SUMMARY AND CONCLUSION
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Surgery and/or radiation are the first choice to treat primary solid tumor, but by the time it is detected, cancer cells may have already spread to other organs. Any resulting metastases are often so widely spread or unapproachable that successful remedy for them is impossible. The conventional therapy of etoposide is less effective because of its narrow therapeutic index. The various known side effects are, decreased white blood cell count with increased risk of infection, and decreased platelet count with increased risk of bleeding, mild nausea and vomiting, loss of appetite and fetal damage, if pregnancy occurs while taking etoposide.

YIGSR- a synthetic pentapeptide corresponding to a partial sequence of the laminin β1 chain, H-Tyr-Ile-Gly-Ser-Arg-NH₂, was reported to inhibit experimental metastasis formation. Literature reveals that YIGSR suffers from drawback same as other bioactive peptides while administered in situ, as it has shorter half life, degraded by enzyme and also has fast renal clearance. To improve the antimitastatic activity, cyclization and D-amino acid substitution of the peptide was carried out, however the problems remained unsolved. The EILDV, pentapeptide sequence of fibronectin have anti-adhesion activity. In the last decade the biological activity of the EILDV has been established by scientist. Till date the use of the same in the drug delivery has not been evaluated. This is the first attempt, in which the EILDV has been used as ligand to target the cancerous cells. To improve the activity of YIGSR and EILDV, novel drug delivery systems has to be developed, for that the liposomal system is the best suitable option to tag these peptides as ligand.

Liposomes are vesicles composed of a phospholipid bilayer in which pharmaceutical agents, enzymes and hormones can be encapsulated. Resemblance of the membrane structure to cell membranes makes liposome's non-immunogenic and diversifies intake methods. Thus the liposomes are suitable candidate for attachment of the peptide as ligand to its surface. Numerous methods have been utilized in previous research to increase the interaction between liposome and cell. In order to achieve the targeting of liposomes, which carry drugs or other bioactive molecules, it is indeed necessary that these vesicles bind to their target cells with high selectivity and affinity. A fruitful approach consists of attaching a ligand to surface of the liposomes.
which can bind to the specific receptors over expressed by the target cells and thereby increasing the intracellular delivery by endocytosis.

The present study is focused on designing, characterizing and evaluating etoposide loaded liposomes tagged with the peptides like YIGSR/EILDV with the help of linker for the specific binding towards the cancerous cells. The developed system would be further subjected to \textit{in-vitro} and \textit{in-vivo} characterization.

The third chapter, \textit{drug and analytical profile}, discusses the various aspects of drug profile such as physicochemical properties pharmacological activities and different methods of analysis. In the present studies we selected UV method for the analysis of Drug content as well as in vitro drug release. The method was validated using accuracy and precision.

The drug present in plasma as well as tissue was estimated by High Pressure Liquid Chromatography (HPLC). HPLC method condition includes C18 lichrosphere column, mobile phase Acetonitrile- Distilled water [45:55], pH adjusted to 4 with acetic acid, the detection was performed using Fluorescence detector at wavelength $\lambda_{ex}$ 250 and $\lambda_{em}$ 325. The ration time for etoposide was 5.8 min.

Etoposide, topoisomerase II inhibitor categorizes under anticancer drugs. For in vitro estimation Spectrophotometric methods have been developed in PBS (pH 7.4) and with organic solvent (methanol:chloroform–9:1) for its reproducibility. The scanning was performed in a range of 400-200 nm and absorption maxima were observed at 286±1 nm for both the solvent system. The standard plot indicates the etoposide follows Beer’s-Lambert law the drug concentration range 5-50 $\mu$g/mL. The regression ($r^2$) was found to be 0.99 for both the solvent system. These results showed accuracy and precision of the developed method. Reversed phase High Performance Liquid Chromatography (Rp-HPLC) was applied to quantitate etoposide concentration in the biological system includes plasma, liver, kidney, lung, spleen and tumor. The extraction of the drug from the tissues was performed with the help of dichloromethan. The drug peak was eluted in the mobile phase containing Acetonitrile:Distilled water [45:55] and pH adjusted to 4 with the Acetic acid. The
standard plot was drawn between the concentration of drug and area of peak. The retention time was found to be 5.8 min. The peaks were well resolved using the Fluorescence detector. The regressed curve was showed linearity. The values of $r^2$ were found to be 0.99 for plasma, liver, spleen, lung, kidney and tumor homogenate.

The fourth chapter intends to describe the design and characterization of untagged and peptide tagged etoposide liposomes. The liposomes were prepared by thin film hydration technique. The lipids used to prepare liposomes include Egg Phosphatidyl Choline [EPC], Soya Phosphatidyl Choline [SPC], Disteroyl Phosphatidyl Choline [DMPC], Dimyristoyl Phosphatidyl Choline [DPPC], Hydrogenated Soy Phosphatidyl Choline [HSPC], cholesterol and etoposide. Different molar ratios of lipids along with cholesterol and drug were dissolved in organic solvent mixture (Methanol:Chlororform, 2:1). The ratio of total lipids to drug was kept constant (1:10) while the ratio of PC to cholesterol was varied at the ratio of 9:1, 8:2 and 7:3. The parameters like pressure, rpm, hydration time, hydration volume, transition temperature were optimized and kept constant for entire study. The organic solvent was evaporated on rotary evaporator at specified temperature (as per the Tg of the lipids, range 35-65°C). The lipid film was then vacuum dried for 4 h and the lipid film was then hydrated with distilled water above Tg temperature of lipid used for approximately 60 min. The liposomal suspension formed was then employed for particle size reduction using high pressure homogenizer [Aventis, Canada]. For particle size reduction three cycles (60 sec each) of homogenization was performed at 7500 psi at 60°C and the uniform particle size distribution was obtained.

The liposomal suspension was characterized for particle size and percent entrapment efficiency. The unentrapped etoposide was separated by centrifuging the liposomal suspension at 3500 rpm for 10 min, free drug settled down as cake and liposomes present in supernatant. The unentrapped drug and aliquots of supernatant was dissolved in the chloroform methanol mixture (1:9) and absorbance was measured on Shimadzu 1601 UV spectrophotometer at wavelength 286 nm. The percent entrapment was found to be less than 80 % for all formulations. The composition of EPC:CH (9:1) gave maximum entrapment of 78.84 % at the ratio of 9:1 (Drug : Lipid). To improve the percent entrapment efficiency, an attempt was made to formulate charged liposomes by thin film hydration technique followed by high
pressure homogenization. Stearyl amine [SA] served as cationic lipid and Dipamitoly Phosphatidic Acid [DPPA] as anionic lipid to induce charge to the liposome. The entrapped and unentrapped drug was separated by centrifugation and were quantified after by dissolving them in the organic solvent (methanol:chloroform=9:1). The absorbance was measured using Shimadzu 1601 UV spectrophotometer at wavelength 286 nm. There was no considerable increase in the percent entrapment of etoposide in presence of charged lipids observed.

To improve percent entrapment and to obtain particle with narrow range of size distribution, etoposide liposomes were prepared using mixture of lipids (natural and synthetic). The liposome was prepared by thin film hydration technique and the ratio of drug to total mix lipid was varied (1:15, 1:20 and 1:25). Optimum entrapment (98.12±0.80%) and particle size (117.64±12.2nm) was obtained with 1:25 mol ratio. Effect of cholesterol was also evaluated by varying the percentage of cholesterol in the lipids mix. The maximum entrapment was found to be 94 % at ratio of 3.5:4.5:2.5 (EPC: HSPC: CH) and therefore final formulation contained drug to total lipid of 1:27. Stealth liposome was prepared in same manner by incorporating DSPE-PEG 2000 to the optimized lipid composition. Effect of pegylation on the percent entrapment and particle size was evaluated. Mol% of DSPE-PEG 2000 was varied from 0 to 8 and optimum entrapment (92.01±1.27%) was observed at ratio of 5 mol% of DSPE-PEG 2000.

The amount of the polymer (PEG) required for stearic stabilization was optimized using the electrolyte induced flocculation test. In the study, specified quantity of liposomal suspension was diluted to predetermined volume using various molar ratio of flocculating agent (sodium sulphate). The absorbance of the resulting suspension was measured at OD_{420} on UV spectrophotometer. The concentration of the steric stabilizing agents, which allow minimum possible changes in the absorbance when different mol concentrations of the flocculating agent were added to the liposome was selected as optimum concentration and used for further studies. The 5 mol% of the total lipids of DSPE-PEG-COOH was found to be adequate for stearic stabilization.

To provide specificity towards the target, the surface modified liposomes were formulated. Peptide tagging was performed by attaching peptides to liposomes via
linkers to overcome stearic hindrance. The peptide tagged liposomes were prepared by using thin film hydration technique. The linkers N-glutaryl Phosphatidyl Ethanolamine (NGPE) and DSPE-PEG-COOH was synthesized by reacting the carboxyl terminal of the anhydride [glutaric for NGPE and PEG of DSPE-PEG] with amine of DSPE in presence of DCC and TEA at standard condition of temperature (25°C) and at inert atmosphere (in presence of N₂). They were characterized by TLC and FTIR. The disappearance of spot of DSPE in mobile phase 65:35:4 [methanol:chloroform:water] indicates completion of reaction. FTIR spectra confirmed the formation of –CONH- bond between the carboxyl terminal of anhydride and amino terminal of DSPE by showing prominent peak at 1650 cm⁻¹. Conventional liposome (CL) contains NGPE as linker while stealth liposomes (SL) have DSPE-PEG-COOH as linker moiety. The effect of linkers on the percent entrapment and particle size was also studied. NGPE at 6 mol % gave a high entrapment of 92.34±1.16% and particle size 133.4±17.4 was chosen for the further studies. Similarly DSPE-PEG-COOH at 5 mol% gave 91.71±1.11% entrapment and particles 121.2±15.3 nm and used for further studies.

The peptides, Tyr-Ile-Gly-Ser-Arg-NH₂ [YIGSR] and Glu-Ile-Leu-Asp-Val-NH₂ [EILDV], were synthesized by solid phase synthesis. Moreover the peptides were synthesized as amide carboxyl terminal in order to mimic its environment in the native protein. The YIGSR peptide, sequence of laminin glycoprotein present in extra cellular matrix, has affinity for the laminin receptor. Peptides were purified using preparative HPLC and molecular weight was confirmed by Gel permeation chromatography (GPC). Before coupling of actual peptides to the liposomes, a pilot study was run using arginine, the terminal amino acid of YIGSR peptide. The effect of time on the coupling efficacy was performed by quantifying free amine group using TNBS assay. At 12 h, the coupling efficiency was more than 75% for both the linkers. The mol ratio of peptides to linker and coupling time was also evaluated. The 1:1 mol ratio of linker to peptide was selected on the basis of percent coupling.

The freeze drying of liposomes were carried to improve its stability. The type and amount of cryoprotectant required for formation of porous cake having least aggregation and appreciable reconstitution was optimized. Sucrose, mannitol and lactose were used as cryoprotectants and amount was varied on the basis of weight of
total lipid. The sucrose proved to be the best cryoprotectant (5 times by weigh of total lipids) among all three sugars for all formulations evaluated. All etoposide liposomal formulations showed negative zeta potential in a range of -23 to -33 which is suitable for stability.

In vitro release profile was performed using dialysis bag diffusion method in PBS pH 7.4 at controlled condition of temperature (37°C) and agitation 100 rpm. Plain liposomes having EPC provided maximum release. The rate of drug release from various formulations followed the order PL > CL > SL. There was no significant change in the release behavior of peptide tagged liposomes (NLY & NLE; PLY & PLE) and untagged liposome (CL & SL) having linkers; CL compared with NLY and NLE and SL compared with PLY and PLE.

The freeze dried formulations were tested for its stability at 2-8°C and 30°C±2°C. At specified time, samples were removed, reconstituted and characterized for change in percent entrapment and particle size. The favorable temperature for storage was 2-8°C at which minimum changes was seen in the percent entrapment and particle size.

The fifth chapter includes In vitro cell line studies, were performed on the highly metastatic B16F10 melanoma cells. To evaluate the cytotoxicity effect of free and liposomal etoposide formulations, viability tests were performed on highly metastatic B16F10 melanoma cell lines using MTT assay. The cells were seeded in 96 well plates at a concentration 4x10⁴ cells/mL and exposed to free ETO, CL, SL, NLY, PLY, NLE and PLE for 2 h and 24 h at 37°C in humidified condition for 24 h.

IC₅₀ value of the free ETO was 0.015 (mM/mL) at 24 h exposure. In case of untagged liposomes, CL and SL, have IC₅₀ value of 0.024 and 0.026 (mM/mL) respectively. Whereas peptide tagged liposomes showed lower IC₅₀ values of 0.02, 0.021, 0.0092 and 0.009 (mM/mL) for NLY, NLE, PLY and PLE respectively, suggest that the tagged liposomes are more effective than free etoposide and untagged liposomes. At 2 h exposure time the free etoposide had lower IC₅₀ value (0.035 (mM/mL) in comparison to the other liposomal formulations indicated the fast internalization of free drug in the cells.
The encapsulated formulations showed comparable cytotoxicity with free ETO. Among the liposomal formulations, the peptide tagged liposomes showed higher toxicity towards the B16F10 cells when exposed for 24 h.

Extent of inhibitory effect on colony formation was performed by colony formation assay. B16F10 melanoma cells (2x10⁴ cells/mL) were cultured in complete medium and after 24 h cells were treated with free ETO and etoposide loaded liposomal formulations at sub-toxic concentrations (half & one forth of the IC₅₀ value). At 24 h, free ETO gave a percent colony inhibition of 90.49±2.10 % (treated at a dose of half of the IC₅₀ value). The cells treated at dose of half of IC₅₀ value in case of peptide tagged liposomal formulations the percent colony inhibition were 85.57±5.24, 88.15±4.56, 86.21±5.60 and 88.62±3.57 % for NLY, PLY, NLE and PLE respectively. While untagged liposomes, CL and SL had IC₅₀ of 64.95±6.54% and 77.86±4.1% respectively at half of the IC₅₀ dose. Similarly the cells treated at one forth dose of IC₅₀ values showed concentration based inhibition and were lower than the half dose of the IC₅₀ values.

The B16F10 melanoma cells were grown up to 80 % confluency. The monolayer of cells was treated with half and quarter dose of the IC₅₀ concentration of free ETO and etoposide loaded liposomes along with blank liposomes and incubated at 37°C and 5% CO₂ for 24 h. The percent wound width was calculated and the PLY [124.59±12.4%] and PLE [132.67±14.54 %] showed maximum anti-migration activity in comparison to all the liposomal formulations when treated at a dose of half of the IC₅₀ value. The inhibitory effect on migration was concentration dependent when treated for the two different doses of IC₅₀ value (half and quarter dose). The effect on cell morphology was evaluated by performing the Leighton tube assay. The cells were grown on cover slip and treated with free ETO and liposome formulations at ½ and ¼ dose of IC₅₀ value. All the formulations modified the cell morphology.

The adhesion assay was performed to evaluate whether the inherent adhesive property of the peptide remain intact after tagging to liposomes. The cells were harvested, counted by trypan blue exclusion method and diluted to 3 x 10⁵ cells/mL. In terms of percent cell attachment the PLE (51.32±2.7%) and PLY (54.11±4.1%) have...
maximum anti adhesive property among all formulations tested. The free peptides also demonstrated anti adhesive property towards B16F10 melanoma cells at a concentration of 10\(\mu\)g/100\(\mu\)L and the values were 77.50 ± 2.6 % and 71.33 ± 1.9 % for YIGSR and EILDV respectively. The cell cycle analysis was performed by using Fourier Assorted Cell Sorter (FACS) using propidium iodide as staining agent. The formulations retarded growth of the cells in G2 phase. Untreated cells and cell samples of blank formulations showed a typical cell cycle curve where sizable populations of cells were found in the G0-G1 phase, followed by the S phase. Free etoposide and liposomal formulations showed totally different cell population than the untreated cells. The treated cells showed decrease in the G0-G1 phase while increase in the G2-G1 phase, which were contrary to the untreated and the blank sample cells.

Cellular uptake of ETO was determined in B16F10 melanoma cells. The cells were exposed for 2 h to free ETO and liposomal ETO. The cells was then washed with PBS, harvested using saline EDTA and the drug was extracted from the cells and quantify using HPLC. Maximum uptake was found with free ETO (4.50 ± 0.20 \(\mu\)g). Peptide tagged liposomes have cellular uptake of 3.00 ± 0.20, 3.24 ± 0.29, 3.52 ± 0.34, 3.56 ± 0.40 \(\mu\)g [equivalents /10^7 cells] for NLY, NLE, PLY ,PLE respectively. The untagged liposomes have comparable less cellular uptake i.e. CL (2.40 ± 0.12 \(\mu\)g) and SL have (2.11 ± 0.11 \(\mu\)g). The results of cellular uptake studies supports the cytotoxicity effect of the free and liposomal etoposide as the free drug easily internalize in the cells in short period of time and exerts the cytotoxic effect.

The sixth chapter described about in vivo studies of the liposomal formulations. The formulations were selected on the basis of the performance of the formulations in the in vitro cell line studies. The pharmacokinetic study and biodistribution of free ETO and SL, PLY and PLE was carried out in Balb/c mice. Encapsulation increases the half life of the ETO with respect to free ETO. The SL showed maximum half life up to 325 min. Biodistribution studies were performed to determine the accumulation of free ETO and various liposomal formulations in tumor bearing Balb/c mice at a dose 10mg/kg. The samples were withdrawn at 0.25, 0.5 1, 2, 4, 8 and 24 h post injection. Liver showed maximum accumulation of the free and liposomal etoposide. The free ETO accumulation in the liver started reducing with time after 0.5 h while in case of the liposomal formulation [CL, NLY, NLE] maximum concentration was seen even
after 4 h post injection i.e. 42.64 ± 4.80, 38.2 ± 4.26, 39.71 ± 5.63 % respectively. However, the percent dose recovered in case of pegylated liposomal formulation at 8 h post injection was found 18.31 ± 2.88, 19.95 ± 2.93, 19.41 ± 0.95 for SL, PLY, PLE receptively. These observations suggested that pegylated formulation successfully avoid RES uptake and therefore its maximum concentration was observed after 8 hour in place of 4 h post injection as observed in case of the conventional liposome. After 4 h, the spleen uptake of CL was approx. 2 times higher than that of SL. The spleen is the second organ in which the high distribution of the ETO was found. The percent recovery of ETO in spleen was found to be of 25.25±2.76, 30.61± 5.00, 26.69±4.78 % for CL, NLY, NLE and lower recovery was found in case of the stealth liposomes and peptide tagged liposomes having PEG linker (15.00±2.10, 12.03±1.74, 12.54±1.41 %, SL, PLY and PLE respectively). The drug concentration was observed constant in the kidney and lung for the all time for free as well as encapsulated ETO. The maximum drug accumulation in tumor was found after 8 h post injection of SL, PLE and PLY formulations.

The higher amount of ETO even after 8 h post injection in tumor was attributed to targeting nature of the peptide tagged liposomes i.e. YIGSR and EILDV tagged liposomal formulations. The tumor uptake of free ETO was maximum 3.11±0.82 % after 1 h post injection. The tumor uptake of CL was significantly higher than that of free ETO 4.53±0.96 %, 5.84 ± 0.91 % after 2 h and 4 h post injection indicated that the size of liposomes lesser than 200 nm increases blood residency due to Enhanced permeability and retention (EPR) effect. In case of SL the maximum tumor accumulation was found after 8 h post injection i.e. 12.85±2.38 %. Comparative higher tumor retention, particularly in case of peptide tagged conventional liposomes, was found to be 8.88±0.42 % and 10.06±1.37 % for NLY and NLE respectively at 4 h post injection.

In metastatic potential studies (in vitro treatment), colony inhibition was used to evaluate the potential of the liposomal formulations. PLE inhibited colonies to the maximum in comparison to free ETO, SL and PLY. The tumor index was low for PLE which indicate that the EILDV tagged liposomes have higher antimetastic potential.
The experimental metastasis was performed on C57/BL6 mice to evaluate the antimetastatic activity of free ETO and liposomal formulations. In this experiment, the metastatic tumor was induced by inoculating B16F10 melanoma cells via tail vein. PLE executed similar inhibitory effect on lung colonization of B16F10 melanoma cells in comparison to free ETO, SL and PLY as the metastatic potential assay. The histopathological slides showed that the peptide tagged liposomes (EILDV and YIGSR) effectively reduced the tumor in comparison to free etoposide and stealth liposomes.

Conclusion:
Etoposide is an important drug in the treatment of various solid tumors. On administration through conventional drug delivery it has been found to exhibit various side effects. In the treatment of cancer targeting is an important criterion. Over the last few decades use of peptide has been exploited for its application in targeting tumor. In the present study liposomal formulations tagged with peptides (YIGSR and EILDV) were evaluated for its efficiency in targeting tumors (B16F10 melanoma cells).

The liposomes were prepared using lipid combination of EPC:HSPC:CH and employing thin film hydration technique which gave formulations of high entrapment and narrow particle size distribution. The freeze drying method employed on these formulations provided good stability to the formulations. In vitro cell line studies performed on B16F10 melanoma cells revealed that the peptide tagged formulations had comparable cytotoxic, anti-migrational, colony inhibiting, cyopathic and antiadhesive activities to free etoposide.

Biodistribution studies showed that the liposomal formulations provided longer circulation and higher accumulation in the tumor in comparison to free etoposide. The anti metastatic activity evaluated by both in vitro treatment to cells as well as treatment to the tumor induced model showed that peptide linkage improved tumor regression activity in comparison to non tagged liposomes as well as free etoposide. EILDV tagged liposomal formulations gave profound activity of tumor regression in comparison to YIGSR.
The above studies showed that peptide tagging with EILDV has its potential in tumor targeting of etoposide. Further studies using clinical trials can provide a better understanding of application of peptides for tumor targeting of etoposide. The lipids that were used in the studies were limited and further combinations of lipids can be studied for good entrapment of etoposide and to make the formulation cost effective.