Chapter 9
In-vivo Antimetastatic Activity

9.1 Evaluation of antimetastatic activity of pH-sensitive Liposomes of PCL and IH in C57BL/ 6 mice using B16F10 melanoma model.

Animals:

Six to eight weeks old inbred C57BL/6 pathogen free male mice were obtained from Animal House of ACTREC, Kharghar, Navi Mumbai. The animals were housed in well ventilated cages kept in air-conditioned rooms (21 ± 2 °C) during the experiment with 24h day & night cycle. Animals were fed with food pellets and water ad libidum. All the experiments were performed according to the rules and regulations of Animal Ethics Committee.

Figure 9.1. C57BL/6 mice

Melanoma cells:

B16F10 melanoma cells were obtained from the National animal cell repository at NCCS, Pune, India. The cell lines were regularly grown in Iscove's Modified Dulbecco's Medium (IMDM, GIBCO-BRL, Maryland) with non essential amino acids supplemented with 10% heat inactivated fetal bovine serum, FBS (JRH BioSciences), 100 units/ml penicillin and 100 mg/ml streptomycin. The cell lines were maintained at 37°C in a 5% CO_2 humidified atmosphere. For all the experiments, cells were grown in 10% media and the experiments were performed when the cells were approximately 80-90% confluent.

Cells were harvested using saline EDTA from culture plates. The viability of the cell was assessed by Tryphan blue exclusion.

9.2 Tail vein metastasis assay

Each plate containing semiconfluent B16F10 cells were pretreated with PCL / IH and their liposomal formulations with concentration below their respective IC_{50} values (sub-toxic level) for 48 hours. C57BL/6 mice were divided into six groups, each comprising of six animals for each drug. To produce experimental metastasis, C57BL/6 mice were injected intravenously with $1x10^5$ cells (control and formulation treated) in 0.1 ml PBS via tail veins. After 20 days, the mice were sacrificed, their lungs were resected and photographs were taken before fixation in buffered formalin/Bouin's solution. The numbers of metastatic nodules on the surface of the lungs were counted.

The treatments given to six groups were as follows:

- Group 1: Untreated control.
- Group 2: Placebo for conventional liposomes of PCL / IH.
- Group 3: Placebo for pH-sensitive liposomes of PCL / IH.
- Group 4: Plain drug solution of PCL / IH.
- Group 5: Conventional liposomes of PCL / IH.
- Group 6: pH-sensitive liposomes of PCL / IH.

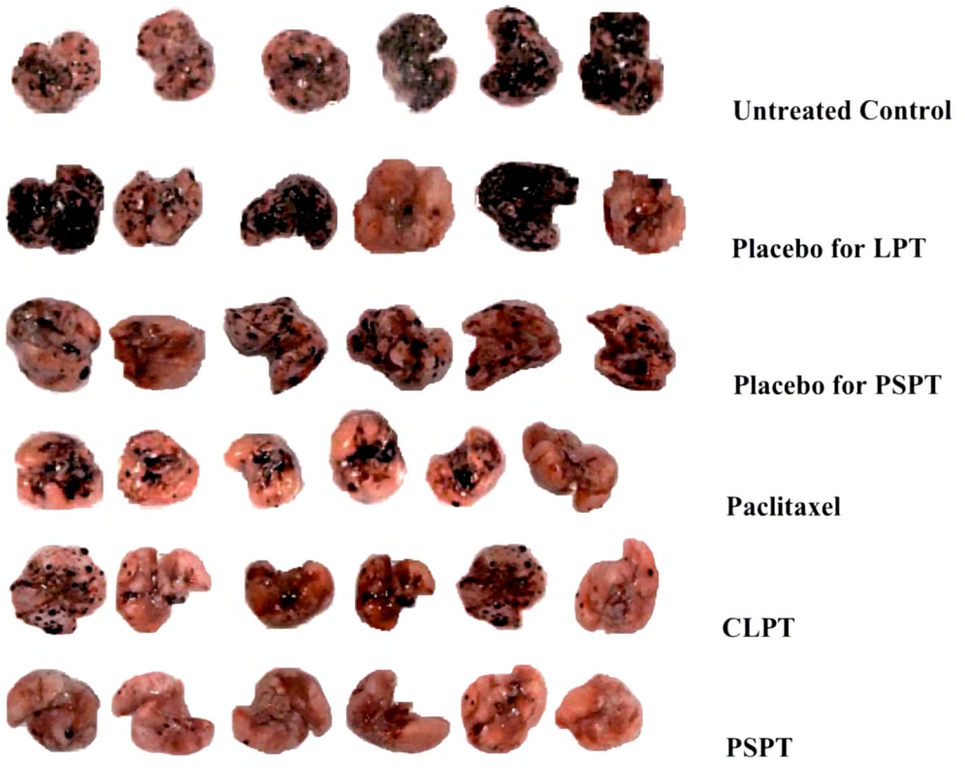

Figure 9.2. Representative example of lungs of C57BL/6 mice showing metastatic colonies formed on i.v. injection of Paclitaxel and its liposomal formulations treated B16F10 melanoma cells.

Table 9.1. *In-vivo* antimetastatic activity of Paclitaxel and its liposomal formulations in C57BL/6 mice.

Formulations	Average number of metastatic colonies	% Inhibition in lung nodule formation
Untreated	198	--
Placebo for CLPT	176	11.11
Placebo for PSPT	164	17.17
Paclitaxel (PCL)	37	81.31
CLPT	43	78.28
PSPT	9	95.45

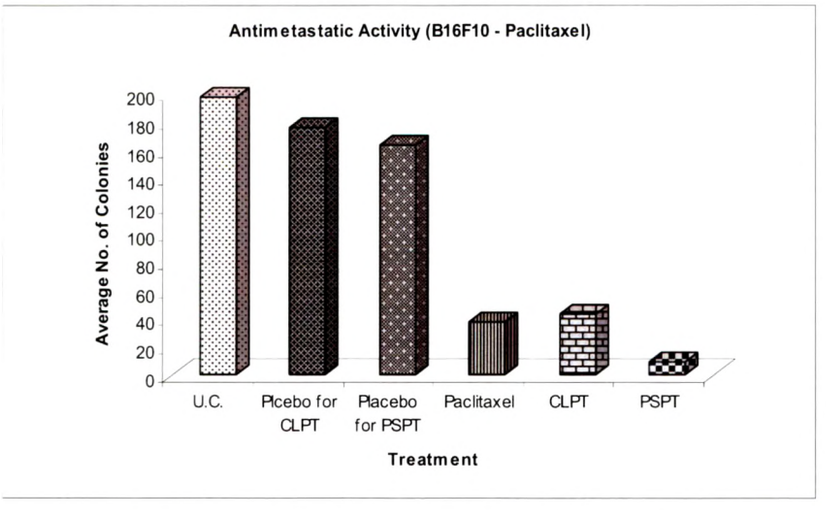

Figure 9.3. Average number of colonies found on lungs of C57 BL/6 mice after B16F10 melanoma cells treated with paclitaxel and its liposomal formulations. ($p < 0.05$)

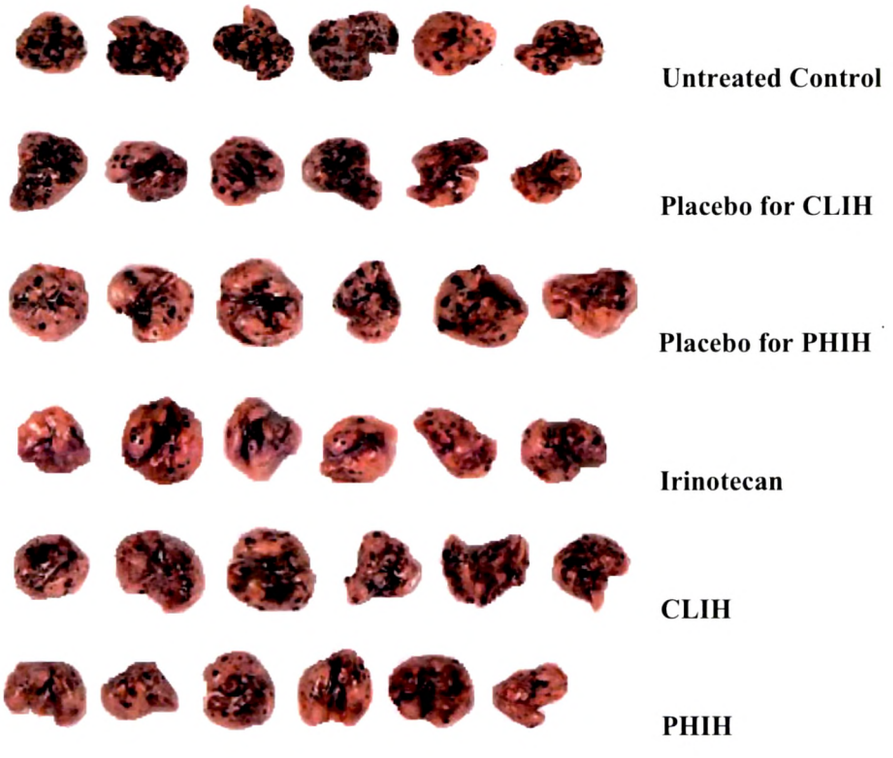

Figure 9.4. Representative example of lungs of C57BL/6 mice showing metastatic colonies formed on i.v. injection of Irinotecan and its liposomal formulations treated B16F10 melanoma cells.

Table 9.2. ***In-vivo*** **antimetastatic activity of irinotecan and its liposomal formulations in C57BL/6 mice.**

Formulations	Average number of metastatic colonies	% Inhibition in lung nodule formation
Untreated	173	--
Placebo for CLIH	158	8.67
Placebo for PSIH	143	17.34
Irinotecan	64	63.01
CLIH	80	53.76
PSIH	47	72.83

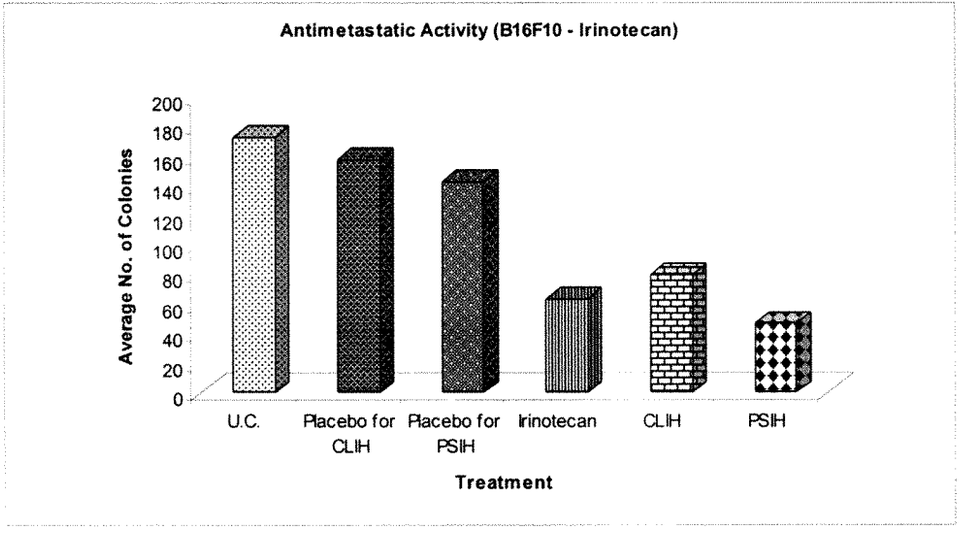

Figure 9.5. Average number of colonies found on lungs of C57 BL/6 mice after B16F10 melanoma cells treated with Irinotecan and its liposomal formulations. ($p < 0.05$)

9.3 Histology

Tissues were fixed in buffered formalin for 24–48 h. After washing in fresh PBS, fixed tissues were processed and embedded in paraffin. Sections (5 milli micron) were collected on microscope slides, deparaffinized and stained with Haematoxyline & Eosin. The images were captured at 10X and 40X magnifications.

Table 9.3. Summary of microscopic findings in lung histology of animals treated with PCL and its liposomal formulations.

Sr. no.	Group and Treatment		Number of animal showing microscopic change					
			U.C.	Placebo for CLPT	Placebo for PSPT	PCL	CLPT	PSPT
	Number of animals examined		6	6	6	6	6	6
	NAD		0	0	0	4	2	4
1.	Area of metastasis	Minimal	-	-	4	2	2	-
		Mild	-	4	-	-	-	-
		Moderate	-	-	2	-	-	-
		Severe	6	2	-	-	-	-
2.	Melanocytes with pigment	Minimal	-	-	4	2	4	2
		Mild	-	2	-	-	-	-
		Moderate	2	2	2	-	-	-
		Severe	4	2	-	-	-	-
3.	Macrophages with pigment	Minimal	-	-	4	-	2	2
		Mild	-	4	2	-	-	-
		Moderate	6	-	-	-	-	-
		Severe	-	2	-	-	-	-
4.	Mitotic figures	Minimal	-	-	4	2	2	-
		Severe	6	6	2	-	-	-
5.	Pleomorphism	Minimal	-	-	4	2	2	-
		Severe	6	6	2	-	-	-
6.	Epitheloid cells	Minimal	-	-	4	2	2	-
		Severe	6	6	2	-	-	-

"-" Examined but no pathological finding.

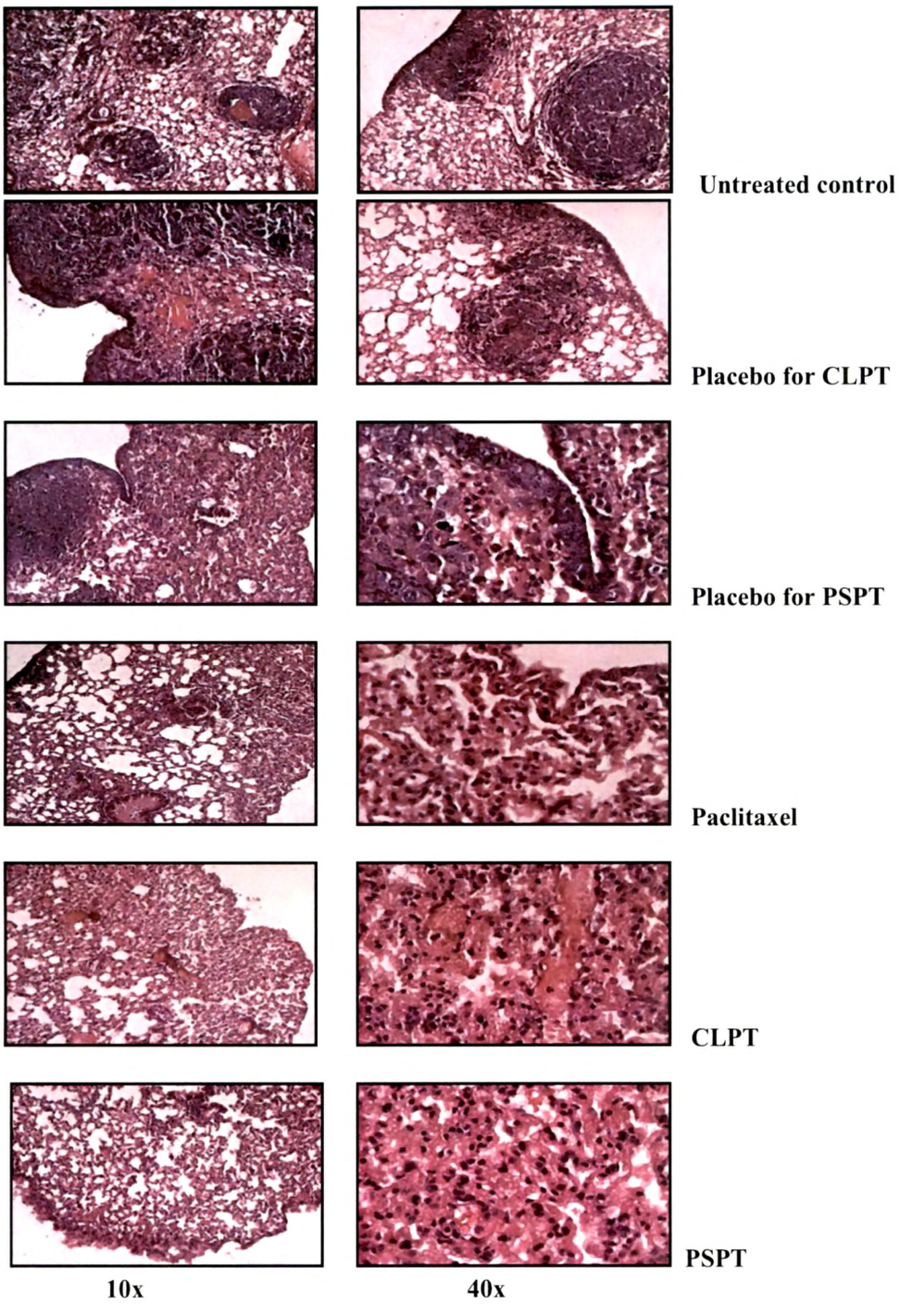

Figure 9.6. Histopathological pictures of lung sections of C57BL/6 mice after treatment with PCL and its liposomal formulations.

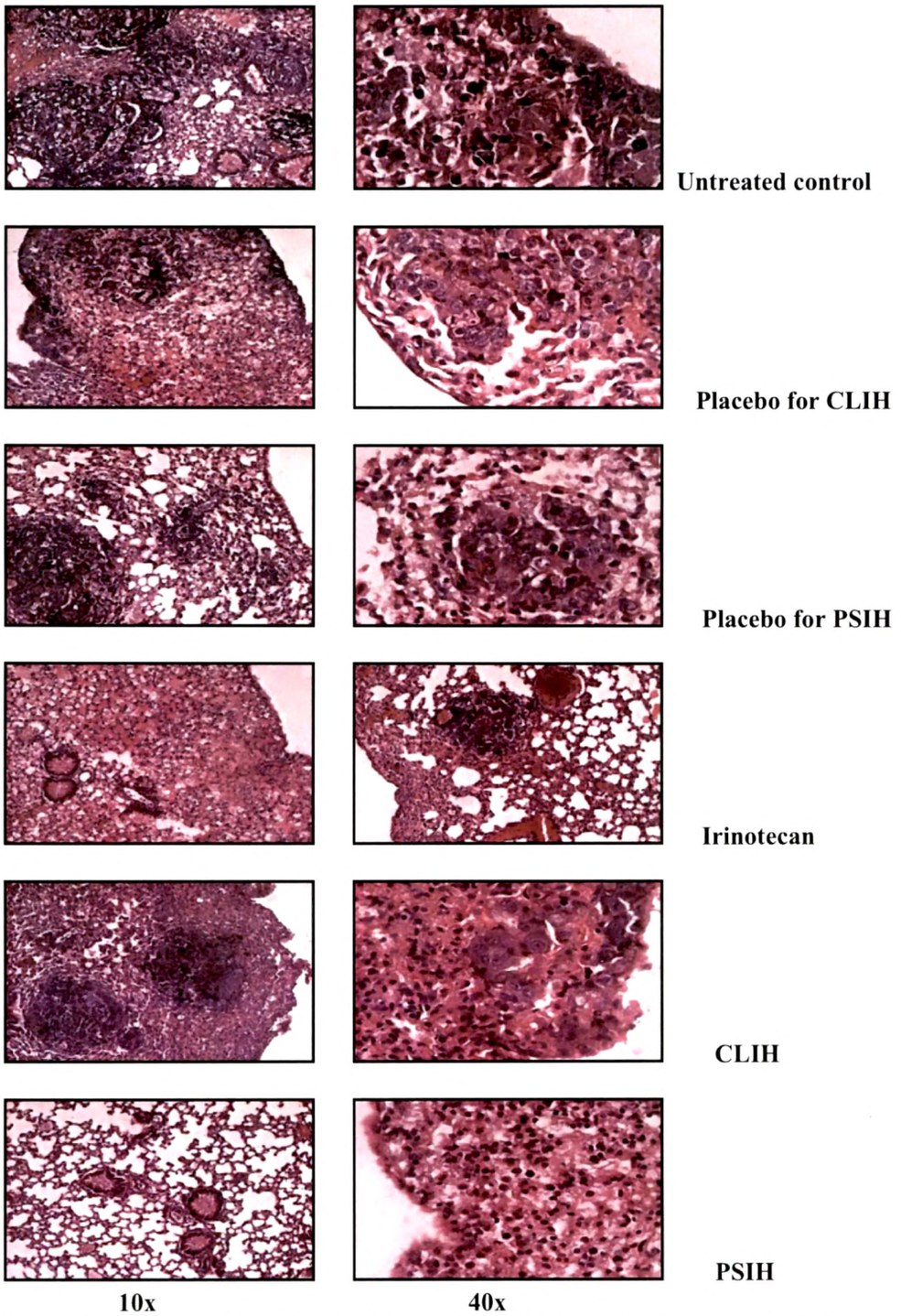

Figure 9.7. Histopathological pictures of lung sections of C57BL/6 mice after treatment with Irinotecan and its liposomal formulations.

Table 9. 4. Summary of microscopic findings in lung histology of animals treated with IH and its liposomal formulations.

Sr. no	Group and Treatment		Number of animals showing microscopic change					
			U.C	Placebo for CLIH	Placebo for PSIH	IH	CLIH	PSIH
	Number of animals examined		6	6	6	6	6	6
	NAD		0	0	0	2	0	4
1.	Area of metastasis	Minimal	-	1	4	-	-	1
		Mild	2	4	-	-	2	-
		Moderate	2	1	2	2	4	-
		Severe	2	-	-	2	-	-
2	Melanocytes with pigment	Minimal	-	2	4	-	-	2
		Mild	-	4	-	2	4	-
		Moderate	2	-	2	-	2	-
		Severe	4	-	-	2	-	-
3.	Macrophages with pigment	Minimal	2	4	-	2	-	-
		Mild	2	-	2	-	4	-
		Moderate	2	-	-	2	2	-
4.	Mitotic figures	Minimal	-	-	2	-	-	2
		Mild	-	-	2	-	4	-
		Moderate	2	4	2	2	2	-
		Severe	4	-	-	2	-	-
5	Pleomorphism	Minimal	-	-	2	-	-	-
		Mild	2	-	2	-	4	-
		Moderate	-	4	2	2	2	-
		Severe	4	-	-	2	-	-
6.	Epitheloid cells	Minimal	-	-	2	-	-	-
		Mild	2	-	-	-	4	-
		Moderate	-	4	2	-	2	-
		Severe	4	-	-	2	-	-

"-" Examined but no pathological finding.

9.4 RESULTS AND DISCUSSION

The most deadly aspect of cancer is its ability to spread, or metastasize. Cancer cells initially group together to form a primary tumor. Once the tumor is formed, cells may begin to break off from this tumor and travel to other parts of the body. This process is metastasis. These cancer cells that travel through the body are capable of establishing new tumors in locations remote from the site of the original disease. Metastasis is a very

complicated process that still has yet to be completely understood. Research is now focused on understanding in what ways cancer cells have mutated to circumvent the body's defenses and freely travel to other locations. Cancers which have metastasized usually indicate a later stage disease, and treatment becomes more complicated, with poorer outcomes. Metastasis most commonly occurs by way of the bloodstream or the lymphatic system. Just like normal cells, cancer cells must have a blood supply in order to function. They have access to the bloodstream just as healthy cells do. This access allows detached malignant cells from the tumor to enter the bodies' general bloodstream. Once in the bloodstream, the cancer cells now have access to every portion of the body. Once metastasis to the lymphatic system has occurred, then the prognosis for cure drops significantly.

Literature survey revealed that the intravenous treatment of some drugs likes PEG-catalase (Hyoudou et. al., 2004), pentoxifylline demonstrated inhibition in the lung homing (Gude et. al., 1996), reduce spontaneous metastasis (Ambrus et al., 1991) and strong inhibition of lung metastasis of B16F10 melanoma cells (Futakuchi et al., 2004). B16F10 cells pretreated for two hours with pentoxifylline, showed decreased ability of the cells homing lung, as compared to untreated cells (Aparna et. al., 2007). Nearly all tumor cells injected i.v. lodge in the lung capillary bed in the first few minutes after injection (Fidler, 1978) and very few (1%) actually form colonies, depending upon the interaction between tumor and endothelial cells. The determining factors like survival, adhesion, invasion or proliferation capacity of pretreated B16F10 melanoma cells in the target organ will change the destiny of the metastatic cancer. Therefore, B16F10 cells were grown in culture in the presence of 10 % serum and treated with drugs and their liposomal formulations before injection in mice to evaluate antimetastatic activity.

***In-vivo* antimetastatic activity of Paclitaxel and its liposomal formulations**

The B16F10 melanoma cells were cultured and treated with sub-toxic (below IC_{50} value) level of PCL and its liposomal formulations (CLPT and PSPT). Placebos for liposomal formulations were also used at the highest concentration of lipid. Post treatment, cell viability was taken using Trypan Blue dye exclusion test and equal numbers of cells (1 x

10^5 cells per mouse) were injected into mice. 20 days later lungs were resected and analysed for metastasis. Resected lungs were weighed and values were noted down. It was observed that pretreatment with PCL and its liposomal formulations significantly inhibited the lung homing of B16F10 cells as seen by decreased number of colonies on the lung compared to untreated control ($p < 0.05$). The lung colonies or metastatic nodules formed on the surface were manually counted for each animal. The representative examples of the lungs are shown in Figure 9.2. The average number of colonies for different formulation treatment (Figure 9.3) and percentage inhibition in lung nodule formation was calculated (Table 9.1). PSPT inhibited the lung nodule formation by 95.45 %. It was found that inhibition of lung nodule formation was in the order of PSPT > PCL > CLPT > placebo preparations. It is evident from the above data that the pretreated B16F10 melanoma cells with PCL and its liposomal formulations inhibited lung homing and thereby enhanced antimetastatic activity. The results obtained are concurrent with the literature surveyed.

Histology

Haematoxyline and Eosin stained sections of the lungs were microscopically observed and photographed. The histopathological micrographs were shown in Figure 9.6. It is important to characterize the histological features and the subtype of tumor, as this predicts biological behavior and cancer-specific survival rate. The metastatic melanoma is characterized by cells with large, irregular nuclei with abundant cytoplasm, nuclear and cytoplasmic pleomorphism, hyper pigmentation, ploidy, epitheloid cells and abundant mitotic figures. Mitotic figures and pleomorphism helps the pathologist to differentiate benign from malignant tumors. Depending upon the area of tumor island and neovascularization it is possible to determine whether the tumor is metastatic or not.

The tissue sections were systematically observed for area of metastasis, melanocytes with pigments, macrophage with pigment, mitotic figures, pleomorphism, epitheloid cells and necrotic foci. Scoring of individual animals showing microscopic changes in their lung was graded accordingly (1-Minimal, 2-Mild, 3-Moderate, 4-Severe). Depending upon the grading points animals were segregated considering the above said parameters. The summary of microscopic findings in lung histology of animals treated with PCL and its

liposomal formulations was depicted in Table 9.3. The untreated cells showed 10-15 tumor islands of variable sizes per lung section whereas PSPT treated cells showed approximately two medium sized tumor islands per lung section ($p < 0.05$ vs. control).

***In-vivo* antimetastatic activity of Irinotecan and its liposomal formulations**

The B16F10 melanoma cells were cultured and treated with sub-toxic (below IC_{50} value) level of IH and its liposomal formulations (CLIH and PSIH). Placebos for liposomal formulations were also used at the highest concentration of lipid used for the study. Post treatment, cell viability was taken using Trypan Blue dye exclusion test and equal numbers of cells (1×10^5 cells per mouse) were injected into mice. After 20 days, lungs were resected and analysed for metastasis. Resected lungs were weighed and values were noted down. It was observed that pretreatment with IH and its liposomal formulations significantly inhibited the lung homing of B16F10 cells as seen by decreased number of colonies on the lung compared to untreated control ($p < 0.05$). The lung colonies or metastatic nodules formed on the surface were manually counted for each animal. The representative examples of the lungs are shown in Figure 9.4. The average number of colonies for different formulation treatment (Figure 9.5) and percentage inhibition in lung nodule formation was calculated (Table 9.2). PSIH inhibited lung nodule formation by 72.83 %. It was found that inhibition of lung nodule formation was in the order of PSIH > IH > CLIH > placebo preparations. It is evident from the above data that the pretreated B16F10 melanoma cells with IH and its liposomal formulations inhibited lung homing and thereby enhanced antimetastatic activity.

Histology

Haematoxyline and Eosin stained sections of the lungs were microscopically observed and photographed. The histopathological micrographs were shown in Figure 9.7. The tissue sections were systematically observed for area of metastasis, Melanocytes with pigments, macrophage with pigment, mitotic figures, pleomorphism, epitheloid cells and necrotic foci. Scoring of individual animals showing microscopic changes in their lung was graded accordingly (i.e. 1-Minimal, 2-Mild, 3-Moderate, 4-Severe). Depending upon the grading points animals were segregated considering the above said parameters. The

summary of microscopic findings in lung histology of animals treated with IH and its liposomal formulations was depicted in Table 9.4. The untreated cells showed 12-15 tumor islands of variable sizes per lung section whereas PSIH treated cells showed approximately 2-3 medium sized tumor islands per lung section ($p < 0.05$ vs. control).

9.5 CONCLUSION

It has been proved that PCL, IH and their liposomal formulations effectively modify or inhibit steps of metastasis such as embolization, adhesion, survival, invasion or proliferation of pretreated B16F10 melanoma cells leading to decreased lung colonization. As the adhesion capacity of the tumor cells modified, the "intimacy" of the interaction between tumor and endothelial cells is decreased. Moreover, pH sensitive liposomal formulations containing anticancer drugs PCL and IH drastically reduced the number of colonies on lungs compared to other treatments and untreated control. It also accounted reduced area of metastasis, melanocytes with pigments, macrophage with pigments, mitotic figures, pleomorphism, and epitheloid cells on lungs. Hence, pH sensitive liposomes containing anticancer drugs offer potentially an effective approach to the antimetastatic therapy of a variety of tumor.

9.6 REFERENCES

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