"DEVELOPMENT OF TARGET BASED THERAPY FOR OVARIAN CANCER"

Summary of the PhD thesis to be submitted to

The Maharaja Sayajirao University of Baroda, Vadodara for the Degree of

Doctor of Philosophy

In

Pharmacy

Submitted by

Ms. PRIYANKA SURESHKUMAR BHATT

M. Pharm

Guided by

Dr. RAJASHREE MASHRU

Associate Professor

Co-guided by

Dr. AMBIKANANDAN MISRA

Professor of Pharmaceutics

Faculty of Pharmacy

The M. S. University of Baroda, Vadodara

May 2017

Name of the Candidate:	Ms. PRIYANKA SURESHKUMAR BHATT
Name of the Research Guide:	Dr. Rajashree Mashru
Name of Research co guide	Prof. Ambikanandan Misra
Subject	Pharmacy
Registration certificate no.	300
Date of Registration	13/8/2013
Title of Thesis	"Development of Target Based Therapy for Ovarian Cancer"
Place of work	Pharmacy Department, Faculty of Pharmacy, The Maharaja Sayajirao University of Baroda, Vadodara – 390001, Gujarat

Ms. Priyanka S. Bhatt	Dr. Rajashree Mashru	Prof. Ambikanadan Misra	Head Pharmacy Department
Candidate	Guide	Co guide	

Forwarded to The Registrar (Examination), The Maharaja Sayajirao University of Baroda, through the Dean, Faculty of Pharmacy, The Maharaja Sayajirao University of Baroda, for further doing the needful.

Dean
Faculty of Pharmacy
The Maharaja Sayajirao University of Baroda

Summary and Conclusion

Cancer can be regarded as the most difficult disease to treat in the present scenario and the therapeutic regimen is in most cases inefficient to achieve complete remission from the disease. Cancer is characterized by abnormal growth of cells which tend to proliferate and spread in an uncontrolled manner. These cells become unrestricted to the normal physiological mechanism of cell senescence and continue to divide and may get metastatized to distal organs also. In women, ovarian cancer (OC) is ranked as the second most prominent cancer of gynaecological origin leading behind the endometrial cancer and is associated with high incidence of mortality. OC is diagnosed annually in nearly a quarter of a million women globally, and is responsible for approx. 140,000 deaths each year. Almost 75% of OC are detected at last stages III or IV thus making the chances of survival poor. According to histogenesis of a normal ovary, the ovarian tumors are categorized depending on their cells of the origin. The three major types of OC include epithelial cell, germ cell and stromal cell type of OCs. Amongst these three, 90% cases are of the epithelial origin. The foremost reason for effectiveness of chemotherapy is due to the fact that most of the cancer are diagnosed at a later stage when the tumor has metastatized. The standard chemotherapy regimen often depends on combination of extremely toxic taxanes and platinum based anti-cancer drugs. However, development of resistance towards chemotherapy and recurrence are often developed mostly in 60 % of the cases at advanced stages of OC. Moreover, non-specific bio-distribution of chemotherapeutic drugs is responsible for severe adverse effects. All the above aspects conclude into a 5-year survival rate of only 20-30% for advanced stage OC patient.

Current, therapeutic approaches for the OC include diagnosis followed by cyto-reductive surgery and chemotherapy. For first-line therapy, cyto-reductive surgery is performed followed by standard chemotherapeutic treatment including platinum compounds (cisplatin and carboplatin) with taxol derivatives (paclitaxel and docetaxel). In spite of several advancements made in the treatment therapy for OC, chemotherapy is

clinically handicapped due to adverse effects, drug resistance, relapse (risk of recurrence) and poor quality of life of patients. Failure of chemotherapy may be due to the tumor developing refractoriness or resistance to the drug. It often originates due to oncogene and tumor suppressor gene mutation, multidrug resistance proteins, p-gp efflux mechanism, altered or mismatched repairing process, failure of apoptosis pathways and mechanisms that inactivate the drug. In most of the cases chances of relapse after the first chemotherapy are frequently encountered in which the standard chemotherapy may not be efficacious. Therefore, current prospects of research in OC treatments are to overcome limitations of both the diagnosis and therapeutic alternatives in OC. Chemotherapy of OC includes anticancer drugs such as paclitaxel, docetaxel, cisplatin, carboplatin, melphalan. In present research work, we have selected Paclitaxel (PTX) which has gained increased importance as a first line anti-neoplastic drug indicated for treatment of ovarian cancer. It is a mitotic spindle inhibitor that enhances polymerization of tubulin to stabilize microtubules at metaphase stage and also interacts with microtubule to prevent their depolymerization leading to cell cycle arrest in anaphase stage. It is a potent inhibitor of cell replication in epithelial ovarian carcinoma, breast cancer, colon, head and neck cancers and non-small cell lung cancer.

Amongst the various carrier systems for drug delivery, liposomes because of their biological inert nature, freedom from antigenic, pyrogenic or allergic reaction and enhanced stability have gained immense importance. Liposomes can encapsulate hydrophilic as well as hydrophobic drug in its aqueous core and lipid bilayer respectively, can be formulated to achieve predetermined release in biological environment. The entrapment of drug in bilayer or core of the liposomes, loading efficiency of drug molecules in the liposomes, release of drug from the liposomes depends on drug to lipid ratio, solubility of drug and it’s partitioning between aqueous and lipidic layer. Liposomes can augment the therapeutic effectiveness of chemotherapeutic drugs, either by increasing the accumulation of drug in tumor cells, improving pharmacokinetic profile, reducing the adverse effects associated with normal tissues damage by following enhanced permeability

and retention (EPR) phenomenon or by utilizing the targeting approaches. It can encapsulate and retain drug molecule to provide higher stability, has long circulation time when PEGylated and can be designed to achieve site specific delivery. Chemotherapy by use of PTX and PTX encapsulated liposomes presents the following major limitations: PTX has limited aqueous solubility and thus, currently supplied with Cremophor EL ® (CrEL)/ (polyethoxylated castor oil) and dehydrated alcohol to improve its solubility for intravenous administration (Taxol®). The vehicle has been observed to cause serious, life-threatening anaphylactoid reactions. The short half-lives, large volumes of distribution and rapid elimination of Taxol® from the body is responsible for partial drug accumulation in the tumor areas with comparatively high drug exposure in normal organs of the body. Non-specific bio-distribution of PTX leads to high toxicity and low therapeutic indices of drugs. Taxol® has short-term physical stability, as out of the aqueous media, some particles slowly tend to precipitate and exhibits incompatibility with the compounds of some intravenous (I.V.) infusion sets. It is observed that the amount of drug loaded in the bilayer is limited and further, for lipophilic drugs such as PTX, that gets incorporated in bilayer may lead to crystallization during preparation and in bilayer that destabilize the membrane leading to premature release of the encapsulate. Conventional PTX liposomes have previously been investigated and found to have low carrier mediated toxicity compared to Taxol® and tested successfully on various *in vivo* experimental models. PTX loading of 3-3.5 mole % (paclitaxel to phospholipids) has been found to be stable for weeks to months. Thus, the loading efficiency of a hydrophobic drug such as PTX in the lipid bilayer usually relies on the drug:lipid molar ratio. It is hard to get high loading efficiency in the lipid bilayers because the space offered by lipid bilayer is limited, and a large amount of hydrophobic drug molecules can destabilize the structural integrity of liposomal bilayers. When liposomes are used as drug delivery systems, it is important that the therapeutic agent is efficiently and adequately encapsulated.

Active targeting of liposomes to tumor cells is generally attempted by conjugating ligands to the liposomal surface which allow a specific interaction with the tumor cells. A

number of proteins are exclusively expressed or greatly over expressed by tumor cells as compared to normal healthy cells. The surface of the liposomes can be decorated with targeting ligands to augment the cellular uptake by receptor mediated endocytosis. Among the different approaches of active targeting, immunoliposomes using antibody or antibody fragment as a targeting ligand and a lipid vesicle as a carrier for both hydrophilic and hydrophobic drugs, is a fascinating prospect in cancer therapy. Immunoliposomes have the potential to transfer large numbers of drug molecules to tumor cells, and drugs delivered via immunoliposomes have antitumor activities similar to or greater than those of the drug alone. The ability of immunoliposomes to target tumor cells overcomes many limitations of conventional liposomes and provides a novel strategy for tumor-targeted drug delivery.

In present work, an attempt has been made to pre-encapsulated an anti-cancer drug, PTX, in cyclodextrin before loading into the liposomes. The PTX and PTX-DM-β-CD (paclitaxel-Heptakis (2,6-di-O-methyl)-beta-cyclodextrin) inclusion complex have been loaded into phospholipid bilayer and aqueous core of PEGylated liposomes respectively (Figure 1). The strategy was furthered by developing PEGylated immunoliposomes decorated with an antibody fragment to the distal end of PEG chain, for site specific delivery of PTX in OC. In present research, we explored Fab′ fragment of anti-FSHR antibody as a targeting ligand to graft on the surface of liposomes. Thus, immunoliposomes double loaded with PTX and PTX- DM-β-CD inclusion complex is hypothesized to actively target OC cells that may reduce side effects of current chemotherapy and may improve intra-tumor drug accumulation for efficient therapy of OC.

Use of validated analytical method is crucial and fundamental component in formulation development. Analytical method required to estimate paclitaxel during preparation, optimization and characterization of its inclusion complex, conventional PEGylated liposomes, double loaded PEGylated liposomes and immunoliposomes were developed and partially validated for aptness of the analysis. The methods for analysis and estimation of paclitaxel in *invitro* media and plasma using RP-HPLC, phospholipids using

Stewart method, plasma proteins using BCA protein estimation, cell lysate proteins using Bardford method, sulphydryl groups estimation using Ellman's assay and 6-coumrin using spectrofluorometer were performed, calibration curves were prepared, regression equation were defined and the methods were partially validated for their linearity, interday and intraday precision and accuracy. In all the cases, the %RSD was 3.0%.

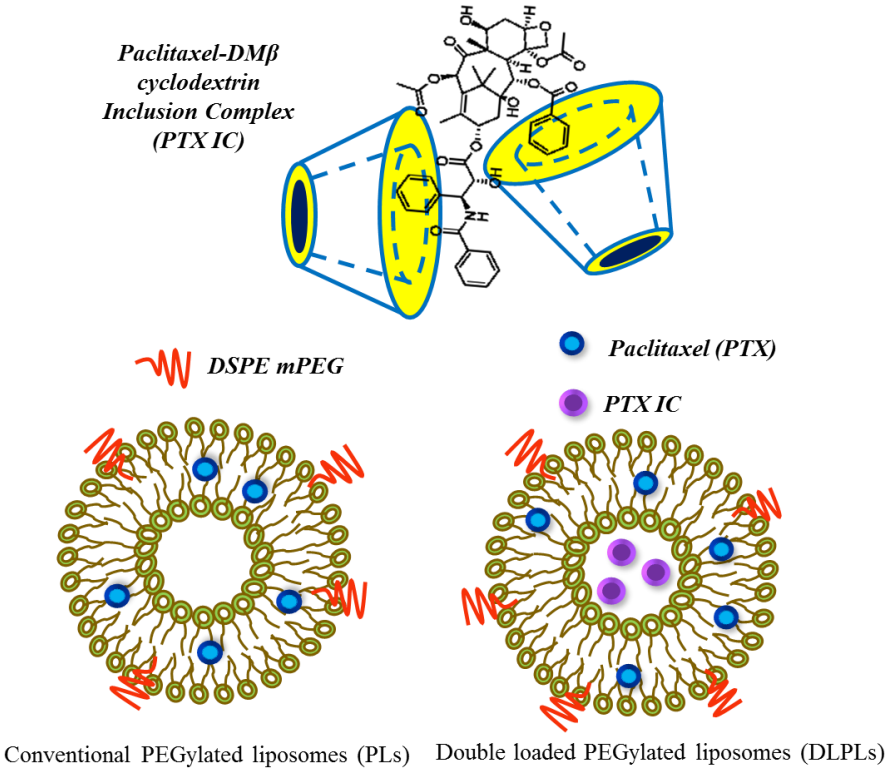

Figure 1: Double loaded PTX PEGylated liposomes

Based on the literature report, DM βCD was selected for inclusion complex (IC) formation as it solubilized the PTX to greatest extent. It was also demonstrated that approx. 100% of added drug got molecularly encapsulated in DMβCD employed at the concentration range tested and further showed linear increase in solubilizing PTX as its aqueous concentration was increased. Further, the increase in solubility did not impacted the cytostatic activity of PTX *in vitro*. PTX-DMβCD ICs were formulated using a modified

co-solvent lyophilization method at varying molar ratios of PTX: DMβCD from 1:1 to 1:25. Based on the highest entrapment of PTX of almost 100% in ICs and physical stability of ICs, the 1:20 ratio was optimized and selected. Solubility of pure PTX in water was found to be 0.38±0.05 µg/mL at 25 ºC while after DMβCD complexation, its solubility increased to 11.1±0.22 mg/ml at PTX: DMβCD molar ratio of 1:20. Formation of complex was confirmed by DSC, SEM, XRD, circular dichroism (Figure 2) and FTIR (Figure 3). PTX-DMβCD ICs in lyophilized form was found to be stable at 2-8 °C for three months.

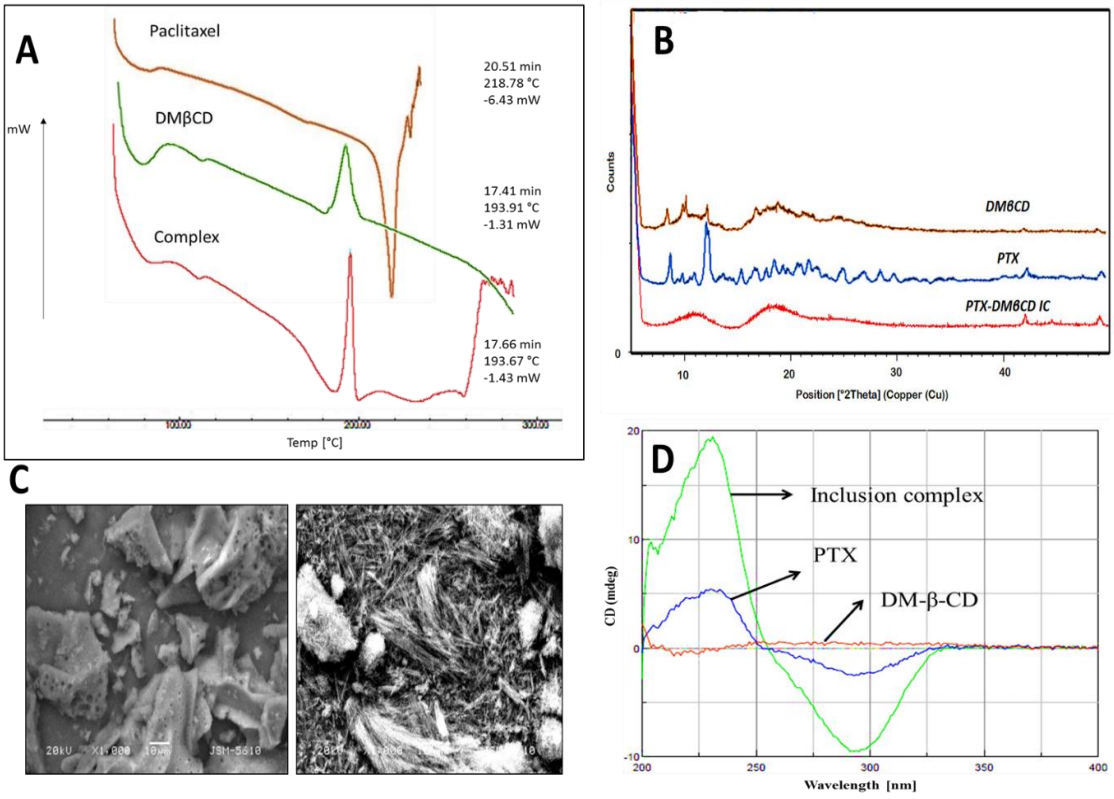

Figure 2: (A) DSC thermograms. (B) X-ray diffraction patterns (C) SEM images PTX-DMβCD ICs (left) and PTX (right) (D) Circular dichroism spectrum

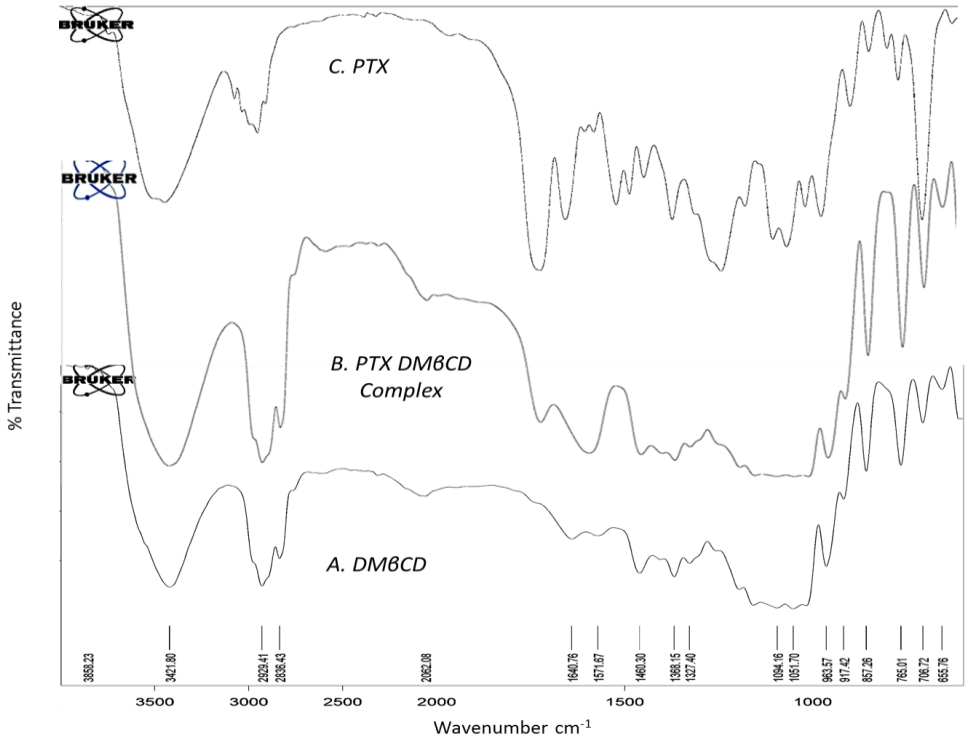

Figure 3: FTIR spectra of (A) DMβCD, (B) PTXD-β-CD ICs and (C) PTX.

For preparation of PEGylated double loaded liposomes, firstly conventional liposomes (CLs) composed of HSPC, Egg PC and Cholesterol incorporating PTX only in bilayer were prepared and optimization of formulation component levels was carried out using D-optimal design. The optimized molar ratio of HSPC: EggPC: Cholesterol:: 22:43:30 with PEGylated lipid at 5 mol% level gave particle size of ~150nm and entrapment efficiency of ~93%. Using this above ratio, PEGylated conventional liposomes (PLs) and PEGylated double loaded liposomes - containing PTX (DLPLs) in aqueous core of liposomes complexed with cyclodextrin along with loading in bilayer, were prepared. Effect of using varying mole% of PEGylating lipid on stability (particle size, zeta potential and protein adsorption) was evaluated using sodium sulphate inducted flocculation test and invitro serum protein adsorption study. The study demonstrated that at 5mole% level of DSPE mPEG2000, the liposomal systems were adequately stable to changes in particle size

due to aggregation and to minimal adsorption of protein that is predicted to prevent their opsonization *invivo* thus favouring the long circulation systemically. Other physicochemical characterization for both the liposomal systems included estimation of their particle size (~150-160nm), zeta potential (-3 to -5 mV), cryo-TEM (confirming unilamellar spherical character), drug loading (~5.8 mole% for DLPLs) and entrapment efficiency (~93%). Further, DSC and FTIR were performed. Herein, the loading for DLPLs was found to be double as compared to PLs as well as CLs. As the DLPLs consisted of cyclodextrin that are known to leach cholesterol from the membranes leading to their destabilization, liposomal membrane integrity test was carried out using carboxyfluorescein dye (CF) incorporation test. Impact of cholesterol incorporation on the stability against leaching was assessed. The incorporation of cholesterol was found important to maintaining integrity of the system as evidenced from the % initial CF latency of 81% for DLPLs containing cholesterol as compared to 68% for DLPL without cholesterol. The prepared PLs and DLPLs were lyophilized to obtain dry powder and stability studies was carried out for DLPLs formulation both in solid state and for suspension form after reconstitution. The liquid state form was stable till two-month time point at 2-8°C, thus indicating that the even after reconstitution, the formulation could maintain desired characteristic for administration. Further, lyophilized form was proposed as drug product presentation for shelf-storage of the developed formulation.

For functionalization of the prepared DLPLs, DSPE mPEG 2000-Maleimie linker was employed at concentration of 1 mol%. Confirmation of functionalization was done by carrying out Ellman's assay which demonstrated 50% efficiency/presence of free thiol group / maleimide functionality available for antibody conjugation. To the above functionalized liposomes, a Fab′ fragments of anti- FSHR monoclonal antibody was armed over the liposomal surface. For preparation of Fab′ fragment, pepsin digestion was carried out to separate Fc portion followed by treatment with reducing agent-DTT (dithiothreitol) that resulted in two Fab′ fragments each of which contains one antibody binding site. Thus, in this work, we generated a purified and immunoreactive $F(ab')_2$ fragments of anti-FSHR

monoclonal antibody using Pierce™ $F(ab')_2$ Micro Preparation Kit containing 25-250 µg of IgG. The entire process of antibody fragmentation and recovery of Fab' fragment for conjugation was confirmed by SDS page and silver staining method. The immunoliposomes were prepared by incubation of Fab′-SH fragments with functionalised PEGylated liposomes loaded with paclitaxel in cold room overnight. The concentration of Fab′ fragments conjugated over liposomal surface was estimated by Bradford protein estimation method which was found to be ~ 13.5 ± 0.15 µg/ml (0.0116 mM/ml of total lipids) thus, % Fab′ fragments conjugated over liposomal surface was found to be 90.49±2.96%. ILs were further characterized for particle size, zeta potential, entrapment efficiency and release of drug and it was found that these characteristics of the liposomal system were similar to that of DLPLs.

To access the performance of the prepared immunoliposomes, invitro cell line studies was carried out. We have evaluated our formulation in suitable ovarian cancer cell lines that express FSHR (Caov3 and OVCAR3). However, it was also important to confirm that our targeted formulation exhibits selectivity and specificity, thus we also incorporated a FSHR negative cell line (SKOV3). In all the above three cell line, FSHR expression was determined by confocal microscopy and flowcytometry using mouse anti-FSHR monoclonal primary antibody followed by incubation with a FITC conjugated goat anti-mouse secondary antibody. The results confirmed that FSHR expression levels of Caov3 cells were higher than OVCAR3 cells and no expression was detected in SKOV3 cells. Immunoreactivity of the prepared ILs were determined against Fab′ in Caov3 cells using flow cytometry and it was observed that after conjugation with liposomes, Fab′ have remained intact and also maintained the binding characteristic to the receptor. The MTT assay was performed to assess cell cytotoxic potential of marketed formulation Taxol®, blank liposomes and the prepared formulations, ICs, PLs, DLPLs and ILs, in ovarian cancer cell lines SKOV3 and Caov3. It was observed for SKOV3 cells, that at the end of 24 hr, the IC_{50} value of ILs were 2.35, 1.26 and 1.04 folds lower compared to Taxol®, PLs and DLPLs respectively and at the end of 48 hr, the IC_{50} value for ILs was found to be 4.44,

2.65 and 1.05 folds lower than the above three treatment groups. Similarly, for Caov3 cells, at the end of 24 hr, IC_{50} value of ILs were 8.09, 4.45 and 3.37 folds lower compared to Taxol®, PLs and DLPLs respectively and at the end of 48 hr, the IC_{50} value for ILs was found to be 10.8, 7.8 and 3.5 folds lower than the above three treatment groups. To prove, whether immune-tagging improves cell specific uptake of liposomes in FSHR expressing cells, cell uptake studies were carried out using confocal microscopy in SKOV3 and Caov3 cells which showed higher uptake in Caov3 cells (Figure 4). Further, to demonstrate the immune-targeting potential of liposomes, plain (non-targeted) and anti-FSHR antibody targeted coumarin loaded liposomes were prepared and the uptake in Caov3 cell lines was evaluated over time period of 15 min, 30 min, 45 min and 60 min, which demonstrated higher uptake in case for targeted formulation. The above results were also confirmed by flowcytometric analysis at various time points.

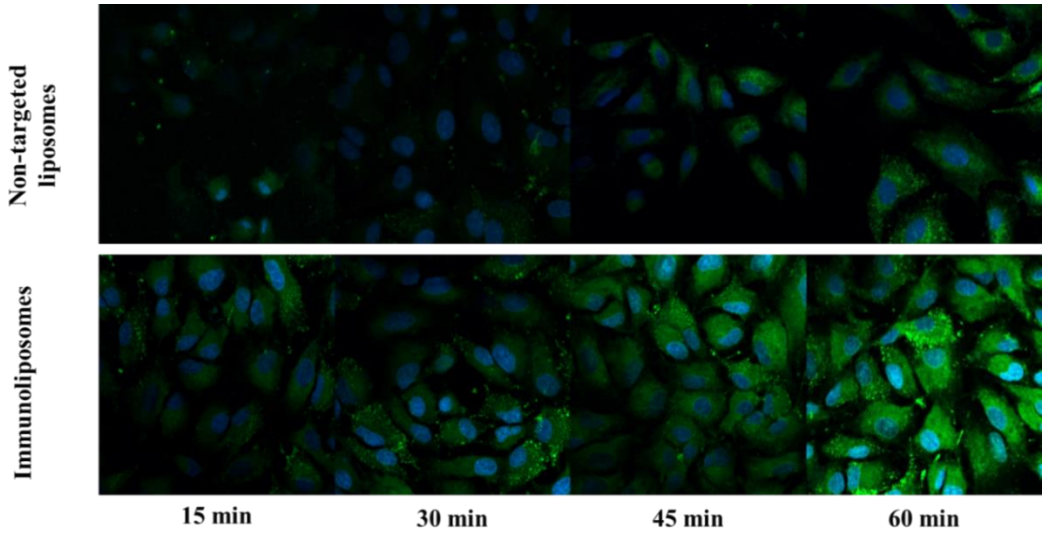

Figure 4: Specific uptake of coumarin 6–loaded non-targeted PLs and immunoliposomes (ILs) in FSHR-expressing Caov-3 cells incubated with 10 µg/mL of PLs and ILs for 15, 30, 45, and 60 min.

For assessment of migration of cell and to testify the potential *in-vivo* anti-angiogenic activity, wound scratch assay was performed on Caov3 cells. For untreated well (control), the recovery of wound occurred to greatest extent covering the entire surface of wound

made at initial time point. In treatment group for DLPLs and ILs, concentration dependent inhibition of wound recovery was observed. At equimolar concentration of treatment given to the cells by DLPLs and ILs, the % recovery of the wound was 1.3 times and 2.3 times lower than Taxol® respectively indicating the superior performance of liposomal formulation in inhibiting cell proliferation. The quantitation of cell death after treatment with various formulations was carried out by FACS after staining with propidium iodide. From the values obtained for SKOV3 cells and Caov3 cells, the highest amount of cell death was seen in ILs group for Caov3 cells (Figure 5).

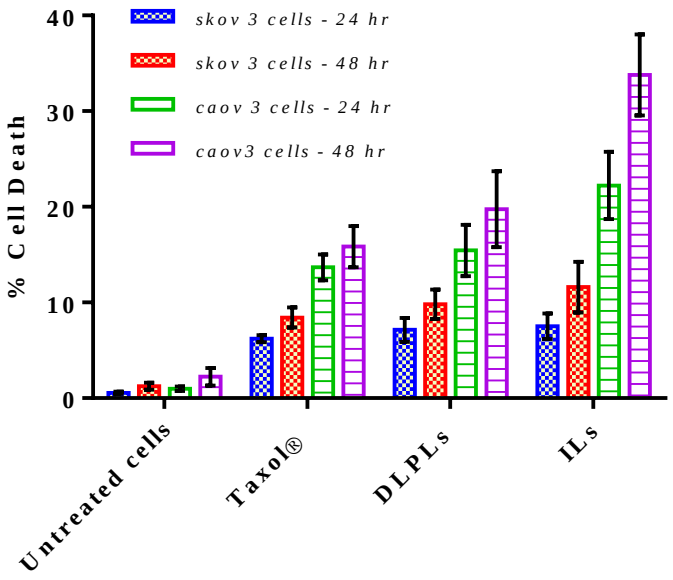

Figure 5: % cell death after 24 and 48 hr treatment with different formulations in SKOV3 and Caov3 cells

Evaluation of cell cycle in the Caov3 cell line, which is a receptor positive cell line indicated superior results of arrest of cells in G2/M phase. For Taxol®, the amount of cells in G2/M increased by 13 % at the end of 24 hr and 6 % at the end of 48 hr. In case of prepared liposomal formulation, the effect was more pronounced leading to an increase to 22% and 13% for DLPLs and 28% and 16% for ILs at the end of 24 hr and 48 hr

respectively. Further, an increase in the cell death was observed as indicated by the increase in percentage of cells in subG1 stage of cell cycle. Performance of DLPLs were similar in Caov3 and SKOV3 cell line leading to almost around 22% and 12% of arrest in G2/M stage, however, for targeted liposomal formulation i.e. ILs, the amount of cells in G2/M was highest for Cao3 cells at the end of 24 hr and 48 hr indicating that the targeted formulation was able to better get internalized in the cells due to receptor mediated uptake (Figure 6).

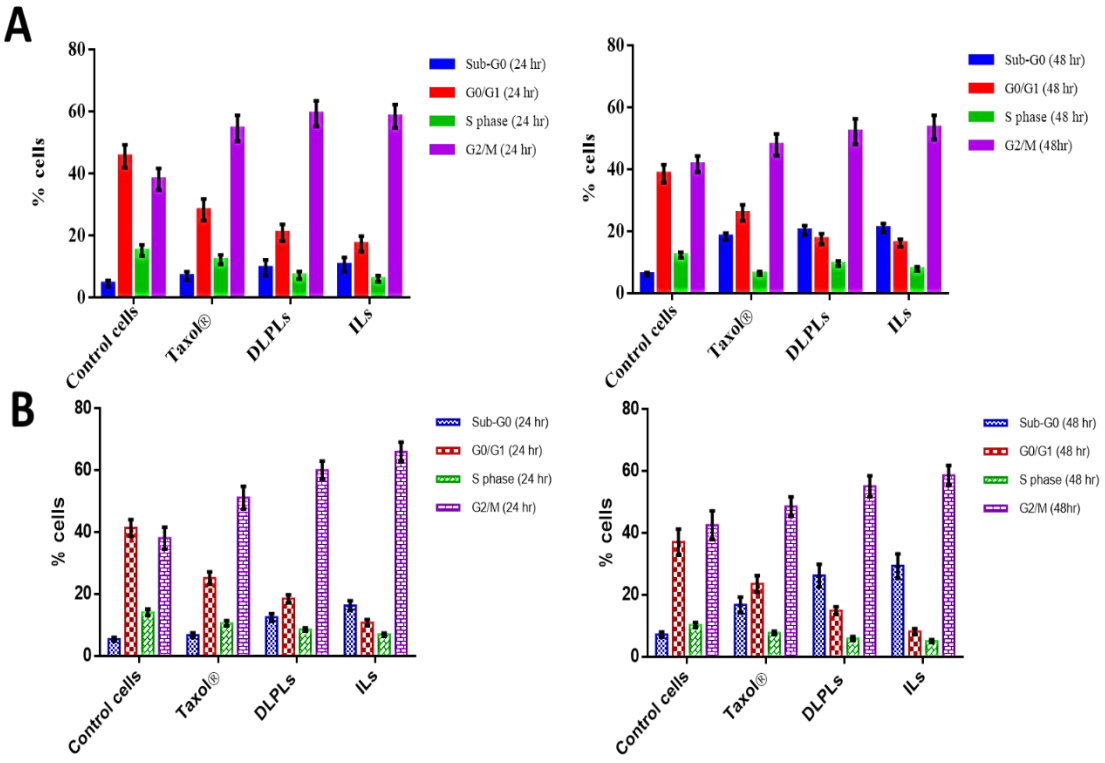

Figure 6: % Cell in various phase of cell cycle after 24hr and 48 hr treatment of (A) SKOV3 cell line and (B) Caov3 cells with Taxol®(PS), DLPLs and ILs

Finally, to access the *invivo* performance of liposomal system, hemolysis study, acute toxicity study and pharmacokinetic study were performed. Hemolysis assay is a useful *in vitro* hematological test as an indicator of toxicity and physiologic compatibility

of the formulation. At highest concentration tested, i.e. 5 mg/ml, for Taxol® hemolysis was ~48.5 %, whereas that for DLPLs and ILs was only 12.10 % and 11.59% respectively ($p<0.05$). The relative % hemolysis for both the formulations (DLPLs and ILs) at 0.5 and 5 µg/ml were approximately 4.5-fold and 4-fold lower respectively than the corresponding tested concentration of the Taxol® (Figure 7).

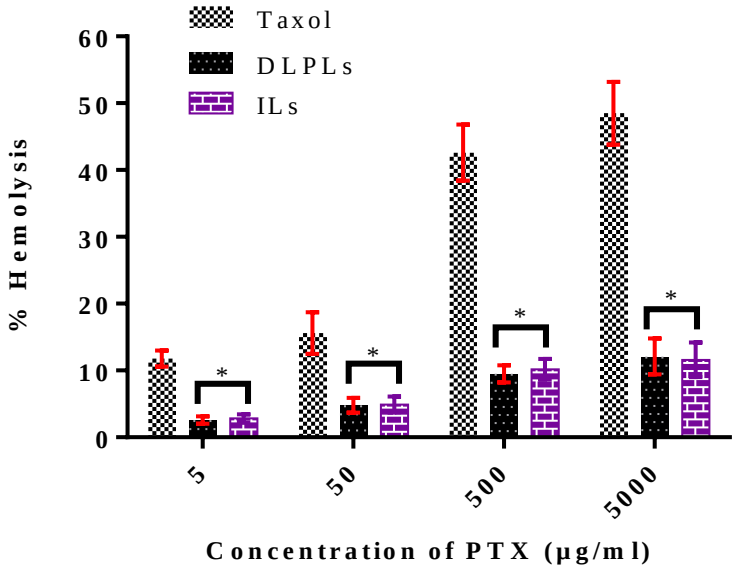

Figure 7:Hemolytic potential of DLPLs and ILs in comparison to Taxol® (*p<0.05)

The plasma concentration-time profiles of PTX after single intravenous bolus administration of the PTX formulations (5 mg/kg) were characterized in Sprague Dawley rats. PTX in Taxol® was quickly eliminated after intravenous administration with MRT and $t_{1/2}$ of 3.61 and 2.82 hr, respectively while the values of MRT and $t_{1/2}$ in the treatment groups significantly increased to 12.89 hr and 12.24 hr for DPLPs and 10.68 hr & 12.46 hr for ILs respectively. This indicates that DLPLs and ILs were able to act as PTX reservoirs in blood giving a prolonged release profile. Consequently, the resultant values of AUC in the DLPLs and ILs treatment groups were significantly increased of the order of 4.37 and

4.10 folds compared with those in the Taxol®. Acute toxicity study was carried out to identify toxicity profile of DLPLs in comparison to Taxol®. The study was conducted in the dose range of 6 to 192 mg/kg and was administered by intra venous route (i.v). MTD for DLPLs and Taxol® was found to be 120 and 12 mg/kg, respectively. The LD_{50} was found to be 136.9 mg/kg and 19.7 mg/kg, respectively for DLPLs and Taxol®. Thus, the MTD and LD_{50} for DLPLs were 10-folds and 7-fold higher compared to Taxol®. The results confirmed the safety of PTX-liposome formulation in healthy female BALB/c mice than Taxol® formulation after a single I.V. dose.

Simultaneous incorporation of drug in liposomal core by pre-encapsulation of hydrophobic drug in form of complex with cyclodextrin along with incorporation in bilayer presents promising strategy for improving the loading efficiency for therapeutic anti-cancer agent like PTX that has non-ionizable functional group and thus presents a formulation challenge of low loading in conventional liposomes as well as associated stability issues. At the employed molar complexation ratio of PTX to DMβCD, stable ICs were obtained and were successfully incorporated in liposomal core during preparation by thin film hydration method. The DLPLs were superior in performance compared to Taxol® with prolonged release profile, a low hemolytic potential on RBCs and higher cytotoxicity as well as anti-proliferative activity on cancer cell line. Pharmacokinetic advantage for the double loaded system was evidenced from the longer circulation time and increase in plasma concentration in rats along with a lower toxicity potential tested in mice in comparison to Taxol®. The results show that the prepared double loaded liposomal systems can address the issues of low loading, hemotoxicity, and specific targeting to the tumor cells that are the main limitations of the currently marketed PTX formulation. Such double loading approach for encapsulation of hydrophobic drugs in the form of water soluble form in the liposomal core can be utilized to improve the loading and may also serve to decrease the total quantum of dose required to be injected during chemotherapy. The use of fragment of monoclonal antibody, devoid of immunogenic potential or non-specificity to interaction with other receptors, and to target only specific receptors overexpressed in cancer can be

explored to direct chemotherapeutic drug to sites only where they are required. The present research provides a feasible approach to achieve these objectives. The optimistic outcome of invitro studies proves the potential effectiveness of the formulation in ovarian cancer.