Chapter 8

SUMMARY AND CONCLUSION

8.1 Summary

Breast cancer is the most frequently diagnosed form of cancer and second leading cause of death in women. The incidence of breast cancer is still rising, and the disease remains largely incurable once it becomes malignant. Estrogen is important factor in development of breast cancer. When it binds with estrogen receptor, it becomes activated resulting in stimulation of tumor growth. This receptor has been found to be overexpressed in about 70-80% of the breast cancer patients. Despite the significant progress in the development of anticancer technology, there is still no common cure for patients suffering from with this malignant disease. Traditional chemotherapy relies on the premise that rapidly proliferating cancer cells are more likely to be killed by a cytotoxic drug. However, in reality, cytotoxic agents have very little or no specificity as very less concentration of drug reaches tumor site, which leads to systemic toxicity. Current treatment methods either reduce estrogen levels or block estrogen-receptor using aromatase inhibitors or anti-estrogens. However, the lack of specificity of currently available chemotherapeutic agents constitutes a major obstacle to the treatment of cancer. In addition, the long-standing problem of chemotherapy is the lack of tumor-specific treatments. Therefore, targeting is required to ensure high efficiency of the drug at tumor site with reduced side effects when dealing with drugs that are presumed to kill cancer cells along with healthy cells. The drug loaded polymeric nanoparticles can be tagged with specific ligands like monoclonal antibodies by synthetic or biochemical means to form a tumor-selective conjugate. Administration of such conjugates leads to the accumulation of drug loaded nanoparticles preferentially in the tumor without significant distribution to normal tissues, leading to high specificity and reduced systemic toxicity. The reduction or prevention of side effects can also be achieved by controlled release. Nanoparticulate drug delivery systems provide a better penetration of the particles inside the body as their size allows delivery via intravenous injection or other routes. The nanoscale size of these particulate systems also minimizes the hypersensitive reactions at the injection site. nanoparticles may be targeted to the growing vasculature serving the growing cancer or to the cancer cells themselves. Targeted delivery utilizes unique phenotypic features of diseased tissues and cells in order to concentrate the drug at the location where it is needed. Active targeting utilizes biologically specific interactions including antigen–antibody and ligand–receptor binding and may seek drug uptake by receptor mediated endocytosis through association of the drug or drug carrier with such antigen or ligand.

Estrogens and progesterone are important factors in the development of breast cancer. The majority of breast tumors found in postmenopausal women contain estrogen receptor (ER) while tumors in younger women often lack this protein. Approximately 70% of all breast cancers retain the estrogen receptor α (ERα, encoded by ESR1) and the progesterone receptor. When estrogen receptor binds estrogen, it becomes activated and can interact with the genes of the cell, resulting in the activation of selected sets of responsive genes. This results in changes in the synthesis of specific RNA's and proteins involved in the regulation of cell proliferation, differentiation and physiological functions. Although normal breast tissue retains estrogen receptor, the amount of this protein produced in positive breast carcinomas is significantly higher. This may account for some of the differences seen in the abnormal growth of various tumors and tumor cell lines when compared to normal breast tissue development.

An alternate strategy to endocrine therapy, which specifically inhibits binding of E2 to the ER, is to inhibit the production of E2 by blocking the cytochrome p450 aromatase enzyme, the rate-limiting enzyme that converts androgens (i.e., testosterone and androstenedione) to estrogens (i.e., E2 and estrone). Anastrozole is one of the second generation Type II (nonsteroidal) aromatase inhibitors which act by binding reversibly to the enzyme and competitively inhibiting binding of the substrate androstenedione. Steroidal inhibitors are competitive-substrate mimics of androstenedione. Exemestane, an irreversible steroidal inhibitor, which bind with high affinity to the binding site of aromatase and are converted to a covalently bound intermediate. The benefits of using aromatase inhibitors over tamoxifen, are believed to be the complete deprivation of E2 and thus better efficacy for ERα-positive breast cancer. Recent clinical data have clearly demonstrated that anastrozole is more effective than tamoxifen as first-line treatment in patients with metastatic breast cancer. Two very small trials demonstrated a significantly increased clinical response rate in patients with ER-positive, HER2-positive cancers treated with preoperative aromatase inhibitors compared to preoperative tamoxifen. This led to a widely accepted hypothesis that aromatase inhibitors were a better choice than tamoxifen in patients with ER-positive, HER2-positive cancers. Hence, the present investigation was aimed at conjugation with estrogen receptor antibody with the objective of ligand based targeting of nanoparticles. The objective of this study is to develop nanoparticulate drug delivery system composed of biocompatible and biodegradable polymers like poly(lactic co-glycolic acid) and poly(ε-caprolactone), using anticancer drugs (anastrozole, exemestane) for enhancing tumor uptake by ligand (antibody) specific breast targeting and thereby, reducing systemic side-effects of the drug.

Analytical method for the estimation of drugs

The reported HPLC method was used to analyze the content of anastrozole and exemestane separately with some modifications. The standard calibration curve was developed in mobile phase in the range of 1 to 50 µg/ml and linearity was obtained. The retention time obtained was 5.2 ± 0.1 min for anastrozole and 5.0 ± 0.1 min for exemestane.

Preparation of Conjugates and nanoparticles

Caprolactone was successfully polymerized to Polycaprolactone containing carboxylic group and this was confirmed by TLC, FTIR and GPC. Both the polymers (PLGA and PCL) were PEGylated using amine-PEG-carboxylic acid by amide bond formation between carboxylic group present in polymers and amine group present in PEG. Free carboxylic group in PEG was further utilized for formulation of antibody conjugated nanoparticles (Immunonanoparticles) by conjugation with amine group present in antibody. Each reaction step as well as purification step were monitored by TLC using 100% ethyl acetate as a mobile phase and iodine as a spotting reagent. PLGA-PEG and PCL-PEG were successfully characterized by FTIR, NMR and GPC.

Anastrozole loaded PLGA nanoparticles were optimized for high entrapment efficiency and lowest particle size using 3^3 full factorial design. The independent variables were drug:polymer ratio, polymer concentration and surfactant concentration. The optimum formulation offered by the software (Design Expert 8.0) based on desirability was found at 0.68, 1, and -1 level of drug:polymer ratio, polymer concentration in organic phase and surfactant concentration in aqueous phase respectively. The results of dependent variables from the software were found to be 61.39% for percentage drug entrapment and 144.68 nm for particle size at these levels which is as per our desired criteria. The calculated desirability factor for offered formulations was 0.990. The optimized batch of anastrozole loaded PLGA nanoparticles was found to have zeta potential of -30.7 ± 1.4 mV. DSC and TEM studies indicated absence of any drug-polymer interaction and spherical nature of nanoparticles, respectively. Pegylated nanoparticles were optimized based on percentage drug entrapment, particle size and phagocytic uptake studies on THP1 cells by FACS. From the results, it was observed that as the amount of PEG increased, percentage drug entrapment, particle size and zeta potential decreased. Anastrozole was successfully loaded in pegylated PLGA nanoparticles prior to Ab conjugation (61.29 ± 1.18%) and no significant loss in drug content was observed after surface functionalization with Ab (60.62 ± 0.54%). However, slight increase in particle size was observed which increased from 126.6 ± 4.1 to 140.4 ± 3.7 nm with slight decrease in zeta potential from -24.1 ± 3.4 to -23.6 ± 4.0 mV. The in vitro drug release studies from different nanoparticulate formulations were performed in PBS pH 7.4. Pegylated nanoparticles showed faster release as compared with non-pegylated nanoparticles. Within 3 h, 82.56 ± 0.62% drug release occurred from plain drug suspension, whereas only 24.14 ± 0.31% and 28.90 ± 1.03% drug released from PLGA and pegylated PLGA nanoparticles, reaching 48.02 ± 0.56% and 60.29 ± 0.85% after 120 h and 64.9 ± 0.24% and 83.04 ± 0.55% after 240 h from PLGA and pegylated PLGA nanoparticles, respectively indicative of sustained release. In-vitro drug release showed biphasic pattern exhibiting Fickian diffusion based release mechanism.

Anastrozole loaded PCL nanoparticles were optimized for high entrapment efficiency and lowest particle size using 3^3 full factorial design. The optimum formulation offered by the software based on desirability was found at 1.0, 0.69, and -1.0 level of drug:polymer ratio, polymer concentration and surfactant concentration respectively. The results of dependent variables from the software were found to be 70.21% for percentage drug entrapment and 200 nm for particle size at these levels which is as per our desired criteria. The calculated desirability factor for offered formulations was 0.965, which was near to 1 and indicates suitability of the designed factorial model. The optimized batch of anastrozole loaded PCL nanoparticles was found to have zeta potential of -32.1 ± 1.1 mV. DSC studies indicated spherical nature of nanoparticles and DSC thermogram of drug, polymers, physical mixtures and nanoparticles indicated no chemical interaction amongst them. In vitro release of anastrozole from plain drug suspension and nanoparticles after 3 h exhibited 82.56 ± 0.62% drug released from plain drug suspension, whereas only 21.36 ± 0.41% and 26.57 ± 1.81% drug release occurred from PCL nanoparticles and pegylated PCL nanoparticles, reaching 44.14 ± 0.69% and 56.71 ± 0.67% after 120 h and 58.22 ± 0.99% and 82.21 ± 0.74% after 240 h from PCL and pegylated PCL nanoparticles, respectively indicative of sustained release.

The drug release from nanoparticles followed biphasic release model with an initial burst release in about first 3 h followed by sustained release for more than 240 h. The regression coefficient of the plot of log M_t/M_∞ versus log t for nanoparticles was found to be 0.935 and 0.957 with value of release exponent (n) as 0.273 and 0.275 for PCL and pegylated PCL nanoparticles. The n value is the release exponent which characterizes the transport mechanism and if its value is less than 0.5, it indicates Fickian release.

Exemestane loaded PCL nanoparticles were optimized for high entrapment efficiency and low particle size using Box-Behnken design. The independent variables were drug:polymer ratio, amount of polymer and volume of organic phase. The optimum formulation offered by the Design Expert 8.0.3 software based on desirability was found at 0.43, -0.68, and 0.27 level of drug:polymer ratio, amount of polymer and volume of organic phase, respectively. The calculated desirability factor for offered formulations was 1, which indicated suitability of the designed factorial model. The results of dependent variables from the software were found to yield 83.96% percentage drug entrapment and 180.51 nm particle size at these levels. Data analysis using student's 't' test revealed that there was no statistically significant difference ($p<0.05$) between experimentally obtained values and predicted values by regression analysis and hence, it confirms the utility of the established contour plots and reduced polynomial equation in the preparation of nanoparticles. The optimized batch of exemestane loaded cPCL nanoparticles was found to have zeta potential of -33.8 ± 2.1 mV. TEM image showed discrete spherical particles with monodisperse size of about 100 nm. DSC studies indicated absence of any drug-polymer interaction. Phagocytic uptake of PCL nanoparticles was compared with pegylated nanoparticles containing different quantity of PCL-PEG (5, 10, 15and 20%) in terms of mean fluorescence intensity. Pegylated nanoparticles displayed resistance to phagocytic uptake when compared to non-pegylated nanoparticles. Among various tested formulations, PCL-PEG (20%) showed minimum uptake by THP1 cells. After 1 h, uptake of PCL nanoparticles was 1.33, 2.30 and 9.06 times more than PCL-PEG (5%), PCL-PEG (15%) and PCL-PEG (20%) nanoparticles. Exemestane was successfully loaded in pegylated PCL nanoparticles prior to Ab conjugation and no significant loss in drug content was observed after surface functionalization with Ab (82.11 ± 1.79%). However, slight increase in particle size was observed. The particle size increased from 168.9 ± 2.9 to 179.8 ± 4.1 nm with slight decrease in zeta potential from -27.1 ± 0.9 to -24.3 ± 1.2 mV. Poly dispersity index before and after Ab conjugation was less than 0.1 indicating unimodal distribution of particles. Pegylated PCL nanoparticles showed faster release as compared with non-pegylated nanoparticles. Within 3 h, 71.36 ± 1.23% drug release occurred from plain drug suspension, whereas only 20.06 ± 1.31% and 24.88 ± 1.13% drug released from PCL and pegylated PCL nanoparticles, reaching 44.89 ± 1.3% and 52.22 ± 3.1% after 120 h and 70.67 ± 1.76% and 83.26 ± 0.85% after 240 h from PCL and pegylated PCL nanoparticles, respectively indicative of sustained release.

Exemestane loaded PLGA nanoparticles were optimized for high entrapment efficiency and low particle size using Box-Behnken design. The optimum formulation offered by the Design Expert 8.0.3 software based on desirability was found at 0.41, -0.09 and 0.75 levels of X_1, X_2 and X_3 respectively. The calculated desirability factor for offered formulations was 1, which indicated suitability of the designed factorial model. The results of dependent variables from the software were found to yield 78.37% percentage drug entrapment and 162.28 nm particle size at these levels. Calculated t value ($t_{calculated}$) for percentage drug entrapment and particle size was found to be 0.6411 and 0.0699, respectively which was less than the tabulated t value of 2.9199 which indicates that there was no statistically significant difference ($p<0.05$) between experimentally obtained values and predicted values by regression analysis and hence confirms the utility of the established contour plots and reduced polynomial equations in the preparation of nanoparticles. The optimized batch of exemestane loaded cPCL nanoparticles was found to have zeta potential of -29.5 ± 1.4 mV. TEM image reveals that the particles were discrete, round and uniform in shape with diameters in the range of 80–100 nm. DSC studies indicated absence of any drug-polymer interaction. In vitro release of exemestane from drug suspension and nanoparticles showed 71.36 ± 1.23% drug release occurred from plain drug suspension, whereas only 25.18 ± 0.56% and 20.21 ± 0.23% drug released in 3 h from PLGA and pegylated PLGA nanoparticles, reaching 48.05 ± 0.94% and 54.25 ± 0.23% after 120 h and 71.4 ± 1.23% and 73.9 ±0.86% after 240 h from PLGA and pegylated PLGA nanoparticles, respectively indicative of sustained release. The drug release from nanoparticles followed biphasic release model with an initial burst release for about 3 h followed by sustained release for more than 240 h. The regression coefficient of the plot of log M_t/M_∞ versus log t for PLGA and pegylated PLGA nanoparticles was found to be 0.935 and 0.984 with value of release exponent (n) as 0.271 and 0.312, respectively indicating Fickian release..

No significant difference between predicted and observed responses was observed in check point analysis with very less normalized error in all the optimization statistical designs.

Nanoparticles were lyophilized using different cryoprotectants like trehalose, sucrose, lactose and mannitol. Out of these, sucrose was found to be the best cryoprotectants causing minimum increase in particle size after re-constitution.

A comparative expression of estrogen receptor in breast cancer cell lines (MCF7 and MDAMB231) was estimated by Western blot analysis. The results confirmed the presence of estrogen receptors in MCF7 cells and absence of the same in MDAMB231 cells.

The 6-coumarin dye acts as a better fluorescent probe due to its low pH-dependent solubility and high fluorescence activity. Using the intrinsic fluorescence property of 6-Coumarin, a comparative analysis of intracellular uptake behavior of 6-Coumarin loaded immunonanoparticles, PEGylated and non-PEGylated nanoparticles were performed in MCF7 cell line by flow cytometry (Quantitative) and fluorescence microscopy (Qualitative). The relative extent of cellular uptake of native 6-Coumarin, 6-Coumarin loaded PEGylated and non-PEGylated nanoparticles, and 6-Coumarin loaded immunonanoparticles was calculated in terms of mean fluorescence intensity (MFI) exhibited by the cells. The fluorescence microscopy results clearly indicate that cellular uptake of nanoparticles were in the order of immunonanoparticles > PEGylated nanoparticles > non-PEGylated nanoparticles > 6-Coumarin which was confirmed by quantitative uptake studies by flow cytometry.

Flow cytometry studies indicated the relative extent of PLGA nanoparticles, pegylated PLGA nanoparticles and immunonanoparticles was calculated using mean fluorescent index (MFI). MFI of pegylated PLGA immunonanoparticles was 1039.10 ± 17.77 which was more than 3 times than that of PLGA nanoparticles (318.27 ± 21.69) after 180 min. Furthermore, pegylated PLGA immunonanoparticles showed about 2 times more uptake when compared with pegylated PLGA nanoparticles (599.95 ± 25.17) at same time point due to receptor mediated endocytosis. The MFI of pegylated PCL immunonanoparticles is 1261.62 ± 39.27 which is about 2 times than that of PCL nanoparticles (730.19 ± 38.68) after 180 min. Furthermore, immunonanoparticles showed about 1.32 times more uptake when compared with pegylated PCL nanoparticles (956.45 ± 33.45) at same time point. Intracellular uptake was found to increase with time for all formulations from 60 to 180 min. The intracellular uptake of nanoparticles was also dependent on the incubation time. The fluorescence intensity increased gradually with the incubation time and so the uptake of nanoparticles. Results clearly indicates the enhanced cellular uptake of immunonanoparticles than un-conjugated nanoparticles.

In vitro cytotoxicity studies were carried out at different concentration of nanoparticles (0.03, 0.3, 3, 15 and 30 μM) and incubated for different time periods (6, 24, 48 h) with drug solution, blank nanoparticles and drug loaded PEGylated, non-PEGylated and immunonanoparticles. The toxicities by drug loaded immunonanoparticles were found to be significant as compared to drug loaded PEGylated and non-PEGylated nanoparticles in MCF7 cells. Toxicity by all the drug loaded nanoparticulate systems was significant when compared with drug solution in MCF7 cells.. The IC_{50} value for ER positive cells treated with PLGA immunonanoparticles was found to be 165.28, 52.44 and 27.95 times lower than anastrozole, anastrozole loaded PLGA nanoparticles and PLGA-PEG nanoparticles respectively, after 6 h of treatment. Exposure for 48 h further lowered the IC_{50} value by 3.46 times to 0.0828 μM which was 8 times less in comparison with PLGA-PEG nanoparticles.

The IC_{50} value for ER positive cells treated with PCL-PEG ImmunoNPs was found to be 158.2, 26.73 and 4.27 times lower than ATZ, ATZ PCL NPs, PCL-PEG NPs respectively, after 6 h of treatment. Exposure for 48 h further lowered the IC_{50} value by 6 times to 0.05 μM which was more than 6.6 times less in comparison with ATZ PCL-PEG NPs. To confirm receptor mediated endocytosis, cytotoxicity studies were also performed on receptor negative cells (MDAMB231) and results clearly indicated that the effect of different NPs on MDAMB231 cells when compared to MCF7 cells. No significant toxicity was found on MDAMB231 cells. Thus, the enhanced cytotoxic activity of PCL-PEG ImmunoNPs for MCF7 cells as compared with MDAMB231 cells clearly demonstrates the high affinity of ImmunoNPs towards ER positive cancer cells.

In vitro cytotoxicity studies were carried out at different concentration of nanoparticles (0.03, 0.3, 1.5, 3 and 15 μM) and incubated for different time periods (6, 24, 48 h) with drug solution, blank nanoparticles and drug loaded PEGylated, non-PEGylated and immunonanoparticles. The IC_{50} value for ER positive cells treated with exemestane loaded PLGA-PEG Immunonanoparticles was found to be 7.5, 4.4 and 2.1 times lower than exemestane, exemestane PLGA nanoparticles, exemestane PLGA-PEG nanoparticles respectively, after 48 h of treatment. The IC_{50} value for ER positive cells treated with Immunonanoparticles was found to be 4.7, 3.1 and 1.8 times lower than that of exemestane, exemestane PCL nanoparticles and exemestane PCL-PEG nanoparticles after 48 h exposure respectively. To confirm receptor mediated endocytosis, cytotoxicity studies were also performed on receptor negative cells (MDAMB231) and results clearly indicated that the effect of different nanoparticles on MDAMB231 cells when compared to MCF7 cells. No significant toxicity was found on MDAMB231 cells. Thus, the enhanced cytotoxic effect of immunonanoparticles could be attributed by higher uptake via receptor mediated endocytosis.

Different drugs are known to have a variety of pharmacological actions, including their effect on genes controlling the cell cycle and induction of pro- and anti-apoptotic genes. Drug loaded nanoparticles inhibit cell growth and proliferation primarily through cell cycle arrest and apoptosis inducing mechanism. Cell cycle analysis demonstrates the arrests of cell cycle by drug solution and drug loaded nanoparticulate formulations at different phases like G1 phase, S phase or G2 phase.

Results of cell cycle analysis demonstrated that anastrozole treated cells showed stronger arrest at G0/G1 phase as compared to nanoparticulate formulations. immunonanoparticles showed lesser percentage of cells at G0/G1 phase (50.1%) as compared to 69.9% and 63.6% cells in PBS (control) and anastrozole treated cells. However, exposure to immunonanoparticles showed stronger arrest at G2/M phase (29.3%) which was almost 1.7 times higher than anastrozole drug solution. The treatment with anastrozole, anastrozole PCL-PEG nanoparticles, Immunonanoparticles to MCF7 cells showed a S, G2/M arrest of (16.4, 17.2%), (14.2, 24.8%), and (19.6, 29.3%) compared to control (12.7, 16.0%).

Results of cell cycle analysis demonstrated that exemestane treated cells showed stronger arrest at G0/G1 phase as compared to nanoparticulate formulations. Immunonanoparticles showed lesser percentage of cells at G0/G1 phase (45.3%) as compared to 68.6% and 60.2% cells in PBS (control) and exemestane treated cells. However, exposure to PLGA-immunonanoparticles showed stronger arrest at G2/M phase (34.2%) which was almost 1.6 times higher than exemestane drug solution. The results indicated that exemestane loaded PCL Immunonanoparticles showed a significant G2/M arrest compared to control, exemestane solution and PCL-PEG nanoparticles. The arrest was almost 1.6 times greater than the drug solution which correlates with the findings that ligand conjugated nanoparticles were more effective in controlling the cell cycle phases as compared to drug solution in MCF7 cells.

To assess the ability of immunonanoparticles to induce apoptosis in MCF7 cell lines, apoptosis study was conducted using standard Annexin V staining procedure. The intensity plot of FITC vs. Texas Red was sectioned in four quarter to differentiate stained and unstained cells. Based on four quarters percentage of cells in early apoptotic cells (FITC positive and PI negative), late apoptosis (FITC and PI positive), necrotic (FITC negative and PI positive) and live cells (FITC and PI negative) were calculated. The results demonstrated higher fraction of total cell death in MCF7 cell line treated with immunonanoparticles than un-conjugated nanoparticles or native drug in solution following site-specific sustained release pattern. No difference in total cell death treated by immunonanoparticles or un-conjugated nanoparticles in MDAMB231 cells was observed. The augmented apoptotic activity of immunonanoparticles in receptor positive cells (MCF7) can be correlated with the cellular uptake results showing higher endocytosis of immunonanoparticles.

The results showed significant fraction of necrotic, early and late apoptotic population of cells after exposure with anastrozole solution, anastrozole loaded PLGA nanoparticles, PLGA-PEG nanoparticles and immunonanoparticles. Control group treated with PBS showed only 1.6% and 3.6% cells in apoptotic phases. After 24 h of exposure with anastrozole solution, only 5.4% cells were in apoptotic phase (early and late apoptosis) whereas exposure with anastrozole loaded PLGA nanoparticles, PLGA-PEG nanoparticles and immunonanoparticles showed 32.6%, 43.7% and 60.9% cells in apoptotic phase. With increase in exposure time to 48 h, the percentage of cells in early apoptotic phase decreased, at the same time percentage of cells in late apoptotic phase were found to be increased. This is possibly due to conversion of early apoptotic phase into late apoptotic phase

The treatment with different nanoparticles formulations to MCF7 cells showed a significant increase in necrotic and late apoptotic population after 24 h. whereas after 48 h of exposure the necrotic population got decreased with an increase in early and late apoptosis explaining apoptosis as the possible mode of cell death. The population statistics after 24 h treatment of PCL-PEG nanoparticles, immunonanoparticles exhibited a necrotic and late apoptotic population of (6.7 and 17.7%) and (12.3 and 22.9%) respectively compared to the drug solution (1.0 and 3.9%). However, 48 h exposure showed an increase in early and late apoptotic population of (0.9 and 33.8%) and (8.9 and 44.9%) than the drug solution (0.7 and 5.3%). Previous studies explained the role of estrogen in inducing breast cancer proliferation and prevention of apoptotic stimulation. Estrogen deprivation or exposure to anti-estrogens (aromatase inhibitors) leads to apoptotic cell death in MCF7 tumors.

The results showed significant fraction of necrotic, early and late apoptotic population of cells after exposure with exemestane solution, exemestane loaded PLGA nanoparticles, PLGA-PEG nanoparticles and PLGA-PEG Immunonanoparticles. Control group treated with PBS showed only 0.2% and 0.4% cells in apoptotic phases. After 24 h of exposure, exemestane PLGA nanoparticles, PLGA-PEG nanoparticles and PLGA-PEG Immunonanoparticles showed 13.0%, 26.5% and 36.6% cells in apoptotic phase which increased to 17.3%, 42.3% and 67.5%, respectively. After 24 h of exposure with exemestane PCL nanoparticles, PCL-PEG nanoparticles and PCL-PEG immunonano-particles showed 9.8%, 25.7% and 35.4% cells in apoptotic phase which increased to 16.6%, 41.6% and 70.2% after 48 h respectively.

The results showed significant fraction of necrotic, early and late apoptotic cell population after exposure (24 and 48 h) to all exemestane formulations whereas control group treated with PBS showed only 0.2% and 0.4% cells in apoptotic phases. After 24 h of exposure with exemestane PCL nanoparticles, PCL-PEG nanoparticles and Immunonanoparticles, 9.8%, 25.7% and 35.4% cells were seen in apoptotic phase which increased to 16.6%, 41.6% and 70.2% respectively after 48 h.

The higher anti-proliferative activity of immunonanoparticles than PEGylated/non-PEGylated formulation in MCF7 cells is the outcome of decreased cell cycle progression. No significant difference in anti-proliferative activity by immune-nanoparticles and PEGylated/non-PEGylated formulations in MDAMB231 cells were observed. However, greater efficiency of immunonanoparticles in arresting more number of receptor positive cells (MCF7 cells) can be explained on the basis of the intracellular drug levels at the site of action (due to enhanced cellular uptake following receptor mediated endocytosis) for a longer period of time (following sustain release) than drug and unconjugated nanoparticles.

The result depicts the presence of a significant fraction of early apoptotic, advanced/late apoptotic and necrotic cell population following treatment with drugs, drug loaded PEGylated nanoparticles and Immunonanoparticles. In MCF7 cells, a higher population of early apoptotic cells and late apoptotic cells was observed in the case of immunonanoparticles but un-conjugated nanoparticles showed less early apoptotic or late apoptotic cell population. Disclosing the effect of immunonanoparticles in inducing apoptosis in receptor positive cell line (MCF7), flow cytometry results depict a higher fraction of apoptotic cells in the case of cells treated with immunonanoparticles than with PEGylated nanoparticles or drug solution. It is noteworthy to emphasize that MCF7 cells treated with immunonanoparticles showed augmented apoptotic activity compared to MDAMB231 cells which shows receptor mediated uptake of immunonanoparticles.

Radiolabeling of nanocarrier systems was performed to study their organ distribution pattern and its fate in vivo. The iodination of tyrosine and ER Ab was successfully performed and found to be stable even after 24 h. The nanoparticles were radiolabeled with ^{125}I and these radiolabeled complexes were intravenously administered to study blood clearance and biodistribution in mice model. In vivo animal studies results showed clear advantage of immunonanoparticles over other non-targeted formulation in term of lower blood clearance and high uterus concentration and prolong plasma half life. The biodistribution studies showed that immunonanoparticles distributed more in uterus which contains ER than other organs. This remarkable targeting efficiency was resulted because of affinity of ER antibody towards these organs. Thus, here we conclude that these nanoparticulate systems will work as a novel tool for ER positive tumors and can be used to deliver therapeutically active substance successfully in conditions such as breast cancer.

8.2 Conclusion

The goal of this study was to design PEGylated and antibody conjugated PLGA/PCL nanoparticulate system for targeting of anticancer drugs, anastrozole and exemestane to breast tumor. Both formulations were PEGylated for longer circulation half life and better tumor retention. FTIR, NMR and GPC reports demonstrated the successful conjugation. DSC studies indicated absence of any interaction. In vitro release studies indicated sustained release property of nanoparticles. Sucrose was found to be the best cryoprotectant for these formulations as it showed good reconstitution property without significant change in particle size. Images of TEM has shown that the particles are spherical in shape and uniform in size distribution with no visible aggregation which is in relevance with data obtained from particle size analysis by Malvern Zetasizer. Phagocytic uptake studies of PEGylated and non-PEGylated nanoparticles on human acute monocytic leukemia cell line, THP-1 showed significant reduction in macrophage uptake of PEGylated nanoparticles and thus providing longer circulation half life. In vitro cell line studies have been performed to evaluate effectiveness of the formulation on cancer cells. Immunonanoparticles showed significant increase in cell cytotoxicity, apoptosis, cell uptake, and cell cycle arrest in comparison to only drug solution or PEGylated nanoparticles.

However, we need to conduct elaborate toxicological studies, preclinical studies and further investigations in human beings under clinical conditions before they can be commercially exploited.