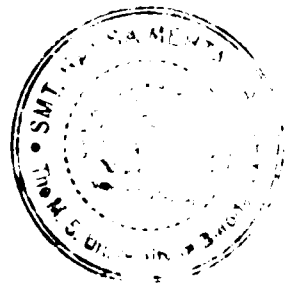

Summary & Conclusion

In clinic, when patients have been diagnosed with cancer, metastasis had already existed in most cases. Even for those patients without detectable metastasis, micrometastasis will eventually lead to a severe disease status after the removal of primary cancer by surgery. Therefore, to control the metastasis beyond primary tumor is a very important concern to prevent the progression of cancer. Systemic chemotherapy, which is considered to be one of the most effective ways to control metastasis at present, has been used with little success because most of the currently used drugs delivered in this way are quickly destroyed or inactivated in blood and liver after administration. Therefore, in the metastatic setting, one must carefully weigh toxicity vs. the possibility of survival prolongation.

During the various stages of metastasis, metastasizing tumor cells encounter various host cells (platelet, lymphocytes, or endothelial cells) and/or extracellular matrix (ECM) and basement membrane components (fibronectin, vitronectin and laminin). The adhesive interaction of tumor cells with components of ECM is regulated by various adhesion molecules and blocking of these interactions prevents tumor metastasis. The characteristic core sequence present in adhesion proteins (laminin, fibronectin, vitronectin) which demonstrated to contribute cell adhesion, spreading and migration of cells has been identified. YIGSR and EILDV are the pentapeptides recognized as the core sequence of laminin and fibronectin respectively for adhesion to laminin and integrin cell receptor present on the tumor and showed inhibition in tumor metastasis by competitively disturbing the function of integrins on the cell surface as substrate analogs. Literature reveals that these peptides suffer from drawback upon intravenous administration due to its shorter half life, degradation by enzyme and rapid renal clearance. A prominent strategy to explore the use of such antimetastatic peptide as a targeting agent is to conjugate it on surface of liposome or polymeric nanoparticles loaded with anticancer drug.

Polymeric micelles made up using amphiphilic block copolymer have received a great deal of attention now a day for delivery of hydrophobic drugs. Majority of research work for anticancer drug delivery has been done using stealth polymeric micellar systems for passive accumulation inside tumor due to well known enhanced permeability and retention (EPR) effect, but they are still cleared by RES system.

Consequently, insufficient uptake at tumor sites will decrease the therapeutic benefit of the administered drug dose and limiting the maximum dosage that can be safely applied. This limitation prevents drug-loaded micelles from achieving the potential therapeutic effects they might otherwise attain. At this juncture, functionalized block co-polymeric micelles will be an advanced option which could serve the purpose of actively tumor targeted anticancer delivery with attachment of various ligands such as folate, thiamine, peptides and proteins on the surface of micelles.

Present study was focused on the formulations of functionalized polymeric micelles of PEG-PCL di-block copolymer and attachment of peptides YIGSR-NH_2/EILDV-NH_2 on the surface of micelles by post insertion technique. Further the peptide conjugated formulations were evaluated for their *in vitro* and *in vivo* performance with comparison to plain drug and stealth micellar formulations.

The third chapter ***analytical method development*** includes the method of analysis of ETO, Methoxy Polyethylene glycol (Mol wt. 2000 & 5000) and peptides (YIGSR-NH_2/EILDV-NH_2). Analytical method of ETO was developed in acetonitrile and phosphate buffer saline (PBS) pH 7.4 for estimation of percent drug entrapment and *in vitro* release studies. The method was validated for accuracy, precision and linearity. In both the solvent system, scanning was performed between 400-200 nm and the absorption maxima were found at 284 nm. Furthermore, the regression coefficient derived from standard plot of both the solvent system was found 0.99 in the concentration range of 10 to 80 μg/ml. The results indicated that ETO follows the Beer's-Lambert law in the range of tested concentrations. Analytical method for intracellular ETO was developed by reversed phase HPLC method using ACN: water: glacial acetic acid (35: 61: 1, v/v/v) and detection was performed by UV detector set at 254 nm using diazepam as an internal standard. The calibration curve was developed using ETO spiked in cell lysate of B16F10 cells and the regression coefficient was found 0.99. The retention time of ETO and diazepam was found 5.25 and 4.74 minutes and peaks were found well resolved. Developed method was evaluated for accuracy, precision and extraction efficiency. The method was method found accurate and precise while the extraction efficiency was found near to 70 %. Estimation method of methoxy PEG-OH of molecular weight 2000 and 5000 was

developed by colorimetric method at 525 nm using UV visible spectrophotometer. The regression coefficient in both the cases was found of 0.99. Quantification method for peptide YIGSR-NH_2 and EILDV-NH_2 was carried out by TNBS (2,4,6-trinitrobenzene sulfonic acid) assay. The free amine group of peptide reacted with TNBS and formed a chromogenic derivative, which was measured by UV visible spectrophotometer at 345 nm. A linear relationship was observed with regressed coefficient near to one in both the peptide.

The fourth chapter ***Synthesis and characterization of PEG-PCL di-block copolymer*** covers synthesis and characterization of different molecular weight copolymers. The synthesis of CH_3O-PEG-PCL and HOOC-PEG-PCL of various molecular weight were carried out using CH_3O-PEG-OH/ HOOC-PEG-OH (molecular weight 2000/5000) with monomer ε-caprolactone (CL) using HCl in diethyl ether as activator. Synthesized copolymers were characterized by ^{1}HNMR, GPC and FTIR. The ^{1}HNMR spectra exhibited characteristic peaks of PEG and PCL; moreover the molecular weight calculated using integration ratio of peak at δ 4.05 ppm due to PCL blocks and peak at δ of 3.65 ppm due to the PEG blocks were found relatively closer to those of the theoretical molecular weight. GPC chromatograms of copolymers exhibited unimodal peak and the molecular weight obtained were in accordance with molecular weight obtained by ^{1}HNMR technique. FTIR spectra showed characteristics absorptions bands of PEG and PCL.

The fifth chapter ***Formulation optimization and evaluation of Methoxy PEG-PCL micelles*** includes the fabrication of micellar formulation loaded with drug ETO. Solvent displacement technique was used for formulation of micelles using water miscible solvent acetonitrile and acetone. Compared to acetonitrile, use of acetone resulted in smaller particle size with no any significant change in percent drug entrapment. Moreover, use of acetone will be beneficial in terms of scale-up of formulations due to its lower boiling point. Process parameters like rate of addition of organic solvent and stirring speed was optimized and it was found that these parameters affect largely on particle size and percent drug entrapment. A stirring speed of 1000 rpm and rate of addition of 1 ml/min resulted in to micelles with optimum particle size and percent drug entrapment. Formulation parameters like ratio

of drug to polymer and aqueous phase to organic phase was studied. The percent drug entrapment and particle size was found increased with increase in ratio of drug to polymer. In addition, particle size and percent drug entrapment also found increased with increase in the molecular weight of hydrophobic core part (PCL) of different block length copolymers. This result signifies that the drug encapsulation efficiency depends on the copolymer composition as well as the ratio of drug to polymer. At different ratio of aqueous phase to organic phase, particle size and polydispersity index affected to larger extent and it was due to variation in polymer concentration in organic phase. An increase in ratio of aqueous phase to organic phase resulted in decrease in particle size and polydispersity index upto certain extent, after that particle size and polydispersity index found increased. It was attributed due to lower copolymer concentration which resulted in to higher diffusion of the organic phase into aqueous phase.

Percent drug loading of ETO was also assessed for different molecular weight copolymers. The MPCL220, MPCL235 and MPCL250 micellar formulation showed maximum drug loading of 2.73, 3.91 and 4.24 percent while MPCL550, MPCL570 and MPCL5100 showed 4.23, 4.40 and 5.32 percent drug loading. A practical drug loading obtained with different micellar formulations was found solely dependent on the molecular weight of block copolymer especially the hydrophobic core part and tends to increased with increase in hydrophobic core part molecular weight. Zeta potential of MPCL micellar formulation of different molecular weight was found near to -4 to -5 mV and was due to PEG segment which capped the carboxyl acid end groups of the PCL chains resulted in to low negative zeta potentials.

Critical micelles concentration (CMC), which is considered as important parameter for *in vitro* and *in vivo* stability of micelles was assessed using pyrene as fluorescent probe. The CMC value of all micelles was obtained in between 2.00 to 1.14 X 10^{-3} mg/ml. It was observed that as the molecular weight of hydrophobic part increased, CMC value decreased implies that the hydrophobic PCL blocks mainly affect the CMC. The fixed aqueous layer thickness (FALT) of MPCL micellar formulations were calculated based on Gouy- Chapmann theory. The FALT of micellar formulation was found between 3 to 4 nm and was mainly depend on the molecular weight of PEG

block. Increase in molecular weight of PCL block also resulted in to decrease in FALT due to stronger interaction of PEG chains to core part. Placebo micellar formulations showed increase in FALT compared to ETO loaded formulations might be due to hydrophobic interaction between drug and core part.

In vitro stability studies of MPCL micellar formulations carried out in presence and absence of bovine serum albumin (BSA) in PBS and it was observed that all micellar formulation except MPCL220 remained stable even after 48 h of incubation. The particle size of MPCL220 micelles found increase upto 8.0 and 8.5 fold in presence and absence of BSA respectively. Moreover, the increase in particle size was found to large extent in ETO loaded MPCL220 micelles compared to placebo MPCL220 micelles. The results indicate that except MPCL220 and MPCL220-P, all other micellar formulations showed excellent *in vitro* stability with least adsorption of BSA. PEG surface density/nm^2 of MPCL220, MPCL235 and MPCL250 micelles were found from 0.830 to 0.753 with distance (D) of two neighboring chain from 1.20 to 1.33 nm. MPCL550, MPCL570 and MPCL5100 micelles exhibited PEG surface density between 0.640 to 0.493 with distance (D) from 1.56 to 2.02 nm. Increase in molecular weight of PCL block resulted in to reduction in surface density and increase in distance between two PEG chains, which was attributed due to strengthened hydrophobic interaction between PCL chain lengths, which occupied less molecular number of di-block copolymer to shape a single micelle. ETO loaded micellar formulation exhibited lower PEG surface density compared to placebo formulations due to interaction between drug and PCL core part. The values obtained could be very helpful to understand the *in vivo* behavior of the MPCL micelles.

In vitro hemolysis studies of ETO injection, placebo ETO injection and MPCL micellar formulation were carried out to evaluate the hemolytic potential of formulations. Both ETO injection and placebo ETO injection showed substantial hemolytic properties due to presence of free ETO & surfactants and the effect was found concentration dependent. MPCL220 micellar formulation exhibited higher hemolytic activity compared to other MPCL micellar formulations. This effect was due to rapid release of ETO from MPCL 220 micelles due to its lower molecular weight. The percent hemolysis in micellar formulation except MPCL220 after 24 h of

incubation was between 1 to 5 % at ETO concentration at 200 μg/ml. Based on the results obtained after evaluation of MPCL micelles, MPCL235 and MPCL570 micellar formulation were selected for further studies and the synthesis of carboxyl group ended block copolymer (HOOC-PEG-OH) of similar molecular weight were synthesized.

The sixth chapter covers ***formulation of peptide conjugated PEG-PCL micelles*** and its characterization. Functionalized micelles of PEG-PCL were prepared using a blend of copolymers of similar molecular weight i.e. CH_3O-PEG-PCL & HOOC-PEG-PCL by nanoprecipitation method. Peptide YIGSR-NH_2 or EILDV-NH_2 was conjugated to the terminal carboxyl group of PEG present on surface of micelles using EDC and NHS chemistry. The percent conjugation efficiency of peptide to micelles was determined by quantifying free amine group of peptide by developed TNBS assay. At 3 h of incubation and 1: 1 molar ratio of functional polymer to peptide, maximum percent conjugation of more than 85 percent achieved in both the peptides. Effect of particle size, zeta potential and percent drug entrapment after and before conjugation of peptide also evaluated. Increase in particle size upto 10 nm with a reduction in zeta potential (at pH 7.4) was noticed after conjugation of peptide, signifies that carboxyl groups were consumed for conjugation of peptide. Percent drug entrapment also found decreased after conjugation due to release of drug during dialysis procedure employed in peptide conjugation method.

DSC and X-RD were carried out to investigate the physical state of incorporated drug. It was deduced that ETO was either molecularly dispersed or distributed in an amorphous state in the PCL core. TEM studies indicated micelles formed are of spherical shape. Lyophilization of micelles was carried out to increase the stability of micelles. Sucrose, trehalose and poloxamer-188 were used as freeze drying aids. Use of sucrose or trehalose at various ratios failed to preserve the initial particle size due to crystallization of PEG during freeze drying. Use of poloxamer-188 prevented formation of intra and inter particulate bridges in crystallization process of PEG during freeze during and maintained initial particle size with redispersibility index between 1 to 1.5.

In vitro release studies of micellar formulations were carried by dialysis diffusion technique in PBS pH 7.4 to correlate the predictable drug release *in vivo*. After 12 h, MPCL235, YPCL235 and EPCL235 showed drug release of 22.99 ± 4.76, 16.34 ± 3.56 and 19.38 ± 4.44 percent respectively, while MPCL570, YPCL570 and EPCL570 at the same time exhibited drug release of 17.98 ± 3.98, 9.08 ± 3.01 and 11.69 ± 4.40 percent respectively. A significant change in drug release profile was observed with change in PCL core molecular weight and micelles with high molecular weight PCL core showed retarded drug release. A short term stability studies performed at two different conditions i.e. at 2-8 °C and at 25 ± 2 °C with 60 ± 5 % RH to evaluate the stability of micellar formulation. It was found that at 2-8 °C storage condition, micellar formulation showed good stability in terms of particle size and drug loading preservation compared to 25 ± 2 °C with 60 ± 5 % RH.

The seventh chapter includes ***in vitro cell line studies*** using highly metastatic B16F10 melanoma cell lines. MTT assay was performed to evaluate the cytotoxic effect of plain ETO and micellar formulations. After 24 h, plain ETO exhibited IC_{50} of 0.087 µM which is almost 25 to 50 fold lower compared to all other micellar formulation. An incubation time of 48 h, change in cytotoxicity profile of micellar formulations resulted. MPCL235, YPCL235 and EPCL235 exhibited 0.082, 0.040 and 0.055 µM IC_{50} while MPCL570, YPCL570 and EPCL570 showed 0.120, 0.045 and 0.075 µM IC_{50} after 48 h incubation. Plain ETO at 48 h incubation showed 0.082 µM IC_{50}. A further increase in incubation time to 72 h, the IC_{50} value obtained in plain ETO was 0.043 which is similar to 48 h IC_{50}. The IC_{50} values obtained for MPCL235 and MPCL570 was 0.034 and 0.040 µM after 72 h incubation while YIGSR conjugated micelles YPCL235 and YPCL570 showed 3.07 and 2.15 fold increases in toxicity respectively compared to plain ETO. EILDV conjugated micelles EPCL235 and EPCL570 showed increase in cytotoxic effect of 1.72 and 1.38 fold compared to plain ETO. The results obtained in cytotoxicity studies showed that micellar formulation have potential in release of drug intracellular in controlled manner. Moreover, peptide conjugated micelles exhibited higher cytotoxicity compared to non peptide conjugated micelles suggesting internalization of micellar carrier by receptor mediated mechanism and hence higher amount of drug available inside cells. Placebo micellar

formulation showed no any cytotoxic effect against B16F10 cells implying polymer used for assembly of micelles is safe and nontoxic.

Further invitro cell line studies carried out at subtoxic concentration i.e. at quarter and half IC_{50} dose. Leighton tube assay was performed to see the changes in cell morphology after drug treatment. A significant change in cell morphology was observed in plain ETO and micellar formulations treated B16F10 cells and the effect was concentration dependent. Colony forming ability of B16F10 cells was assessed after treatment with quarter and half IC_{50} dose of plain ETO and micellar formulations for 48 h incubation. Colonies having more than 50 cells were counted and the percent colony inhibition was calculated based on untreated control. Compared to plain ETO, micellar formulation exhibited higher inhibition in colony formation at both the tested concentrations. Peptide conjugated micellar formulation demonstrated higher inhibition in colony formation compared to non conjugated micelles. Cell migration of B16F10 cells were assessed by scratch wound assay model. B16F10 cells were grown to 80 percent confluency in tissue culture plate and a wound was created with help of sterile pipette tips and the wound created cell monolayer was treated with subtoxic concentrations of plain ETO and micellar formulations for 48 h. After drug treatment, cells were incubated further for 48 h for coverage of wound. Zero h plate was fixed immediately after wound creation and considered as reference plate. After measuring cell wound width using Laser Capture Microdissection Microscope [LCMM, Zeiss, Germany], percent relative wound width and percent cell migration was calculated. In untreated control, a B16F10 cell has covered the wound upto 70 percent and was considered as 100 percent cell migration. Compared to plain ETO, micellar formulation exhibited inhibition in migration of cell to greater extent. Both, YIGSR and EILDV conjugated micelles showed higher antimigratory activity compared to non conjugated micelles.

Cell adhesion study was conducted using YIGSR-NH_2 and EILDV-NH_2 as substrate for adhesion of B16F10 cells after treatment with plain ETO and micellar formulations. The results obtained indicated that after drug treatment, a reduction in cell adhesion was observed in all micellar formulation to substrate coated plates. Peptide conjugated micellar formulations YPCL235, YPCL570, EPCL235 and

EPCL570 showed extreme reduction in cell adhesion compared to MPCL235 and MPCL570. The results obtained suggest that after conjugation of peptide to micelles remain biologically active. Confocal microscopy was performed to see the uptake of micelles loaded with 6- coumarin. After 1 and 3 h of incubation, cellular uptake of micelles took place and the fluorescence intensity depended on incubation time. Moreover, peptide conjugated micelles exhibited more pronounced fluorescence compared to non conjugated micelles, indicating receptor mediated uptake of peptide conjugated micelles.

Cellular uptake of plain ETO and micellar formulations were carried out measuring intracellular ETO by HPLC method. Plain ETO due to its solubilized state showed 75.02 ± 5.23 percent cellular uptake which was very high compared to micellar formulations. MPCL235 and MPCL570 showed cellular uptake of 6.43 ± 2.81 & 5.30 ± 1.51 percent. YIGSR and EILDV was conjugated on the surface of micellar formulations at 5 %, 10 % and 20 % surface density and its effect on cellular uptake was studied. It was found that compared to 5 % surface density, 10 % showed near to 1.5 fold higher cell uptake. A further increase in surface density to 20 % showed no significant increase in cellular uptake. The result implies that after certain surface density of ligand, there was no any effect of excess ligand on the cellular uptake enhancement. Flow cytometry studies using PI staining for cell cycle distribution showed arrest of B16F10 cells in G2-M phase and the effect was found concentration dependent. Moreover, peptide conjugated micellar formulation showed higher percent of cell accumulation in G2-M phase due to higher intracellular uptake of drug compared to non conjugated micellar formulations. Placebo formulation showed no effect on cell arrest of B16F10 cells and cell cycle distribution was similar to untreated control.

The eighth chapter ***in vivo studies*** consist of evaluation of developed micellar formulation for their therapeutic effectiveness. Biodistribution and experimental metastasis studies were carried out using Balb/C and C57BL/6 mice respectively. Biodistribution study was carried out in EAT tumor induced animal by radiolabeling method in which plain ETO and micellar formulations were radiolabeled with Tc99 and their radioactivity in various organs were estimated at various time points after

intravenous injections. Before intravenous injection of radiolabeled formulations, amount of stannous chloride required for maximum radiolabeling of formulation was optimized followed by stability studies of labeled complex in normal saline for 24 h. The labeled formulations were stable over a period of 24 h with more than 96 percent radioactivity of labeled complex after 24 h.

Biodistribution profile of micellar formulations observed was totally different to that of plain ETO injection. Micellar formulations showed their three to five fold higher concentration in blood at 24 h post injection, implies that micelles having long circulating properties due to presence of PEG chain on the surface of micelles. Liver uptake of micellar formulations were found near to two fold higher compared to plain ETO. The higher liver uptake by liver was attributed to their particulate form with particle size lower than 100 nm. Plain ETO showed higher spleen uptake compared to micellar formulations. Although, the splenic uptake was found lower compared to liver uptake for all micellar formulations. Compared to plain ETO, a lower lung uptake was observed in micellar formulation might be due to their smaller size, which helped in escaping of micelles from lungs. Renal uptake of micellar formulations were found very low compared to plain ETO, indicating slower renal clearance of micellar formulations. Radioactivity in stomach was found $\leq$1% in all the formulations, indicating the stability of labeled complex *in vivo*.

Tumor uptake of plain ETO was found very poor at all the time point compared to micellar formulations. MPCL235 and MPCL570 exhibited 2.57 and 2.15 fold higher tumor uptake to plain ETO at 1 h post injection and exhibited maximum tumor uptake of 1.78 ± 0.10 & 1.92 ± 0.38 percent respectively after 4 h post injection. YPCL235 and YPCL570 exhibited 12 to 13 fold higher tumor uptake after 4 h post injection compared to plain ETO. While EPCL235 and EPCL570 showed 11.97 & 14.89 fold higher tumor uptake compared to plain ETO. These results suggest that the tumor uptake can be significantly increased by conjugation of peptide YIGSR-NH_2 and EILDV-NH_2 on the surface of micelles which can interact with surface cell receptors and resulting into receptor medicated endocytosis.

Experimental metastasis study was conducted using highly metastatic B16F10 cells which are reported to produce pulmonary metastatic nodules in lungs after intravenous injection of cells. The study was carried out by two different methods to find out the potential of B16F10 cells for induction of metastasis i.e. by pretreatment and post treatment method. In pre-treatment method, B16F10 cells were initially treated with subtoxic concentrations of plain ETO and micellar formulations for 48 h and 0.1 million cells were injected to C57BL/6 mice via tail vein. Mice were sacrificed on 21st day of cell inoculation, lungs were isolated and the numbers of pulmonary metastatic nodules were counted. While in post-treatment method, 0.1 million B16F10 cells in PBS were injected to tail vein of C57BL/6 mice and on second day plain ETO and micellar formulations at dose of drug 2 mg/kg were injected via tail vein. On 21st day of cell inoculation mice were sacrificed, lungs were isolated and the numbers of pulmonary metastatic nodules were counted.

In both the method, inhibition in formation of lung nodules were calculated based on number of lung nodules present in untreated control group. The *in vitro* treated B16F10 cells with plain ETO and ETO loaded micellar formulations exhibited reduction in formation of pulmonary metastatic nodules with maximum effect in peptide conjugated micelles with $P<0.001$. In post-treatment method, the inhibition in formation of nodules was less compared to pre treatment method due to distribution of formulations through out the body. However, micellar formulations exhibited higher inhibition in formation of pulmonary metastatic nodules. Peptide conjugated micelles showed near to 1.75 and 1.40 fold higher inhibitions in metastatic nodules formation compared to plain ETO and non conjugated micellar formulations respectively. The result obtained signifies the significant potential of peptide conjugated micellar drug delivery for effective prevention and treatment of metastasis.

Histopathology studies of lung sections after counting of pulmonary metastatic nodules were carried out by haematoxilin and eosin staining procedure. Compared to untreated control in both pretreatment and post treatment method, plain ETO and micellar formulation treated group showed reduction in the number and size of pulmonary metastatic nodules. Peptide conjugated micelles showed reduction in size of tumor islands to large extent with no any blockage of blood vessels.

Conclusion:

Tumor metastasis is the one of the major causes of morbidity and mortality in patients with solid malignant tumor and is the result of successive interactions between cancer cells and host tissues. Therefore, the compound which can interact with cell surface receptors and competitively inhibit the interaction between tumor cell and extracellular matrix are clinically useful. Peptide YIGSR-NH_2 and EILDV-NH_2 derived from protein laminin and fibronectin respectively responsible for binding to laminin and integrin receptors was utilized as ligand on the surface of polymeric micelles (PEG-PCL) loaded with anticancer drug ETO. YIGSR-NH_2/ EILDV-NH_2 conjugated micelles were showed their potential in effective treatment of metastasis when evaluated *in vitro* using highly metastatic B16F10 cell line. Further, experimental metastasis study results proved that the formulated drug delivery carrier helps in reduction in metastasis of tumor. Biodistribution studies results demonstrated higher tumoral uptake of peptide conjugated micelles compared to plain drug. Overall, this carrier system brings us a step closer with use of antimetastatic peptide like YIGSR-NH_2 and EILDV-NH_2 in prevention and treatment of metastasis.