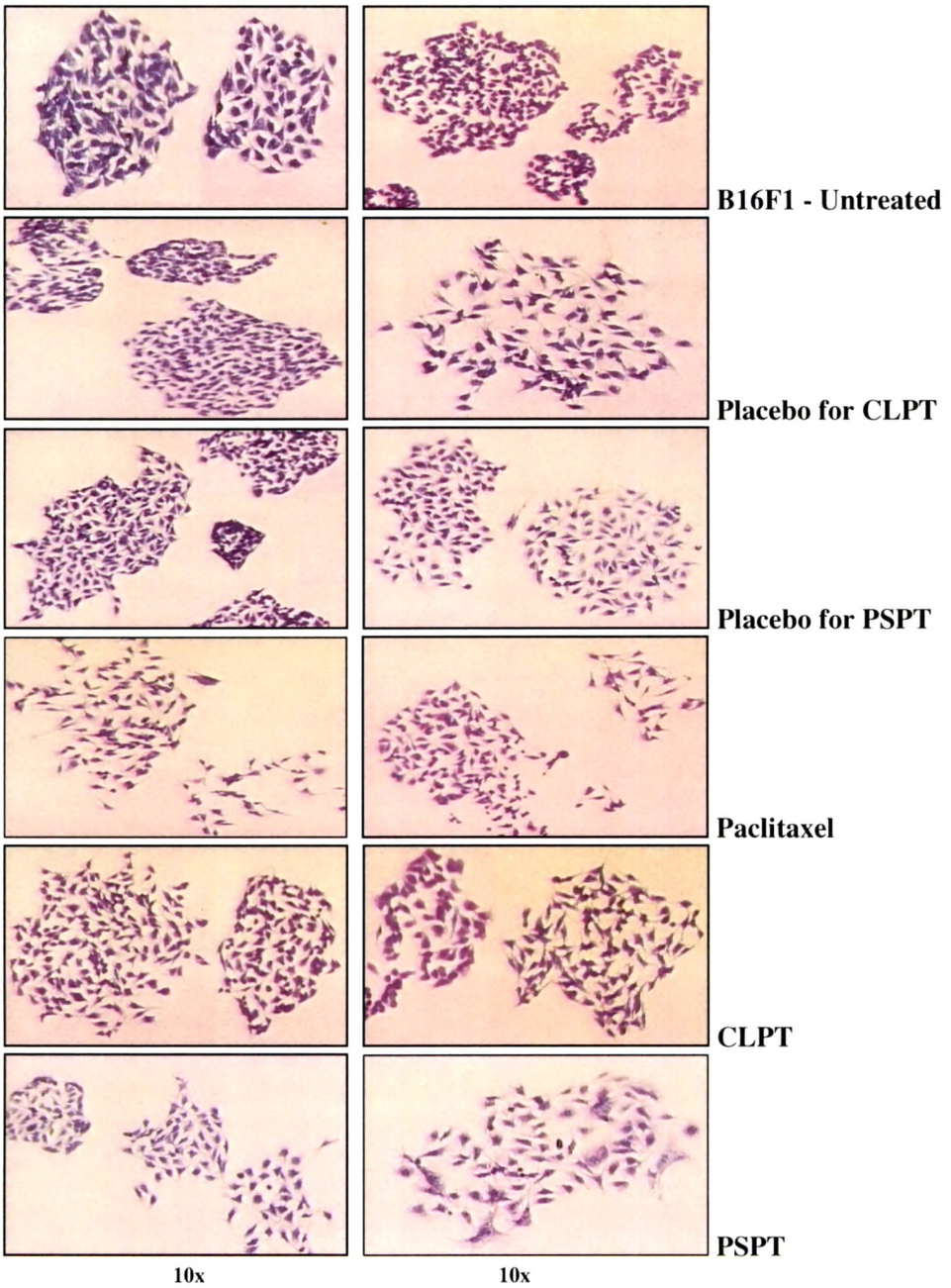


Figure 6.11. Colony formation assay for Paclitaxel and its liposomal formulations in B16F1melanoma cell line.

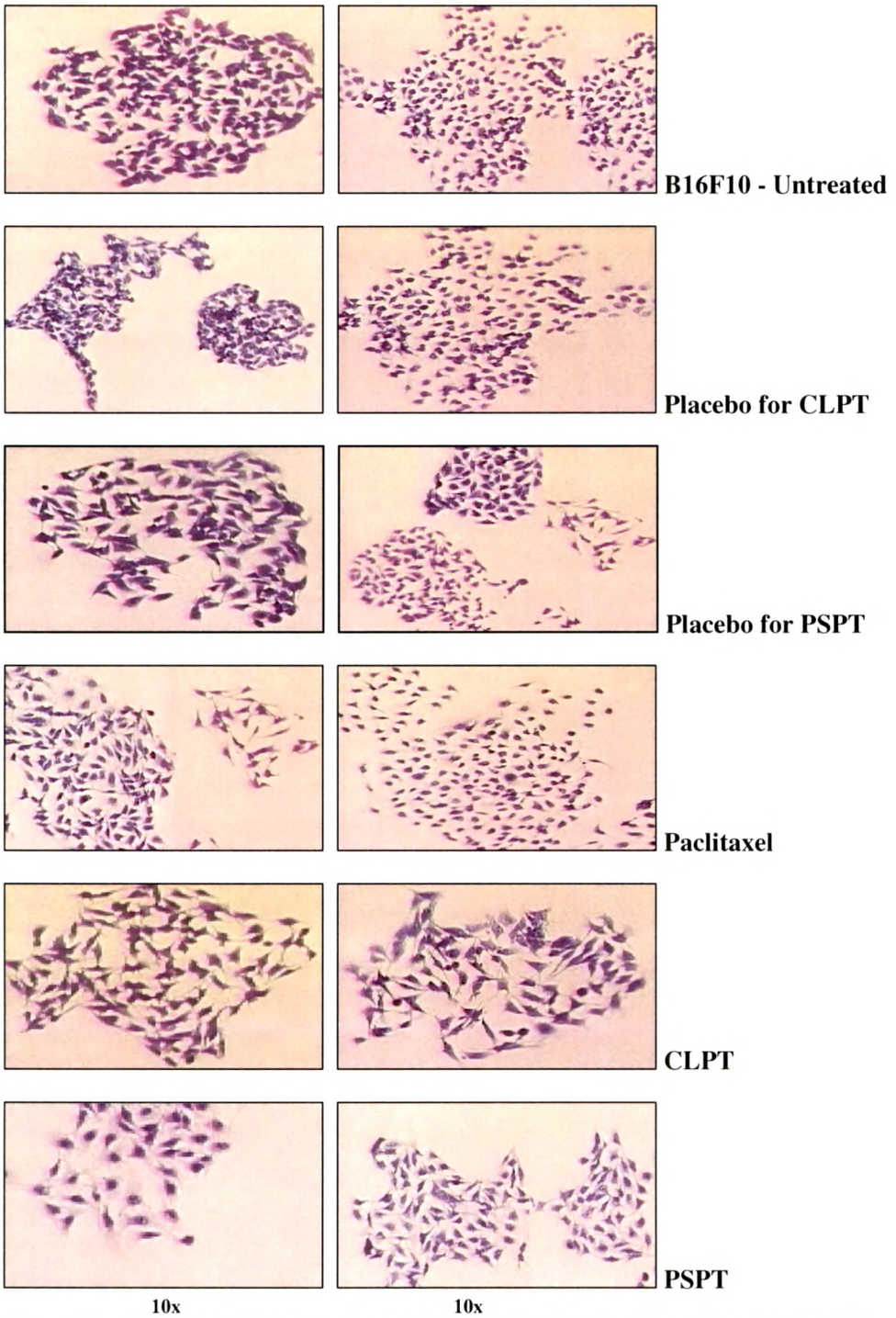


Figure 6.12. Colony formation assay for Paclitaxel and its liposomal formulations in B16F10 melanoma cell line.

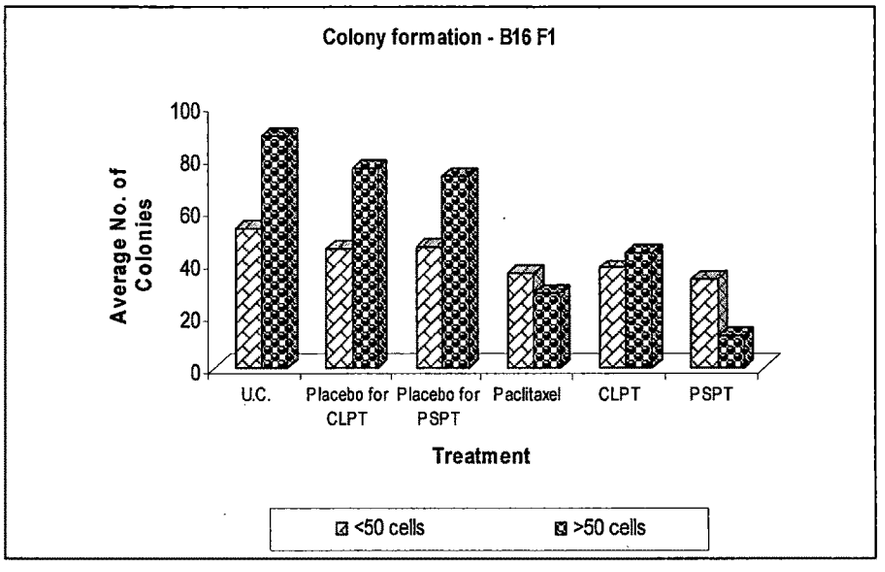


Figure 6.13. Colony formation in case of B16F1 cells incubated with Paclitaxel and its liposomal formulations at concentration below IC_{50} value.

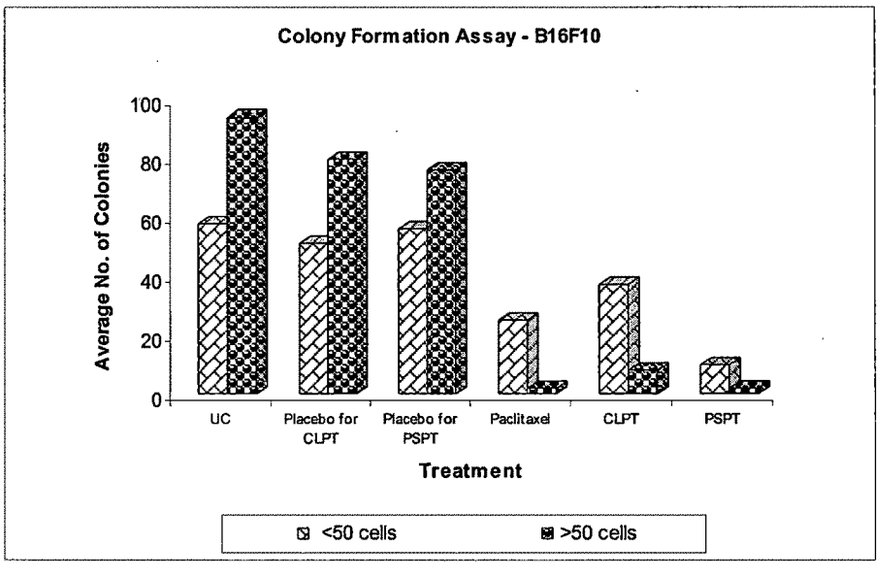


Figure 6.14. Colony formation in case of B16F10 melanoma cells incubated with Paclitaxel and its liposomal formulations at concentrations below IC_{50} value.

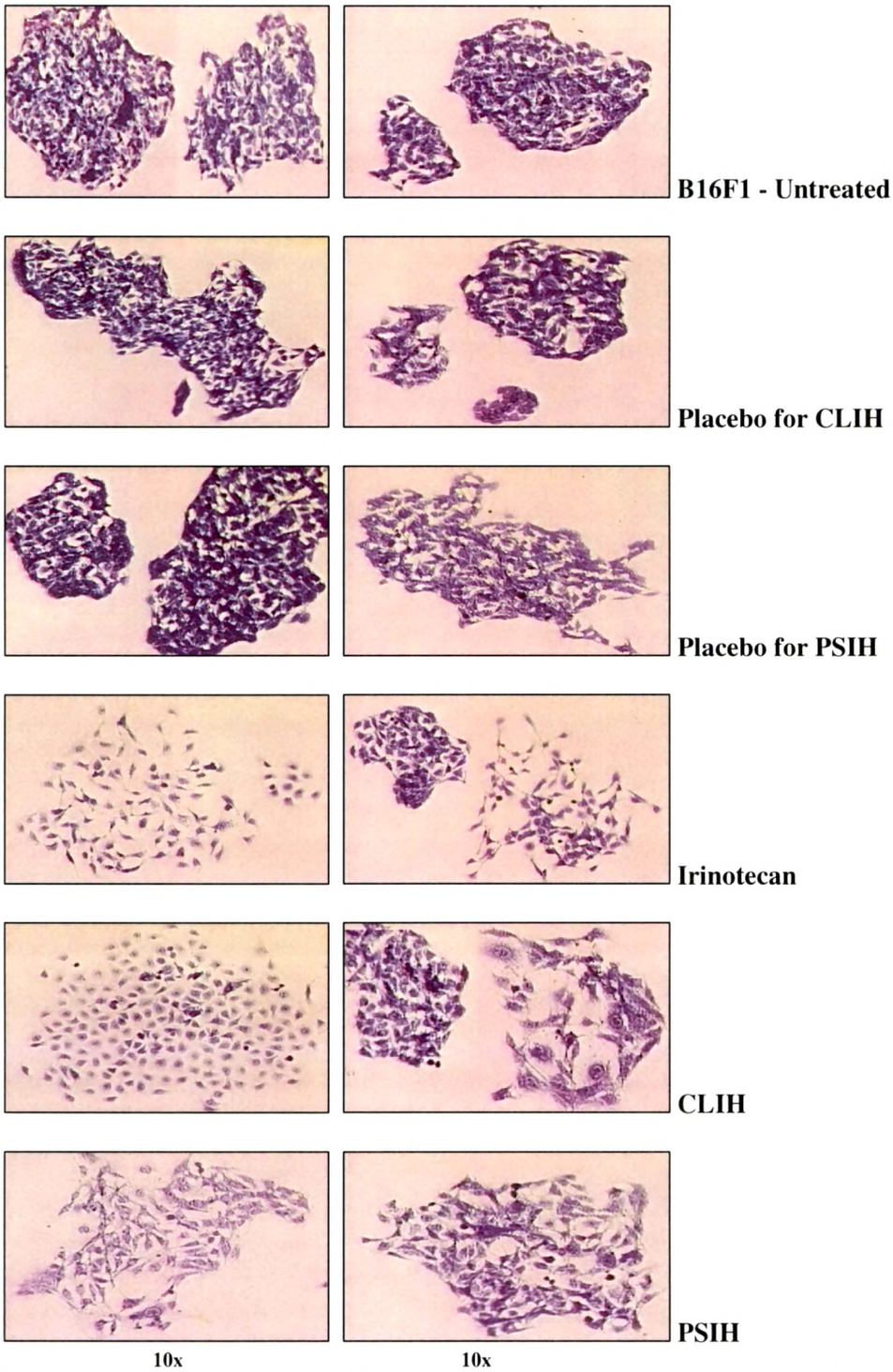


Figure 6.15. Colony formation assay for Irinotecan and its liposomal formulations in B16F1 melanoma cell line.

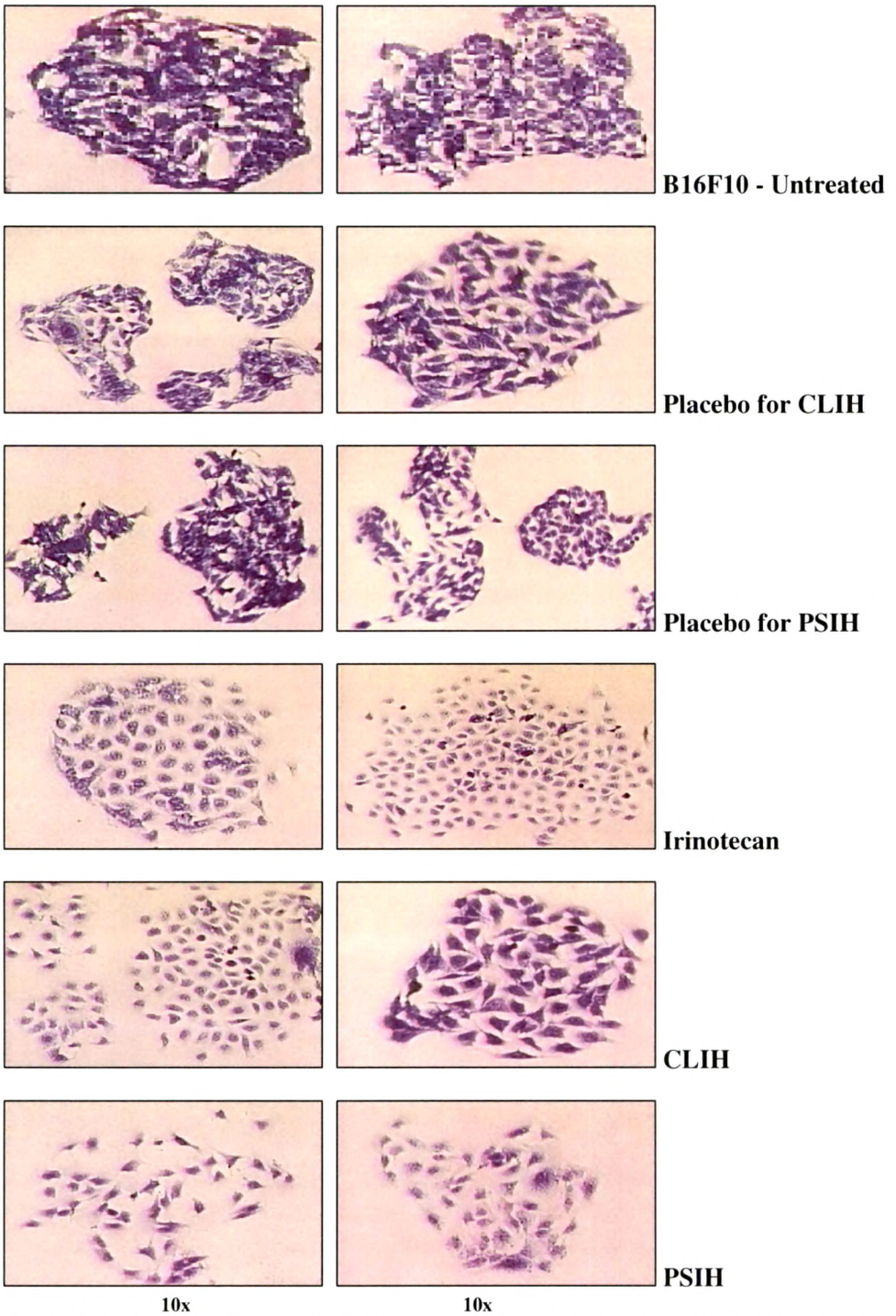


Figure 6.16. Colony formation assay for Irinotecan and its liposomal formulations in B16F10 melanoma cell line.

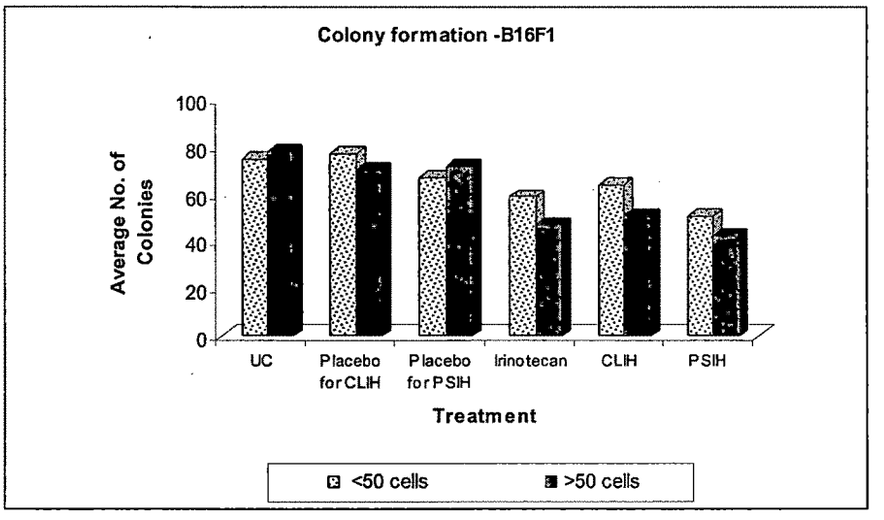


Figure 6.17 Colony formation in case of B16F1 melanoma cells incubated with Irinotecan and its liposomal formulations at concentration below IC_{50} value.

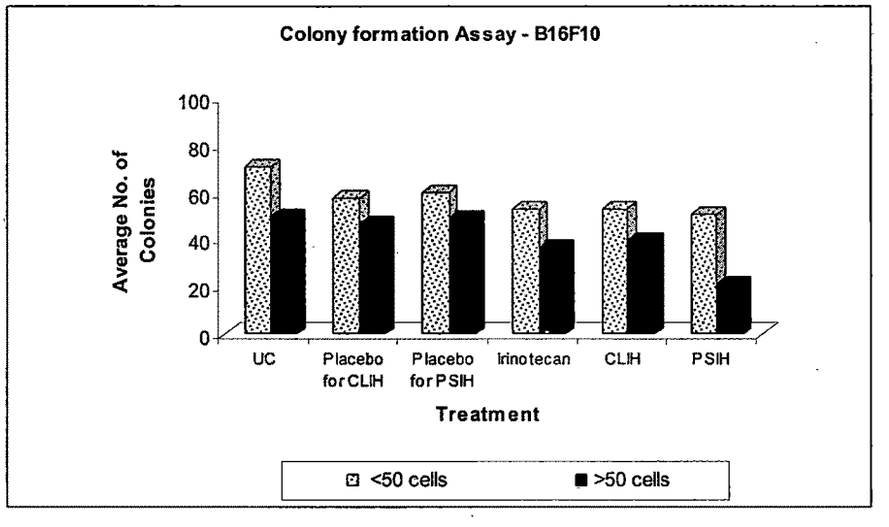


Figure 6.18. Colony formation in case of B16F10 melanoma cells incubated with Irinotecan and its liposomal formulations at concentration below IC_{50} value.

6.6 Cell Motility

Wound healing assays have been carried out in tissue culture for many years to estimate the migration and proliferation rates of different cells and culture conditions. These assays generally involve first growing a confluent cell monolayer. A small area is then disrupted and a group of cells destroyed or displaced by scratching a line through the layer with a micropipette tip/needle. The open gap is then inspected microscopically over time as the cells move in and fill the damaged area. This "healing" can take from several hours to over a day depending on the cell type, conditions, and the extent of the "wounded" region.

Subconfluent culture of B16F1 and B16F10 melanoma grown in 35 mm Petri dishes was treated with different concentrations of PCL / IH and its liposomal formulations below their respective IC_{50} values in complete medium for 48 hours. Cells were also incubated with blank liposomes (placeboes) at the highest concentrations used for the study to evaluate the possible effects of formulation components. After 48 hours treatment, cells were rinsed with PBS. Using a sterile 200 µl plastic pipette tip, three separate uniform, cell-free wounds were made through the cells moving perpendicular to the line drawn in the middle of the Petri culture dish. Cells were maintained on low serum media (1.5 ml; 0.5% serum in DMEM) for another 48 hours. Cells were fixed with methanol and stained with 0.5 % Crystal violet solution. Photographs of each wound were taken 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. Percentage migration and relative wound width was calculated from the above readings.

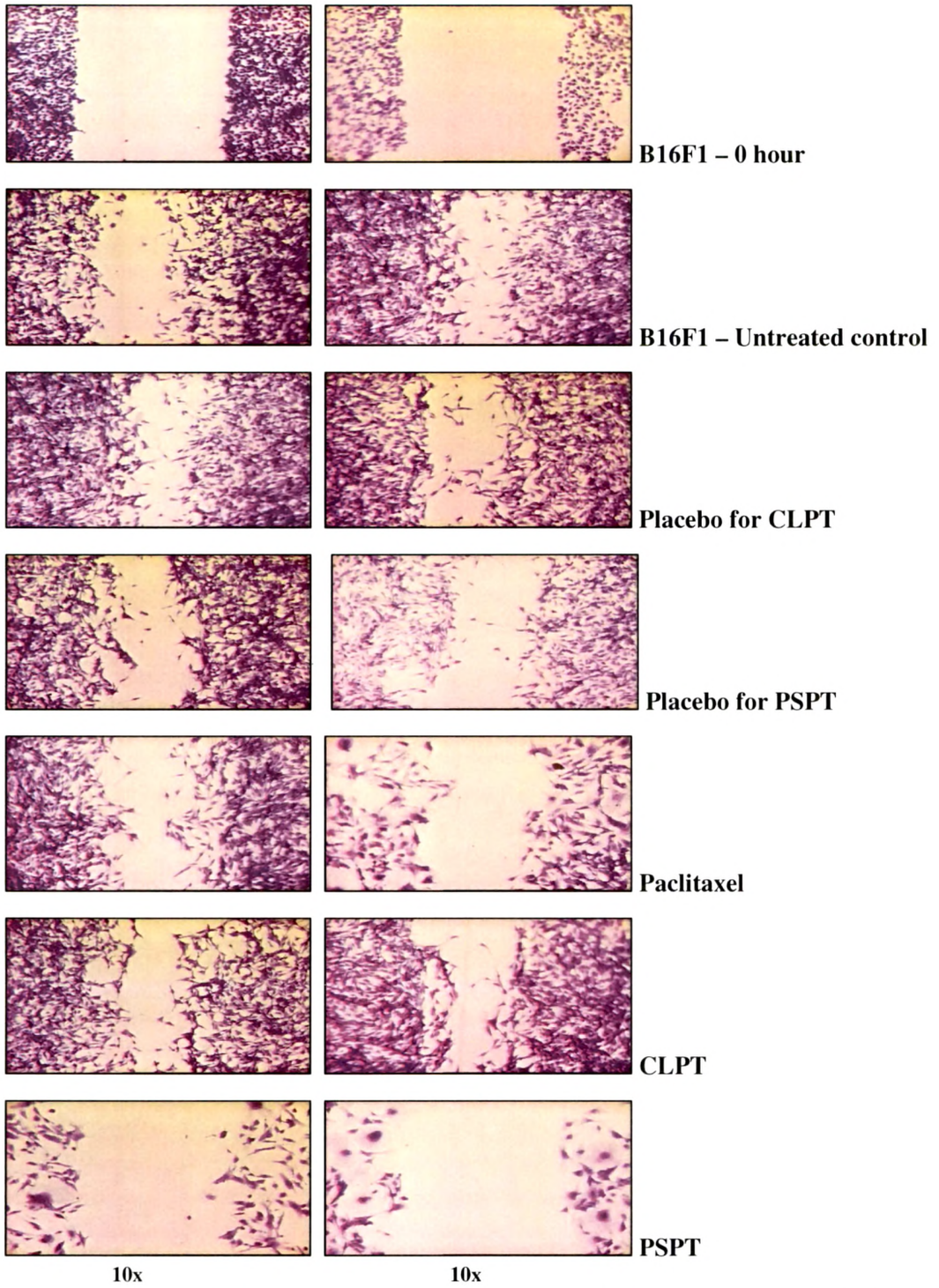


Figure 6.19. Wound assay for Paclitaxel and its liposomal formulations in B16F1 melanoma cells after 48h of incubation.

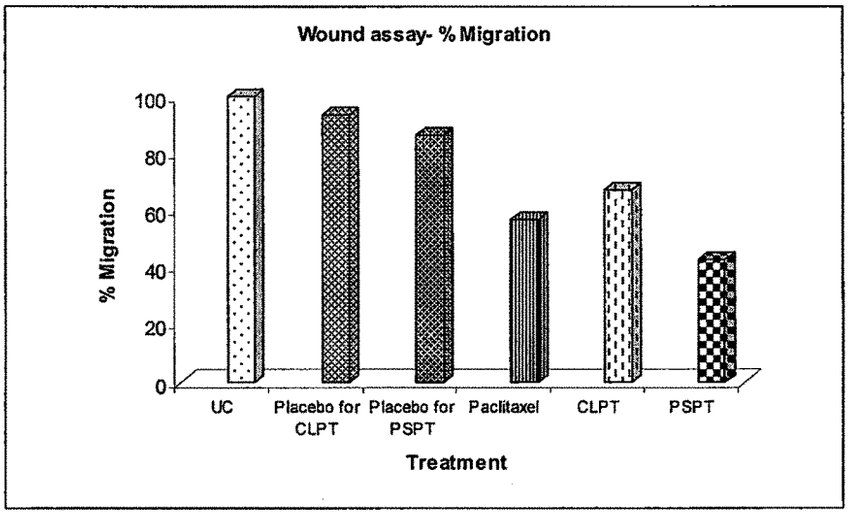


Figure 6.20. Effect of Paclitaxel and its liposomal formulations on cell migration in case of B16F1 melanoma cells after treatment for 48 hours.

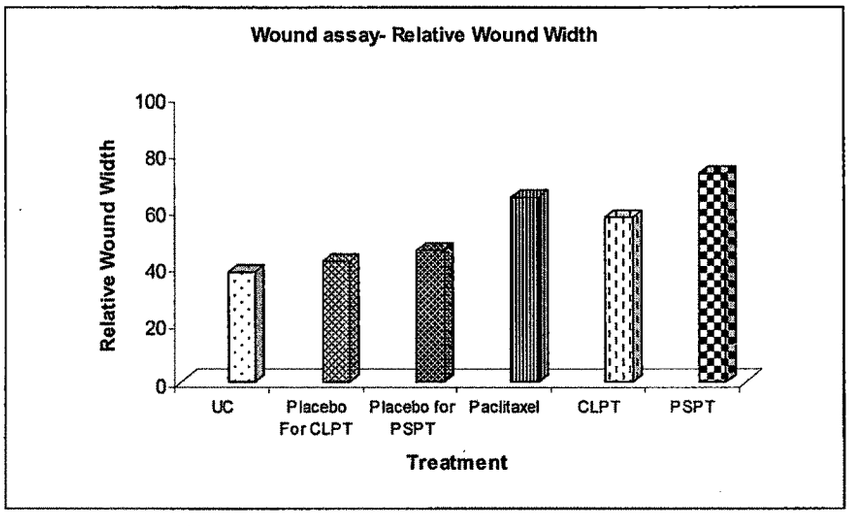


Figure 6.21. Effect of Paclitaxel and its liposomal formulations on relative wound width in case of B16F1 melanoma cells after treatment for 48 hours.

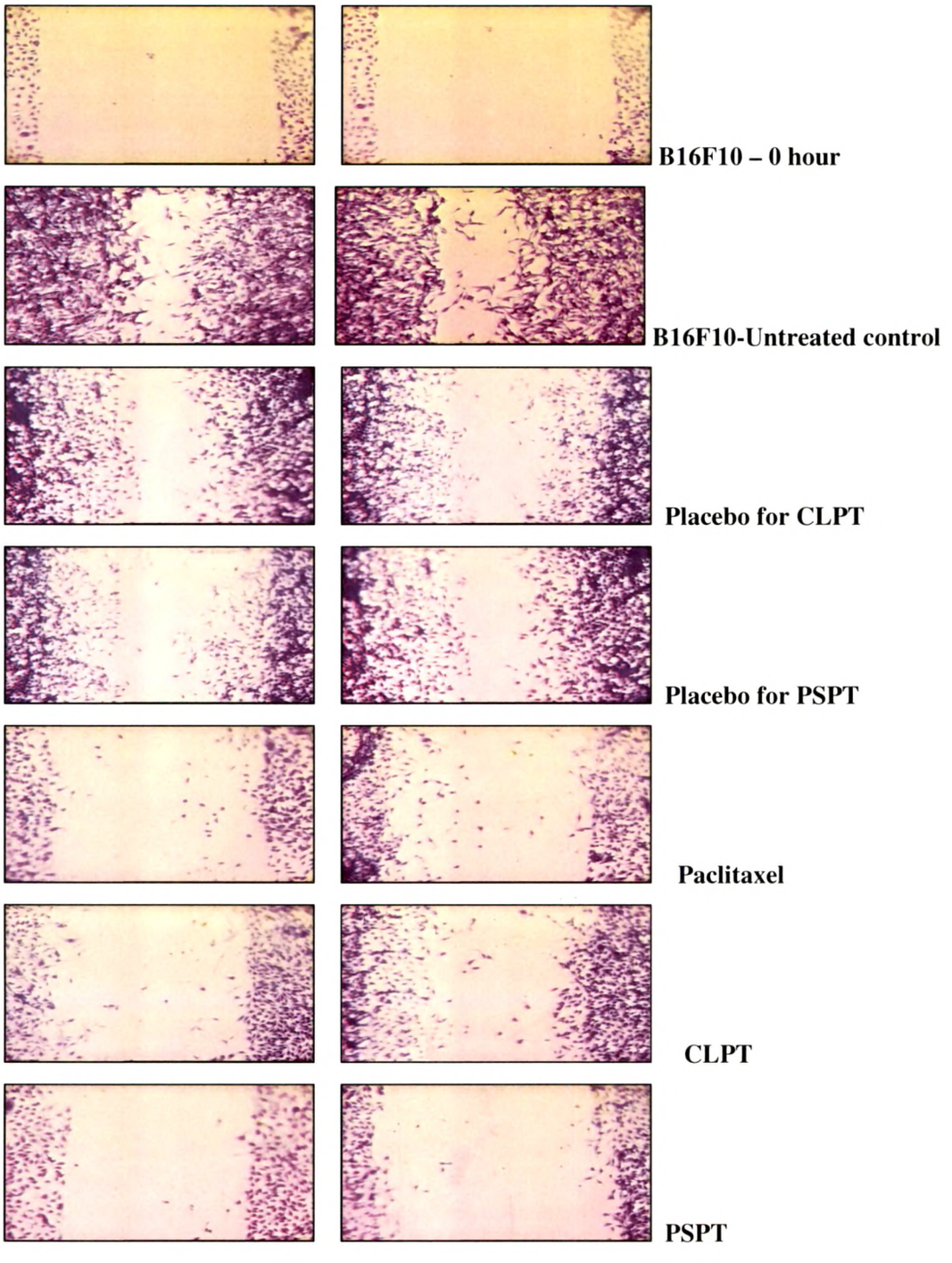


Figure 6.22. Wound assay for Paclitaxel and its liposomal formulations in B16F10 melanoma cells after 48 h of incubation.

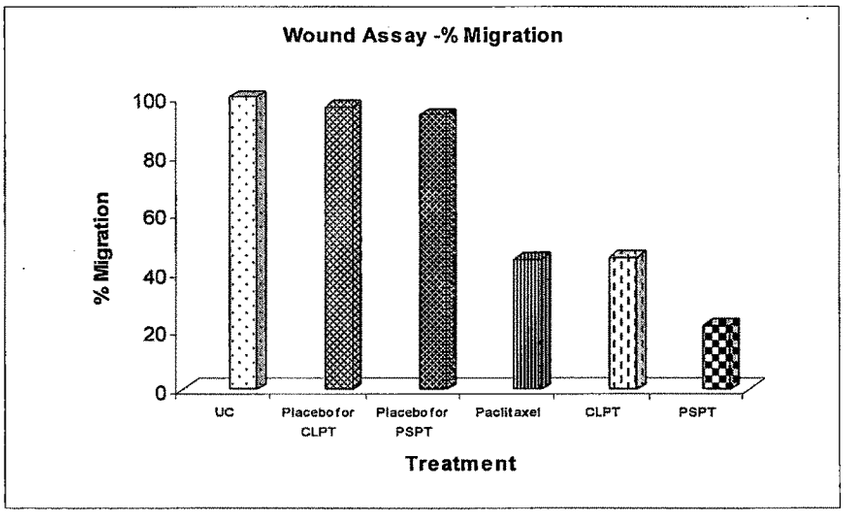


Figure 6.23. Effect of Paclitaxel and its liposomal formulations on cell migration in case of B16F10 melanoma cells after treatment for 48 hours.

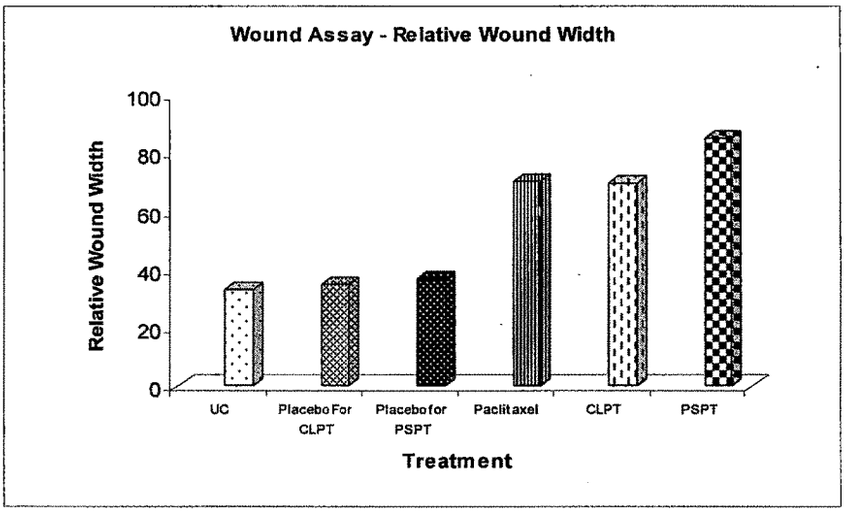


Figure 6.24. Effect of Paclitaxel and its liposomal formulations on relative wound width in case of B16F10 melanoma cells after treatment for 48 hours.

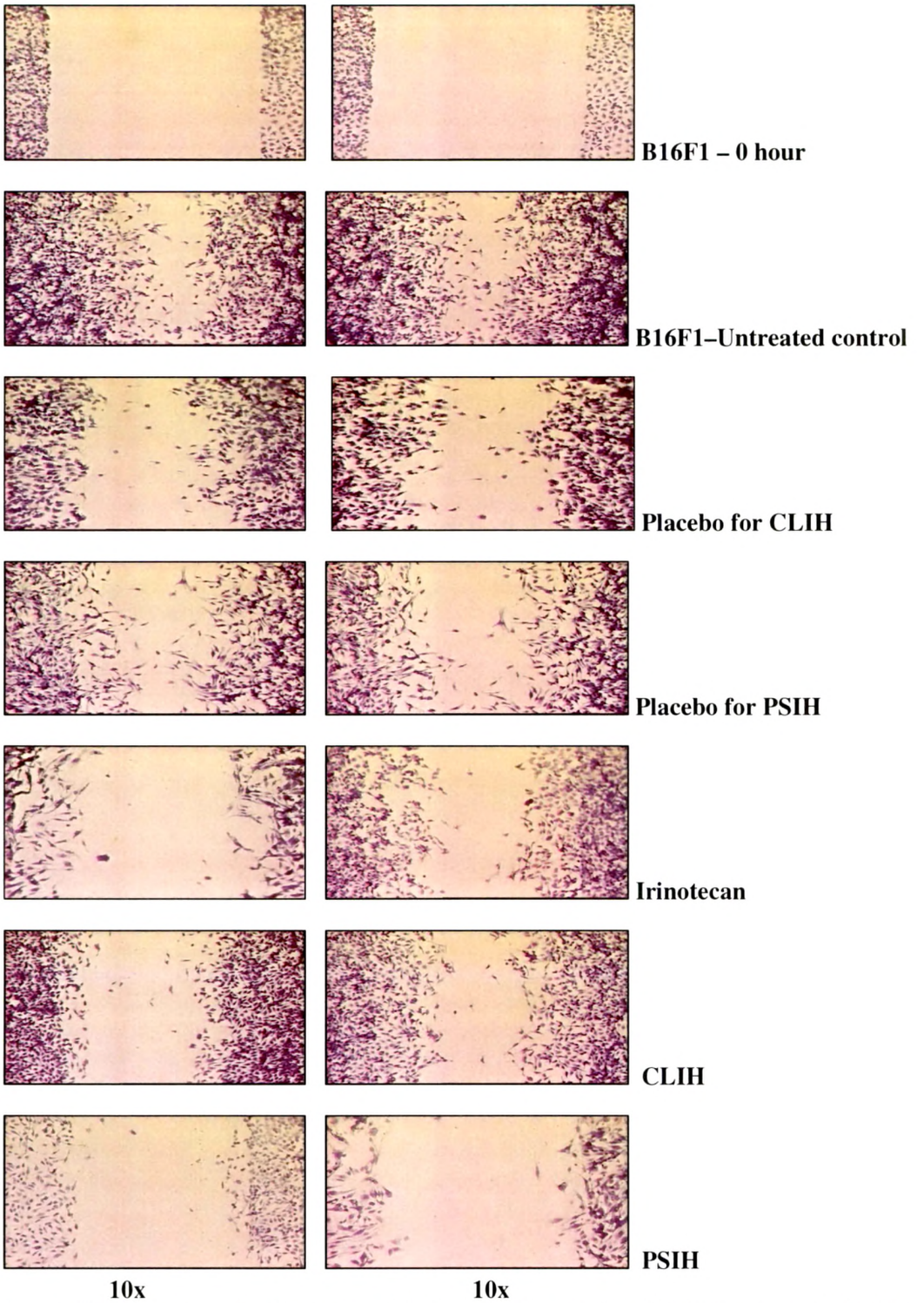


Figure 6.25. Wound assay for Irinotecan and its liposomal formulations in B16F1 melanoma cells after 48h of incubation.

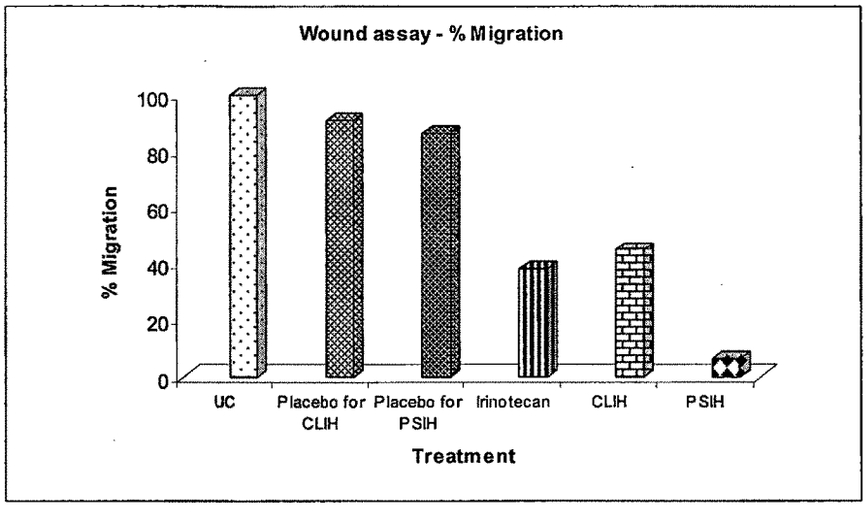


Figure 6.26. Effect of Irinotecan and its liposomal formulations on cell migration in case of B16F1 melanoma cells after treatment for 48 hours.

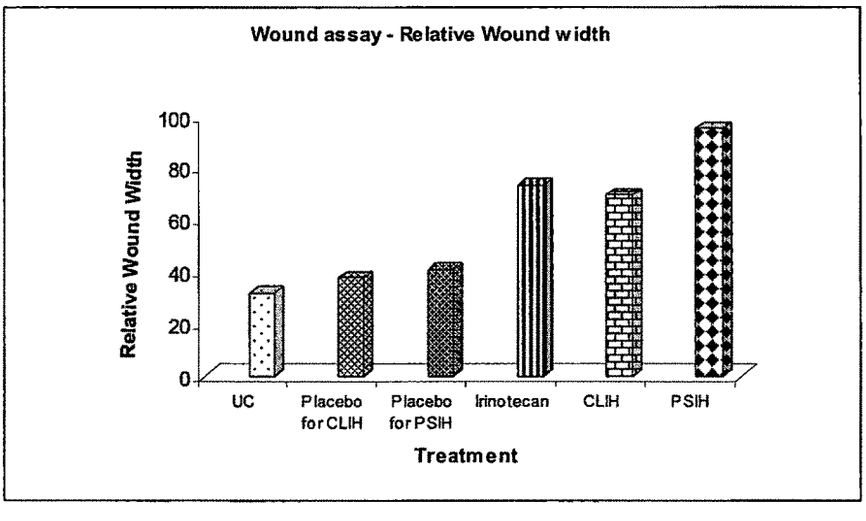


Figure 6.27. Effect of Irinotecan and its liposomal formulations on relative wound width in case of B16F1 melanoma cells after treatment for 48 hours.

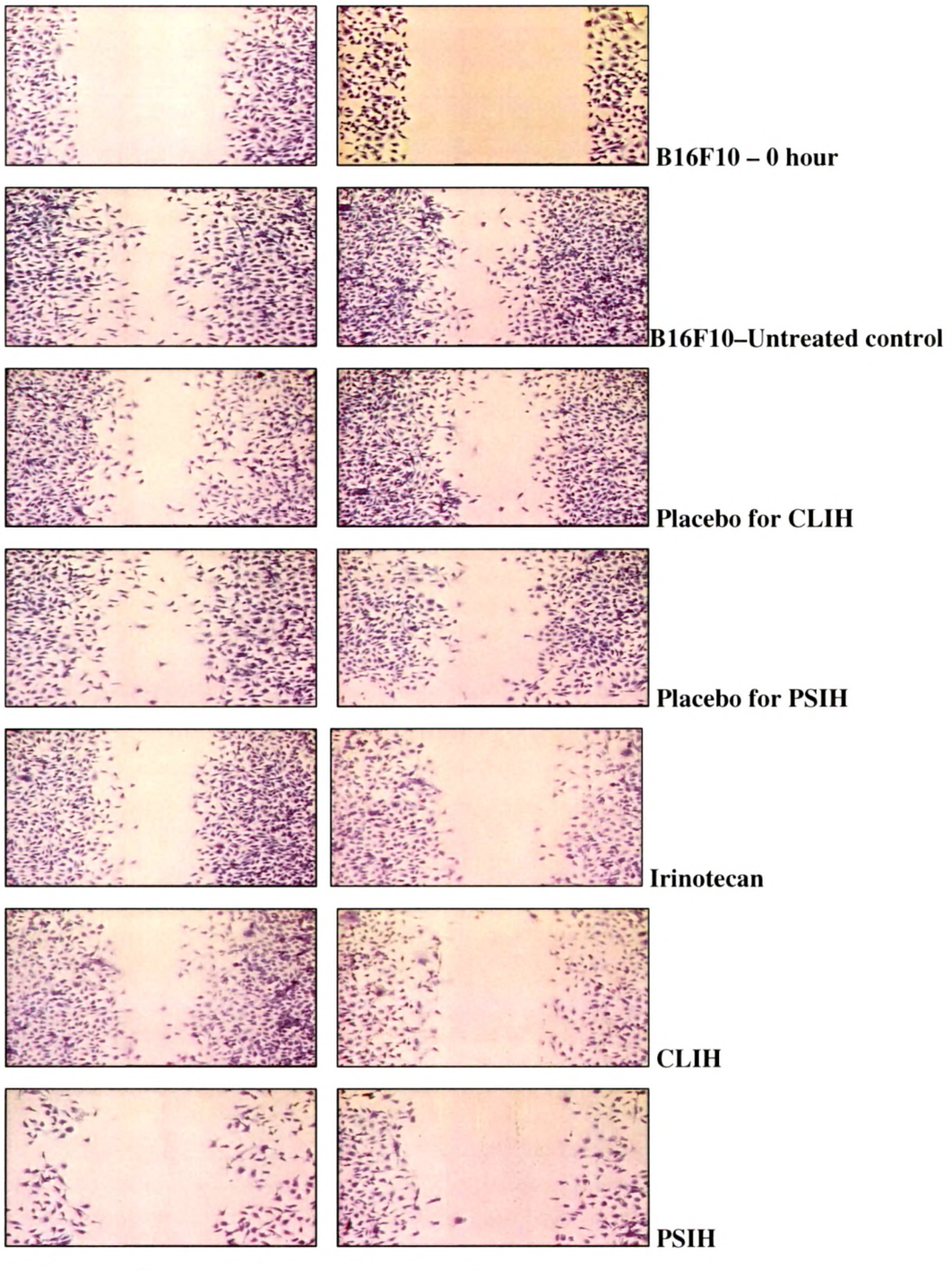


Figure 6.28. Wound assay for Irinotecan and its liposomal formulations in B16F10 melanoma cells after 48h of incubation.

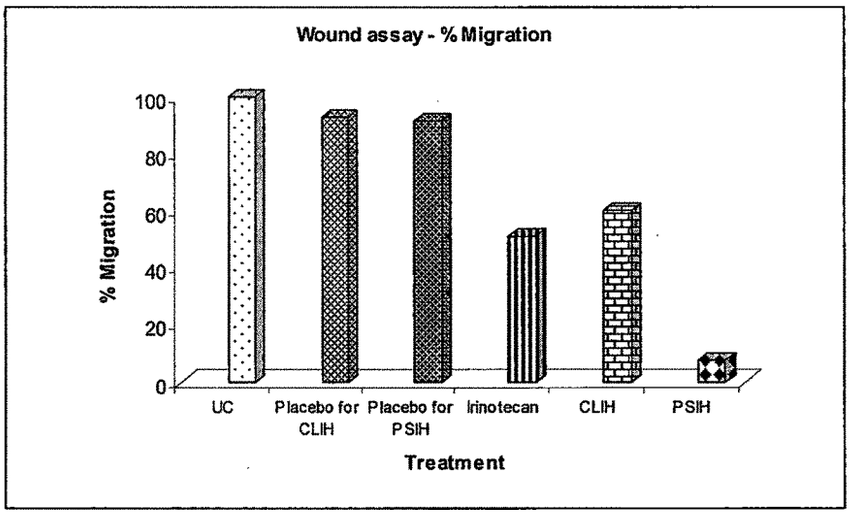


Figure 6.29. Effect of Irinotecan and its liposomal formulations on cell migration in case of B16F10 melanoma cells after treatment for 48 hours.

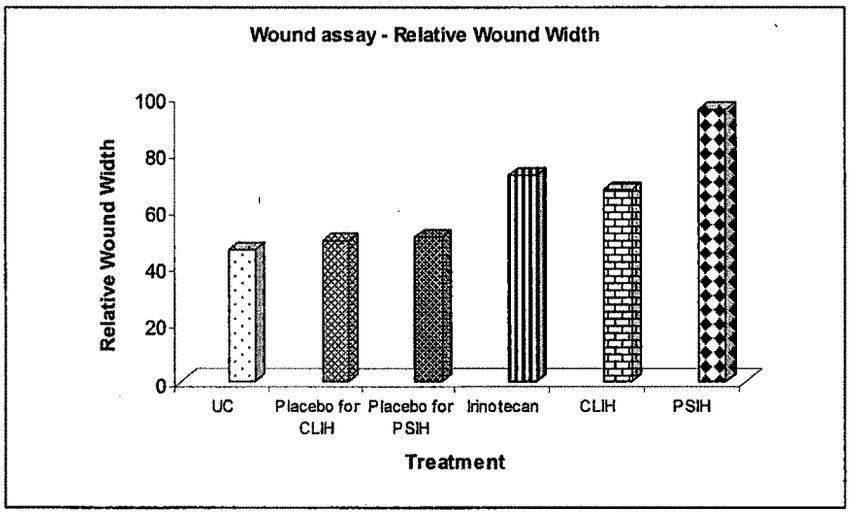


Figure 6.30. Effect of Irinotecan and its liposomal formulations on relative wound width in case of B16F10 melanoma cells after treatment for 48 hours.

6.7 Cell Cycle analysis

A dysregulation of the cell cycle components may lead to tumor formation. Some genes like the cell cycle inhibitors, RB, p53 etc., when they mutate, may cause the cell to multiply uncontrollably, forming a tumor. The cells which are actively undergoing cell cycle are targeted in cancer therapy as the DNA is relatively exposed during cell division and hence susceptible to damage by drugs or radiation.

Flow cytometry [Fluorescence Activated Cell Sorting (FACS) analysis]

Sub confluent B16F1 and B16F10 cells were treated with various concentrations of PCL/ IH and its liposomal formulations at a concentration below their respective IC_{50} values 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 equipped with a 488-nm argon ion laser and analyzed using Modfit (DNA Modeling System) software expressed as fractions of cells in the different cell cycle phases. Samples were run in duplicate and each experiment was repeated three times.

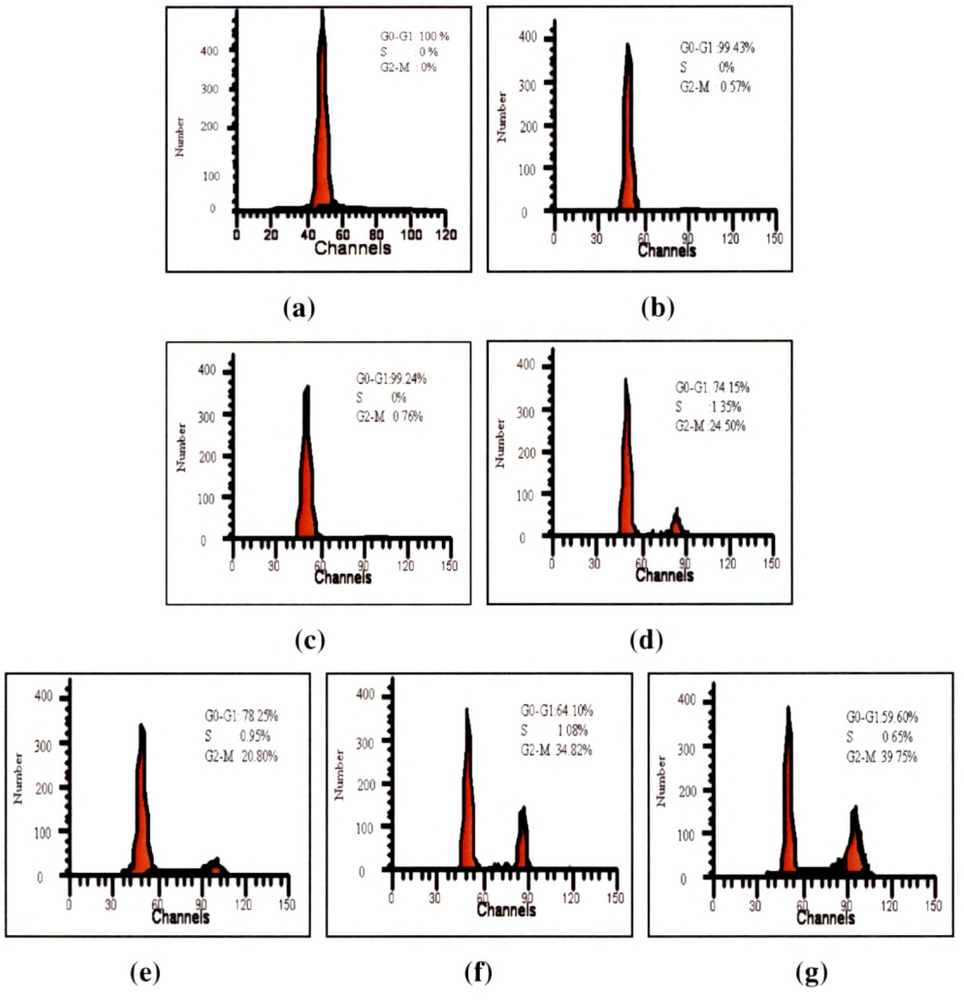


Figure 6.31. Cell cycle analysis of a) Untreated control, b) Placebo for CLPT, c) Placebo for PSPT, d) Paclitaxel, e) CLPT, f) and g) PSPT at concentrations below their respective IC_{50} values on B16F1 melanoma cells after 48 hours of treatment.

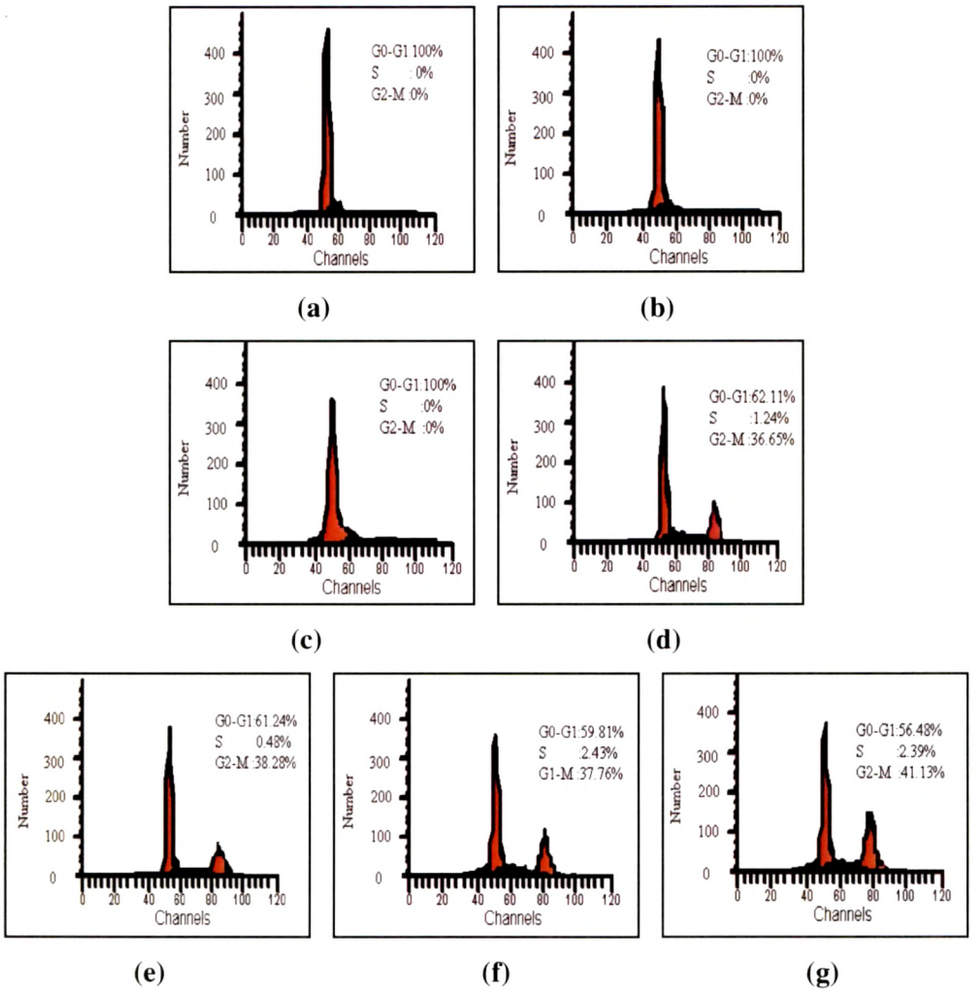


Figure 6.32. Cell cycle analysis of a) Untreated control, b) Placebo for CLPT, c) Placebo for PSPT, d) Paclitaxel, e) CLPT, f) and g) PSPT at concentrations below their respective IC_{50} values on B16F10 melanoma cells after 48 hours of treatment.

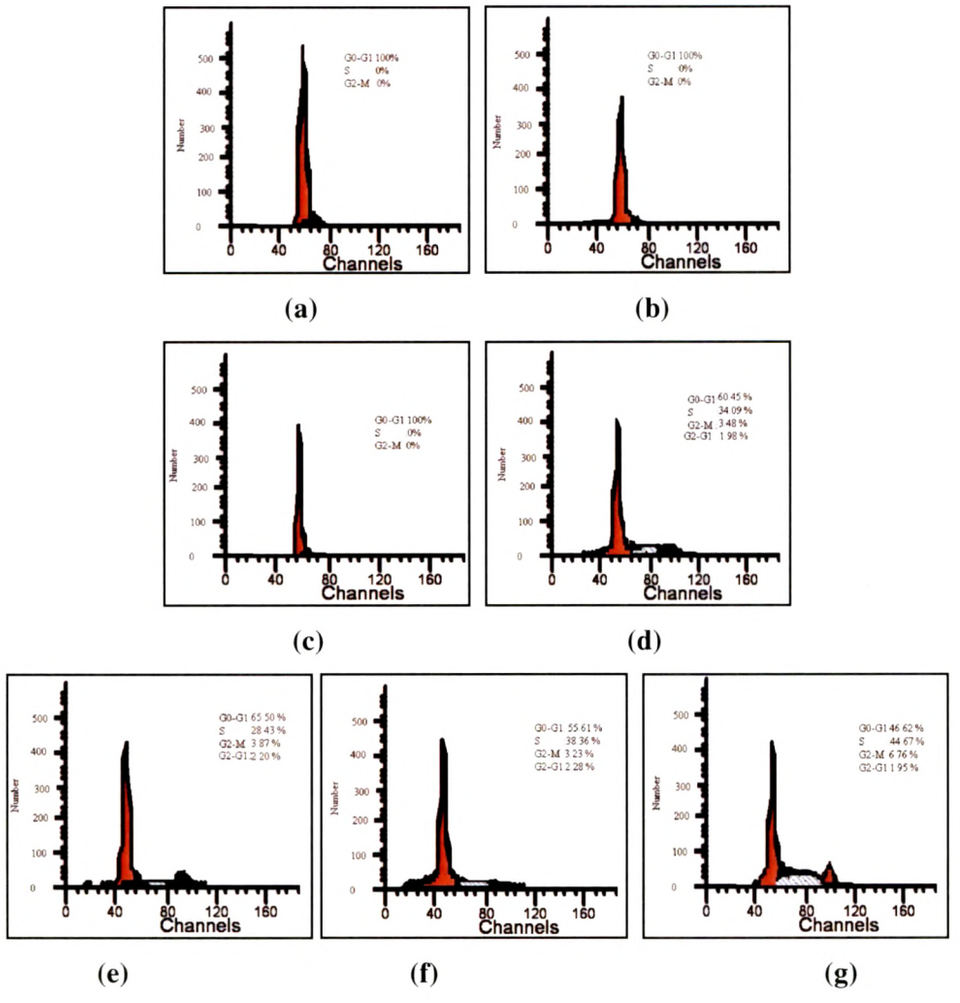


Figure 6.33. Cell cycle analysis of a) Untreated control, b) Placebo for CLIH, c) Placebo for PSIH, d) Irinotecan, e) CLIH, f) and g) PSIH at concentrations below their respective IC_{50} values on B16F1 melanoma cells after 48 hours of treatment.

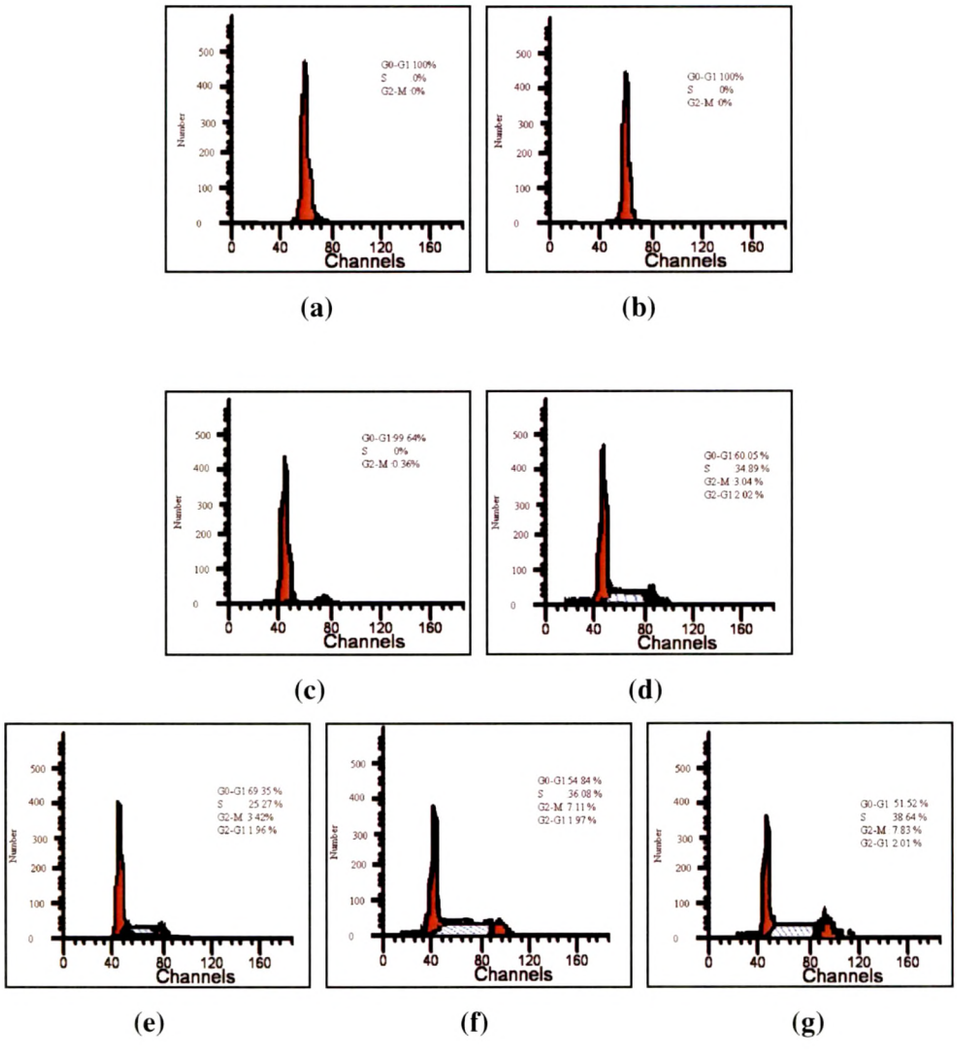


Figure 6.34. Cell cycle analysis of a) Untreated control, b) Placebo for CLIH, c) Placebo for PSIH, d) Irinotecan, e) CLIH, f) and g) PSIH at concentrations below their respective IC_{50} values on B16F10 melanoma cells after 48 hours of treatment.

6.8 RESULTS AND DISCUSSION

The in-vitro cytotoxicity study

The in-vitro cytotoxicity study was carried out as a preliminary study before performing the in-vivo biodistribution of the liposomal formulations containing anticancer drugs Paclitaxel (PCL) and Irinotecan (IH). This was carried out to evaluate the cytotoxic potential of drugs when encapsulated in conventional and pH sensitive liposomes.

Paclitaxel and its liposomal formulations

Cytotoxicity assay

In-vitro cytotoxicity assay was carried out on B16F1 (low metastatic) and B16F10 (highly metastatic) melanoma cell lines revealed that PCL loaded liposomal formulations maintained toxicity comparable with the PCL solution. The result showed that the PCL activity did not reduce in the presence of a lipid carrier. Similar observations have been reported when the anticancer drugs have been encapsulated in lipid carriers (Serpe, *et al.* 2004, Giacomo, *et al.* 2005). 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.

Various concentrations (0.001, 0.01, 0.1, 0.5, 5.0, 12.5. 25 μg/ml) of PCL and its liposomal formulations (CLPT and PSPT) were added to the 96 well flat bottomed plate containing B16F1 and B16F10 cells grown in the DMEM medium. The cytotoxicity was evaluated at 3 time points (24, 48 and 72 h). The cytotoxic effect of PCL was found (72% to 95 %) after 24 h, but appreciable amount of cytotoxicity was found after 48 h incubation whereas, no significant change was observed after 72 h when compared to 48h incubation effect. Hence, evaluation of the cytotoxic potential of the PCL liposomal formulations was carried out for 48 h.

The cytotoxic effect produced by PCL and its liposomal formulations after 48 h of incubation at 37 °C in a humidified incubator (5 % CO_2 and 95 % air) is shown in Table 6.1 and Table 6.2 for B16F1 and B16F10 cells respectively.

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. The cytotoxicity pattern produced by PCL and its liposomal formulations was almost similar and not much significant difference was observed between them in both the cell lines (Figure 6.1 and Figure 6.2).

IC_{50} values for PCL and its liposomal formulations were noted down by plotting the graph of % viability against log concentration and tabulated in the Table 6.3 for both the cell lines. Hundred percent toxicity was observed at 25 µg/ml for PCL and PSPT in case of B16F1 where as 25 µg/ml for PCL and 12.5 µg/ml for PSPT in case of B16F10 melanoma cells. This signifies that the PSPT is more effective than other treatments in the acidic environment of the cancer cells. It can also be concluded from the result that PSPT is more active in highly metastatic B16F10 melanoma cells than low metastatic B16F1 cell line. An increased cell growth inhibition of Paclitaxel was shown using liposomes as carrier (Cabanes et. al., 1998).

From the above study, two sub-toxic concentrations (less than their respective IC_{50} values) from each of PCL solution (0.01µg/ml and 0.1µg/ml), CLPT (0.1 µg/ml and 1.0 µg/ml) and PSPT (0.1 µg/ml and 1.0 µg/ml) were chosen for B16F1 cells and PCL solution (0.01µg/ml and 0.1µg/ml), CLPT (0.5 µg/ml and 5.0 µg/ml) and PSPT (0.1 µg/ml and 1.0 µg/ml) were chosen for B16F10 cells to perform the other following *in- vitro* cell line studies. To evaluate possible effect of formulation components, cells were also incubated with blank liposomes (placebos) at the highest lipid concentrations used for the study which was found to be very less toxic than the vehicle used to dissolve drug at the highest concentration used.

Leighton tube studies

Haematoxyline and Eosin stained cover slips containing B16F1 and B16F10 cells showed significant changes in the morphology of cells. Comparison of various morphological

characteristics of B16F1 and B16F10 melanoma cells on treatment with sub toxic concentrations of PCL solution, liposomal formulations and placebo preparations are shown in Figure 6.5 and Figure 6.6. The untreated samples have the cell membrane and the cellular contents intact. The cellular contents of placebo liposomal preparations treated cells remain intact, inferring that the carrier was non toxic. The cells treated with the sub toxic concentrations of PCL solution showed destruction of the cell wall, nucleus and cell protrusions which resulted in the formation of a large number of dendrites. Cells appeared spindle-shaped with some amount of hypertrophy as compared to control cells which were star-shaped and much more spread out. Similar morphological characteristics are observed with the cells treated with CLPT and PSPT in both the cell lines. The marked change in morphology of the cells treated with PSPT showed that activity of the drug was retained even at sub toxic concentrations.

Confocal microscopy

In-vitro cellular uptake and intracellular distribution of pH sensitive liposome encapsulating FITC-Dextran were evaluated qualitatively by confocal microscopy. Study revealed the cytosolic delivery of the pH sensitive liposomes loaded with fluorescent material. The pictures (Phase contrast and Fluorescent) were taken at different time points like 1h, 24h and 48 h were shown in Table 6.9 and Table 6.10 for both B16F1 and B16F10 melanoma cells. The fluorescent inside the cell was already marked after 1 h of incubation but increased accumulation of fluorescent material inside the cell was observed as the time of incubation increased which indicates the time dependent accumulation. The rapid internalization of fluorescent pH sensitive liposomes could be related to their narrow size distribution and bioacceptable lipid composition (Miglietta et. al., 2000)

Cell proliferation (Colony formation)

The colony inhibition effect of one of the sub-toxic concentration of PCL solution, PCL encapsulated conventional as well as pH sensitive liposomes and placebos for liposomal formulations at the end of 48 hours is described in Figure 6.11 and Figure 6.13 for B16F1 and B16F10 melanoma cells respectively. The colonies were counted depending upon the number of cells clustered to form it i.e. >50 cells or < 50 cells / colony (Figure 6.12 and

Figure 6.14). The colony inhibition study at the end of 48 h showed that the colony inhibition capacity of PSPT was comparable to that of the PCL solution, inferring that the colony inhibiting capacity of the PCL had not declined on encapsulation. The PSPT has shown better inhibition effect compared to PCL and CLPT in both the B16F1 and B16F10 cell lines after 48 h of treatment. In case of PSPT treated plates colonies were less in number and small colonies are observed compared to other treatments. The cell population in those colonies were also very less compared to colonies with other treatments. The percentage colony inhibition for the treatments were in the order of PSPT > PCL > CLPT > Placebos for liposomes. Anticancer drugs encapsulated in nanoparticles have also shown marked colony inhibiting capacities at sub toxic concentrations (Bisht, *et al.* 2007)

Wound assay

Wound assay is an easy method for the analysis of migration of cells (Griffioen and Molema, 2000). Figure 6.19 and Figure 6.22 describes the wounds assay pictures at 0 hour, for untreated control, one sub toxic dose of PCL, CLPT and PSPT along with its placebos for B16F1 and B16F10 cell lines. Percent migration and relative wound width for wounds treated with PCL, CLPT, PSPT and placebos for liposomal preparations after 48 h of treatment was calculated (Figure 6.20 and Figure 6.21 for B16F1 whereas Figure 6.23 and 6.24 for B16F10). 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. The wound widths at 48 h were found to be significantly larger for PSPT than PCL and CLPT ($p < 0.05$). This may be attributed to the pH sensitivity of the formulations and retarded release of PCL from liposomal formulation, which implied that sub-toxic concentrations produced a marked anti-migratory activity at 48 h.

Cell Cycle analysis

The PSPT arrested B16F1 and B16F10 melanoma cells in the G2-M phase of cell cycle similarly as that of plain PCL (Figure 6.31 and Figure 6.32). Analysis of DNA content of Propidium Iodide (PI) stained B16F1 and B16F10 melanoma cells showed significant dose-

dependent inhibition in the G_2-M phase transition on Paclitaxel and its liposomal formulations treatment, which resulted in a clear increase in the percentage of cells in G_2-M phase as compared to the control cells and placebos which are in G_0-G_1 phase in both the cell lines.

Irinotecan (IH) and its liposomal formulations

Cytotoxicity assay

In-vitro cytotoxicity assay was carried out on B16F1 (low metastatic) and B16F10 (highly metastatic) melanoma cell lines revealed that IH loaded liposomal formulations maintained toxicity comparable with the IH solution. The result showed that the IH activity did not reduce in the presence of a lipid carrier. Similar observations have been reported when the anticancer drugs have been encapsulated in lipid carriers (Serpe, *et al.* 2004, Giacomo, *et al.* 2005). As anticipated, the blank liposomal formulations did not produce any significant toxicity, which proves that the cytotoxicity caused by the liposomal IH is due to the released drug and not due to the any component of the liposomal bilayer.

Various concentrations (0.001, 0.01, 0.1, 0.5, 5.0, 12.5. 25 μg/ml) of IH and its liposomal formulations (CLIH and PSIH) were added to the 96 well flat bottomed plate containing B16F1 and B16F10 cells grown in the DMEM medium. The cytotoxicity was evaluated at 3 time points (24, 48 and 72 h). The cytotoxic effect of IH was found (69 % to 94 %) after 24 h, but appreciable amount of cytotoxicity was found after 48 h incubation whereas, no significant change was observed after 72 h when compared to 48h incubation effect. Hence, evaluation of the cytotoxic potential of the IH liposomal formulations was carried out for 48 h.

The cytotoxic effect produced by IH and its liposomal formulations after 48 h of incubation at 37 °C in a humidified incubator (5 % CO_2 and 95 % air) is shown in Table 6.4 and Table 6.5 for B16F1 and B16F10 cells respectively.

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 IH and its formulations (Figure 6.3 and Figure 6.4). The cytotoxicity pattern produced by IH and its liposomal formulations were almost similar and not much significant difference was observed between them in both the cell lines.

IC_{50} values for IH and its liposomal formulations were noted down by plotting the graph of % viability against log concentration and tabulated in the Table 6.6 for both the cell lines. Hundred percent of toxicity was observed at 25 for IH, CLIH and 12.5 μg/ml for PSIH in case of B16F1 where as 12.5 μg/ml for IH, CLIH and 5.0 μg/ml for PSIH in case of B16F10 melanoma cells. This signifies that the PSIH is more effective than other treatments in the acidic environment of the cancer cells. It can also be concluded from the result that PSIH is more active in highly metastatic B16F10 melanoma cells than low metastatic B16F1 cell line.

From the above study, two sub-toxic concentrations (less than their respective IC_{50} values) from each of IH solution (0.01μg/ml and 0.05μg/ml), CLIH (0.01 μg/ml and 0.1 μg/ml) and PSIH (0.01 μg/ml and 0.1 μg/ml) were chosen for B16F1 cells and IH solution (0.005μg/ml and 0.01μg/ml), CLIH (0.01 μg/ml and 0.1 μg/ml) and PSPT (0.01 μg/ml and 0.1 μg/ml) were chosen for B16F10 cells to perform the other following *in- vitro* cell line studies. To evaluate possible effect of formulation components, cells were also incubated with blank liposomes (placebos) at the highest lipid concentrations used for the study. The vehicle used to dissolve drug did not show any toxicity at the highest concentration at which it was used.

Leighton tube studies

Haematoxyline and Eosin stained cover slips containing B16F1 and B16F10 cells showed significant changes in the morphology of cells. Comparison of various morphological characteristics of B16F1 and B16F10 melanoma cells on treatment with sub toxic concentrations of IH solution, liposomal formulations and placebo preparations are shown

in Figure 6.7 and Figure 6.8. The untreated samples have the cell membrane and the cellular contents intact. The cellular contents of placebo liposomal preparations treated cells remain intact, inferring that the carrier was non toxic at the concentration used. The cells treated with the sub toxic concentrations of IH solution showed destruction of the cell wall, nucleus and cell protrusions which resulted in the formation of a large number of dendrites. Cells appeared spindle-shaped with some amount of hypertrophy as compared to control cells which were star-shaped and much more spread out. Similar morphological characteristics are observed with the cells treated with CLIH and PSIH in both the cell lines. The marked change in morphology of the cells treated with PSIH showed that the activity of the drug was retained even at sub toxic concentrations.

Cell proliferation (Colony formation)

The colony inhibition effect on treating with one of the sub-toxic concentration of IH solution, IH encapsulated conventional as well as pH sensitive liposomes and placebos for liposomal formulations at the end of 48 hours is described in Figure 6.15 and Figure 6.17 for B16F1 and B16F10 melanoma cells respectively. The colonies were counted depending upon the number of cells clustered to form it i.e. >50 cells or < 50 cells / colony (Figure 6.16 and Figure 6.18). The colony inhibition study at the end of 48 h showed that the colony inhibition capacity of PSIH was comparable to that of the IH solution, inferring that the colony inhibiting capacity of the IH had not declined on encapsulation. The PSIH has shown better inhibition effect compared to IH and CLIH in both the B16F1 and B16F10 cell lines after 48 h of treatment. In case of PSIH treated plates colonies were less in number and small colonies are observed compared to other treatments. The cell population in those colonies were also very less compared to colonies with other treatments. The percentage colony inhibition for the treatments were in the order of PSIH > IH > CLIH > Placeboes for liposomes.

Wound assay

An easy method for the analysis of migration of cells is Wound assay (Griffioen and Molema, 2000). Figure 6.25 and Figure 6.28 describes the wounds assay pictures at 0 hour, for untreated control, one sub toxic dose of IH, CLIH and PSIH along with its placebos.

Percent migration and relative wound width for wounds treated with IH, CLIH, PSIH and placebos for liposomal preparations after 48 h of treatment was calculated (Figure 6.26 and 6.27 for B16 F1 and Figure 6.29 and Figure 6.30 for B16F10 cells). IH, CLIH and PSIH 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 > CLIH > IH > PSIH where as relative wound width was in the order PSIH > IH > CLIH > Placebos for liposomes when compared with the 0 h wound reading. The wound widths at 48 h were found to be significantly larger for PSIH than IH and CLIH ($p < 0.05$). This may be attributed to the pH sensitivity of the formulations and retarded release of IH from liposomal formulation, which implied that sub-toxic concentrations produced a marked anti-migratory activity at 48 h.

Cell Cycle analysis

The PSIH arrested B16F1 and B16F10 melanoma cells in the S-phase of cell cycle similarly as that of plain IH (Figure 6.33 and 6.34). Analysis of DNA content of Propidium Iodide (PI) stained B16F1 and B16F10 melanoma cells showed significant dose-dependent inhibition in the S- phase transition on Irinotecan and its liposomal formulations treatment, which resulted in a clear increase in the percentage of cells in s-phase as compared to the control cells and placebos which are in G_0-G_1 phase in both the cell lines.

6.9 Conclusion

In-vitro cell line studies revealed that encapsulation of PCL or IH in liposomes did not affect cytotoxicity, anti-proliferative activity and colony inhibiting property. It was also proved that encapsulated drugs showed same kind of morphological changes and cell cycle arrest as that of the plain PCL or IH in both the cell lines. The pH sensitive liposomes encapsulating paclitaxel and irinotecan 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 and IH loaded pH sensitive liposomes can be considered as promising system for *in-vivo* anti-cancer delivery. Further *in-vivo* studies carried on the system can provide vital information in the potency of the pH sensitive liposomes as a potential carrier for the delivery of neoplastic agents.

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