Executive Summary of the Ph.D. Thesis entitled

"FABRICATION OF TARGETED FORMULATIONS TO IMPROVE EFFICACY OF THERAPY IN BREAST CANCER TREATMENT"

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List of contents of Thesis

List of contents of executive summary

1. INTRODUCTION

1.1 Breast cancer

Cancer is generally named after the part of the body into which it originated; thus, breast cancer refers to the irregular growth and proliferation of cells that originate in the breast tissue (1). Breast cancer refers to cancers that arise from breast tissue, most commonly from the milk ducts (inner lining) or the lobules that provide the ducts with milk (2). The breast consists of two main tissue types i.e., glandular tissues and stromal (supporting) tissues. Glandular tissues contain the milk-producing glands (lobules) and the ducts (milk passages) while stromal tissues include fatty and fibrous breast connective tissues (3-5). It also consists of tissue-immune lymphatic system tissue that extracts cellular fluids and waste. Multiple types of tumours can grow in different breast areas.

Breast cancer is the most diagnosed cancer among women and the second leading cause of cancer-related deaths among women affecting about 2.2 million women worldwide (6). Globally, the burden and incidence rates of breast cancer are enormously increasing than the other cancers (7). As estimated by World Health Organization (WHO), by 2050, it is expected that 27 million new breast cancer cases and 17.5 million breast cancer deaths will occur per annum (8). Breast cancer remains a major health issue and currently constitutes a top priority for biomedical research (9). The aggressive nature, early recurrence, rapid metastasis (to secondary sites such as the lung and brain), and poor breast tumour prognosis make the disease enigmatic and incurable (10).

Most cancers arise from benign (non-cancerous) changes in the breast (11). Fibrocystic change, for example, a non-cancerous condition where development of cysts (fluid packets accumulation), fibrosis (scar-like connective tissue formation), and thickening areas with lumpiness, breast pain or tenderness occurs in women (12, 13). Most breast cancers are in the cells which line up the ducts. Most originate in the lobules (lobular cancers) cells, whereas (14) the other tissues start with a small number (15). Most of these tumors are initially dependent upon activation of ERαand Erβ nuclear receptors promotes proliferation and survival of both normal and cancerous breast tissues through transcription of pro-survival genes and activation of cellular signalling (16, 17). Owing to the strong dependency of breast tumorigenesis on the estrogen-ER axis, estrogen suppression and ER antagonists have remained main stay of ER^+ breast cancer treatment for several years (7).

1.2 Current approaches for breast cancer

Traditional breast cancer treatments include radiation therapy- chemotherapy and endocrine therapy, which has improved the therapeutic effect, but the risks and side effects associated with these therapies inhibit the clinical usefulness (18). Most of the commonly used cytotoxic medications are chemotherapeutics that are delivered into systemic circulation (19). The administration of chemotherapeutics of low molecular weight into systemic circulation exhibits rapid clearance, low pharmacokinetic profile, and sub-optimal tissue distribution and only a small fraction reaches the tumour/tumour cell. Hydrophobic polymer loaded chemotherapeutic agents exhibits large volumes of delivery leading to increased accumulation at healthy tissue sites (20).

Endocrine therapies such as selective ER modulators (SERMs), selective estrogen down regulators (SERDs) and aromatase inhibitors are approved as an adjunct therapy for patients with ER^+ breast cancer (15). Aromatase Inhibitors deplete the levels of systemic estrogen by blocking the conversion of androgens to estrogen (21). SERMs compete with estrogen for binding to ER and have mixed agonist/antagonist capacities and are first line of treatment for pre-menopausal women. SERDs such as fulvestrant are said to work by suppressing ER activity by impairing intra-nuclear ER mobility.

1.3 Limitations associated with chemotherapeutic agents

Despite the significant advances in cancer detection, prevention, surgical oncology, chemotherapy and radiation therapy, there is still no common cure for cancer. Chemotherapy is an effective treatment against cancer but undesirable chemotherapy reactions and the development of resistance to drugs which results in multi-drug resistance (MDR) are the major obstacles in cancer chemotherapy.

- ✓ **Lack of selectivity and undesirable side effects:**

Conventional chemotherapy relies on the premise that rapidly proliferating tumor cells are more likely to be destroyed by cytotoxic agents than normal cells. However, these cytotoxic agents have little or no specificity, which leads to systemic toxicity causing undesirable side effects.

- ✓ **Multi drug resistance (MDR)**

Apart from toxicity, chemotherapeutic drug resistance in cancer therapy further limits the usefulness of anticancer agent. Although the mechanism of drug resistance is not clearly understood, however the cancer drug resistance is mainly due to pump and non-pump resistances. Pump resistance is due to ATP binding cassette (ABC) transporters including over-expression of P-glycoprotein (P-gp), a multidrug resistance protein that alter antitumor drug transport mechanisms, multidrug resistance-associated protein such as mutated Topoisomerase II which decrease drug activation and accelerate drug degradation where drug gets inactivated by conjugating with increased glutathione and ABC sub-family G member 2 (ABCG2), which expel drugs from cancer cells. In addition, non-pump resistance is mainly caused by overexpression of antiapoptotic proteins (B-cell lymphoma – 2 [BCL-2]) that prevent apoptosis in cancer cells.

1.4 Nanotechnology in cancer therapy to overcome the drawbacks of conventional therapy

The intrinsic limits of conventional cancer therapies prompted the development and application of various nanotechnologies for more effective and safer cancer treatment as nanotechnology has the potential to revolutionize cancer diagnosis and therapy (63). The increasing interest in applying nanotechnology to cancer treatment is attributable to its outstandingly appealing features for drug delivery, diagnosis and imaging, synthetic vaccine development and miniature medical devices, as well as the therapeutic nature of some nanomaterials themselves (64). Distinctive features of nanotechnology in oncological applications are as follow:

- ✓ Improvement of the drug therapeutic index by increasing efficacy and/or reducing toxicities
- ✓ Targeted delivery of drugs in a tissue-, cell- or organelle-specific manner
- ✓ Enhancement of the pharmaceutical properties (for example, stability, solubility, circulating half-life and tumour accumulation) of therapeutic molecules
- ✓ Enabling of sustained or stimulus-triggered drug release
- ✓ Facilitation of the delivery of bio macromolecular drugs (for example, DNA, small interfering RNA (siRNA), mRNA and protein) to intracellular sites of action
- ✓ Co-delivery of multiple drugs to improve therapeutic efficacy and overcome drug resistance, by providing more precise control of the spatiotemporal exposure of each drug and the delivery of appropriate drug ratio to the target of interest

- ✓ Transcytosis of drugs across tight epithelial and endothelial barriers (for example, gastrointestinal tract and the blood–brain barrier)
- ✓ More sensitive cancer diagnosis and imaging
- ✓ Visualization of sites of drug delivery by combining therapeutic agents with imaging modalities, and/or real-time feedback on the in vivo efficacy of a therapeutic agent
- ✓ Provision of new approaches for the development of synthetic vaccines
- ✓ Miniaturized medical devices for cancer diagnosis, drug screening and delivery
- ✓ Inherent therapeutic properties of some nanomaterials (for example, gold nanoshells and nanorods, and iron oxide nanoparticles) upon stimulation

Nanotherapies that incorporate some of these features (for example, improved circulation and reduced toxicity) are already in use today, and others show great promise in clinical development, with definitive results expected soon. Several therapeutic nanoparticle (NP) platforms, such as liposomes, albumin NPs and polymeric micelles, have been approved for cancer treatment, and many other nanotechnology-enabled therapeutic modalities are under clinical investigation, including chemotherapy, hyperthermia, radiation therapy, gene, or RNA interference (RNAi) therapy and immunotherapy.

1.5 Polymer Lipid Hybrid Nanocarriers (PLHNCs)

Hybrid nanocarriers are combination of two widely employed nanocarrier systems: i) Liposomes and ii) Polymeric nanoparticles, which are widely used in cancer therapy. PLHNCs are utilized as they are biodegradable, non-toxic, and stable and can also be attached by a suitable ligand which helps in active targeting of drugs to target site and reduce their toxicity to normal tissues. PLHNCs can be prepared by several methods like single stap nanoprecipitation, emulsification, sonication, nanoprecipitation self-assembly and two step method. The selection of method is based on ease of preparation, type of drug, batch size, ability to scale up and type of system required.

1.6 Mesoporous Silica Nanoparticles

Mesoporous silica nanoparticles (MSNs) have some unique advantages including high surface area and large pore volume, tuneable particle size (10-1000 nm) and pore diameter (2-30 nm), uniform mesoporosity, flexible morphology, facile surface functionalization and excellent biocompatibility and biodegradation. Textural properties of MSNs provide the possibility to load high amount of drugs within MSNs. On the other hand, there are abundant silanol groups on the surfaces of mesoporous channels and the outer surfaces of MSNs, which facilitate their

surface functionalization. As nanocarriers, mesoporous silica nanoparticles with unique mesoporous structure have been explored as effective drug delivery systems for a variety of therapeutic agents to fight against various kinds of diseases including bone/tendon tissue engineering, diabetes, inflammation, and cancer.

1.7 Folic acid as targeting ligand

Folate receptor-targeting systems have received considerable interest. Folate receptor is a membrane-bound folate-binding protein that, upon folic acid binding, initiates endocytosis and cell absorption. In healthy human cells, the presence of folate sensors is minimal, but it is quite high in tumour cells, particularly solid tumours. This upregulation serves as a possible target for folic acid-based anticancer drug delivery.

1.8 Aim and objectives

The aim of the present investigation was to fabricate targeted formulations to improve efficacy of therapy in breast cancer treatment. The proposed study was planned to achieve an efficient and breast tumor targeted delivery using PLHNCs and MSNs as drug delivery platform with two model drugs (Fulvestrant and Exemestane). It would be able to inhibit the growth of tumor cells by targeting therapeutic moieties to infected cells and prevent the disease progression and metastasis. The MSNs were further added with another molecule that would inhibit the drug resistance by prevention of mutation. Further for making PLHNCs and MSNs target specific, surface modification was done using folic acid for selective folate targeting to breast tumor cells.

The present investigation was proposed to be carried out in following steps:

Step 1: Selection of suitable method for preparation of nanoparticles (PLHNCs and MSNs) based on properties of drugs.

Step 2: Optimization of nanoparticles by Quality by Design approach (QbD).

Step 3: Surface modification of nanoparticles with folic acid.

Step 4: Characterization and evaluation of developed nanoparticles.

Step 5: In vitro cell line studies.

Step 6: In vivo anticancer studies

Step 7: Stability studies

1.9 Hypothesis

It was hypothesized that prepared nanoparticulate formulation in association with attached ligands will achieve targeting of the nanocarriers to tumor cells which will facilitate enhanced cellular uptake and hence greater drug localization in cancer cells.

1.10 Plan of work

1. Literature review and selection of drugs based on persistent problems.

2. Procurement of active ingredients and excipients.

3. Screening of formulation and process parameters for preparation of PLHNCs and MSNs

4. Optimization of PLHNCs and MSNs

5. Drug loading and optimization

6. Characterization of developed nanoparticles viz. particle size, PDI, zeta potential, encapsulation efficiency, drug loading, FTIR, and X – ray diffraction.

7. Surface functionalization with folic acid and characterization of functionalization viz. FTIR, zeta potential and XRD.

8. In vitro drug release studies and release kinetics.

9. In vitro anticancer studies.

10. In vivo pharmacokinetic and tumor regression studies.

11. Stability studies.

2. Research methodology

2.1 Literature Review

An extensive literature review was carried out through peer reviewed journals available on the topic, books, patents, and internet data base.

2.2 Selection of drug

Two different anti-cancer agents, Fulvestrant (FLV) and Exemestane (EXE), widely used for breast cancer treatment, were selected based upon the literature review, physio-chemical properties suitability of the molecule to be entrapped in the nanocarriers.

2.3 Analytical methods

Different analytical methods like UV – Visible spectroscopy and High Pressure Liquid Chromatography (HPLC) were used to estimate drug and excipients and their compatibility. For estimation of FLV and EXE UV visible spectroscopy inside the nanocarrier and for in vitro studies was utilized and for estimation drug in plasma an in vitro cellular uptake studies HPLC method was developed.

2.4 Formulation development and optimization

Drug (FLV and EXE) loaded PLHNCs were prepared using single step nanoprecipitation, self-assembly method. The preliminary optimization was carried out by OVAT analysis followed by Plackett Burman screening design and Box Behnken response surface methodology. For synthesis of blank MSNs optimization was carried out using Box Behnken response surface methodology and further FLV, EXE and quercetin were loaded in blank MSNs using passive loading method.

2.5 Surface modification of developed MSNs

For surface modification, folic acid was used to target the overexpressed folate receptor. The conjugation was carried out by esterification bond cleaving. The conjugation was characterized by FTIR and zeta potential.

2.6 Characterization of developed PLHNCs and MSNs

The developed PLHNCs and MSNs were characterized using different techniques like particle size, PDI. Zeta potential, FTIR, XRD, SEM and TEM analysis.

2.6.1 Particle size and PDI

The particle size and PDI of developed PLHNCs and synthesized MSNs were determined by dynamic light scattering (DLS) with a Malvern Zetasizer (Nano ZS, Malvern Panalytical, UK). Prior to the measurements, all the samples were suitably diluted with double distilled water. The particle size and PDI values were obtained by using polystyrene cells having 10 mm ID, at 25°C. All the measurements were performed in triplicate.

2.6.2 Zeta potential

The zeta potential, reflecting the electric charge on the particle surface and indicating the physical stability of the nanoparticulate systems, of developed PLHNCs and MSNs were measured by determining the electrophoretic mobility using the Malvern Zetasizer (Nano ZS, Malvern Panalytical, UK). Prior to the measurements, all the samples were suitably diluted with double distilled water. Zeta potential was measured using Dip cell with applying field strength 20 V/cm and the average of the zeta potential was given from 30 runs. Smoluchowski approximation was used to calculate zeta potential from the electrophoretic mobility. All measurements were performed in triplicate at 25°C.

2.6.3 FTIR analysis

FTIR spectrum of developed PLHNCs and MSNs were measured with a FTIR spectrophotometer (IR Affinity – 1S, Shimadzu, Japan) in the range of 4000 – 400 cm^{-1} with a scanning resolution of 4 cm^{-1}.

2.6.4 XRD analysis

X – ray diffraction patterns of developed PLHNCs were obtained using X – ray diffractometer (X'Pert PANalytics, Singapore) in which Cu-Kα line used as a source of radiation by operating at the voltage 40 kV and the current applied was 40 mA. Both samples were measured in the 2θ angle range between 5°-50° with a scanning rate of 3°/min and a step size of 0.02°.

2.6.5 Morphology

2.6.5.1 TEM analysis

TEM images of the nanoparticles were obtained on a Technai G2 transmission electron microscope (FEI Company Ltd., Hillsboro, USA) operating at 20-200kV voltage with resolution of 2.4 Ao.

2.6.5.2 SEM analysis

Morphology of the synthesized nanoparticles was characterized using Field Emission Gun-Scanning Electron microscope (JSM 7600F, JEOL, Japan) operated at a voltage of 0.1 to 30.0 kV.

2.7 Evaluation of developed PLHNCs and MSNs

2.7.1 Estimation of encapsulation efficiency and drug loading

FLV and EXE encapsulation in PLHNCs and MSNs respectively were determined by direct lysis method. Briefly, the nanoparticles were dispersed in the medium where drug dissolves and polymer is partially degradable, dispersion was sonicated, centrifuged at 12000 rpm, supernant was collected and measured by HPLC analysis. The percentage encapsulation and drug loading were calculated suing following formula.

$$\%EE = \frac{Amount\ of\ drug\ in\ nanocarriers}{Amount\ of\ drug\ added} \times 100 \quad -----Eq\ (1)$$

$$\%DL = \frac{Amount\ of\ drug\ encapsulated}{Total\ weight\ of\ nanocarrier + drug} \times 100 -----Eq\ (2)$$

2.7.2 *In vitro* drug release

A drug release study was performed using a dialysis bag with molecular mass cut-off of 3000 Da for 15 days. 2 mL of the formulation was filled in a dialysis bag and dipped in receptor media comprising 50 mL phosphate buffer at 37°C. At 2, 6, 12, 24, 48h and subsequent days up to 15th day, 1 mL of sample was withdrawn periodically and fresh media was replaced to maintain sink condition. These samples were analysed using HPLC and the % drug released was calculated and plotted against the time to obtain the release curve. Data of drug release are fitted in zero order, first order, Higuchi, Korsmeyer – Peppas and Hixon – Crowell models determine release kinetic pattern from PLHNCs and MSNs.

2.7.3 Hemolysis studies

For haemolysis study, 1.0 ml blood sample was collected in EDTA solution (30 µl) containing Eppendorf tube from the Sprague Dawley rat by retro-orbital puncture. Blood sample was then centrifuged at 5000 rpm for 10 min at 4 °C to separate the red blood cells (RBCs). The separated RBC pellet was re-suspended in normal saline and plasma components were removed by washing with normal saline (0.9 % w/w Sodium Chloride in water) 3 times before use. Then

0.5 % v/v RBCs were prepared by re-suspending RBC pellet (250 µl) in 50 ml of normal saline. Then 1 ml of RBCs was added to plain drug suspension and PLHNCs and MSNs, 1mg equivalent amount of drug dispersed in 1ml of saline. For positive and negative control, 2.0% Triton-X100 (1ml) and 0.5% saline was used respectively. After treatment (with drug suspension, nanoparticles, positive control, and negative control), RBC dispersion was gently stirred to uniformly disperse RBCs. The treated dispersions were stored at 37°C for 30 min in incubator. After incubation, all the samples were centrifuged at 3000 rpm for 12 min at 4 °C to separate the RBC mass and the solutions were analysed for UV absorbance at λmax of 540 nm against normal saline as a reference solution. Percentage hemolysis was calculated using following equation:

$$\%Haemolysis = \frac{Abs\ (Sample) - Abs\ (Ctrl -)}{Abs\ (Ctrl +) - Abs\ (Ctrl -)}\ x\ 100 - - - - Eq\ (3)$$

2.8 In vitro cell line studies

2.8.1 In vitro cell cytotoxicity studies

The cytotoxicity assay of pure drug and PLHNCs and MSNs was carried out on MDA MB 231 and MCF – 7 cells by MTT assay method. The cells were treated and incubated with drug as well as nanoparticles at different concentrations ranging from 0.1 to 5 µM for 24 hr., 48hr, 72 hrs. After incubation, the cells were treated with 100 µL MTT reagent (5 mg/ml in PBS) and re-incubated for 4 hr., for formazan crystal formation. The reduced formazan crystals were dissolved in 200 µL DMSO, and the absorbance was measured at 570nm using Plate reader (Multiskan, Thermo Scientific). Untreated cells were used as control. The experiment was performed in triplicate for determination of IC_{50} value by using linear regression model. The cell viability was calculated using following equation:

$$Cell\ viability\ (\%) = \frac{Abs\ of\ sample - Abs\ of\ blank}{Abs\ of\ control - Abs\ of\ blank} \times 100 - - - - Eq\ (4)$$

2.8.2 Cell uptake studies

For the cellular uptake study, the nanocarriers were prepared containing Fluorescein Isothiocyanate (FITC). The drug and dye loaded nanoparticles were prepared in concentration of 10% and 0.25% under the dark environment. The cells were grown on 6 well plate in concentration of 10000 cells/well and well was covered with sterile glass coverslip. The cells were incubated for 24 hr. and media was replaced with fresh medium. The cells were treated with plain drug suspension, PLHNCs and MSNs for 3 hrs. and washed with cold PBS (thrice).

Further, the cells were fixed using 4% Paraformaldehyde solution for 30 min, fixed on slide, and observed under CLSM (Zeiss, LSM 900).

2.8.3 Cell apoptosis studies

Cell apoptosis detection was carried out using flow cytometry method. MDA MB 231 cells were inoculated with cell density of 2000 cells/well in 6 well plate, were further incubated for 24 h. The cells were then treated with drug suspension, PLHNCs, and MSNs (IC_{50} value) and incubated for 24 h. Non treated cells were trypsinized using 0.025 % trypsin solution and harvested in 200 µL of binding buffer (1.4 M NaCl, 25mM $CaCl_2$ and 0.1 M HEPES (pH 7.4)) in the form of cell suspension. Immediately, propidium Iodide (8µl) and Annexin V- FITC (5 µl) were added to the cell suspension and gently vortexed for uniform mixing and kept for 20 min in the dark. Cells were analyzed using cell cycle analyzer (FACS CantoII, BD Biosciences, USA). The data were analyzed using FCS Express software (Research Edition).

2.8.4 Cell migration assay

Migration of the cancer cells was assessed using the scratch assay. Briefly, the confluent monolayer of the cells was grown in 6 well-plates at a concentration of 5000 cells/well. The cells were allowed to grow for a period of 24 h. Consequently, scratches were made on the centre of wells using a 200 µL sterile pipette tip. Markings of the scratch were highlighted from the bottom with the help of a marker. The later treatment was delivered to all the wells with the drug suspension, PLHNCs and MSNs at the dose of IC_{50} as found in MTT assay and incubated for 24 h. Images of the scratch assay were captured after the incubation period with Nikon microscope. Captured images were quantified using ImageJ software to assess the inhibitory effect on the scratch width concerning formulation and % scratch closure was then computed.

2.9 In vivo studies

The pharmacokinetic study, biodistribution study and tumor regression studies were carried out as per previously defined method. Female Sprague Dawley (SD) rats (120 – 140 g) were acclimatized at 25°C and relative humidity (RH) of 55-65% under natural light/dark conditions for 7 days before experiment. Mammary tumors were induced using chemical induction method, using MNU (N-methyl, N-nitrosourea) and were given MNU intraperitoneally at a dose of 50mg/kg body weight. After 3 weeks of MNU application, they were palpated twice a week for presence of mammary tumor. Tumor volume was measured.

2.9.1 In vivo pharmacokinetic studies

The animals were divided randomly into three groups of six animals each. The groups received drug suspensions, PLHNC and MSN formulations respectively intravenously. The blood samples were collected from retro orbital plexus (approximately 0.5ml) under mild anesthesia into heparinized micro centrifuge tubes. Plasma was separated by centrifugation at 5000 rpm at 4°C for 5 min. Into 200 µl of plasma, acetonitrile was added to make volume up to 1ml. The samples were vortexed and centrifuged at 5000 rpm for 10 min. The supernant was collected and analyzed using HPLC method. The pharmacokinetic parameters such as peak plasma concentration (C_{max}), half-life ($t_{1/2}$), area under the curve (AUC), time to reach peak plasma concentration (T_{max}) were determined using Kinetica software.

2.9.2 Biodistribution studies

The bio-distribution of drugs was characterized in the tumor bearing rats. The animals were divided into groups of 6 animals each. After 72h of single dose administration, the animals were euthanized, and highly perfused organs such as spleen, liver, kidney, heart, and tumor were isolated and weighed. 30 % of tissue homogenate was prepared and stored at -30°C until further use. 200 µL of homogenate was diluted with 800 µL of ACN to precipitate the protein and the supernant was collected after centrifugation and analysed after suitable dilution by HPLC method.

2.9.3 In vivo anticancer activity

The tumor regression study of PLHNCs and MSNs were determined in previously tumor induced SD rats. As the tumor volume reached ~1000 mm^3, animals were randomly divided into different groups of 6 animals each. Formulations were administered once a week by lateral tail vein injection for 6 weeks and tumor volume was measured after 6 weeks. Blood samples were withdrawn by retro-orbital plexus and the concentration of inflammatory mediators were measured after 3 weeks of treatment for live animals (animals that survived during treatment). The tumor inhibition was calculated by following equation

$$Tumor\ inhibition = \frac{\text{mean tumor volume of model control} - \text{mean tumor volume of treatment group}}{\text{mean tumor volume of model control}} \quad Eq\ (5)$$

2.10 Stability studies

The stability of the lyophilized developed PLHNCs were studied by storage of samples at accelerated conditions (5°C) and at long term stability conditions (25 ± 2°C) for 3 months and 6 months respectively. At regular intervals of 1 months the samples were characterized for particle size, zeta potential and % assay.

2.11 Statistical Data analysis

The results of all the trials are given as mean ± SD. Statistical significance was tested by p test and two tailed student t – test or one way ANOVA. Statistical significance was set at $p<0.05$.

3. Key findings

Different methods were screened for preparation of PLHNCs such as two-step method, double emulsion solvent evaporation and single step nanoprecipitation method to formulate PLHNCs. From this, single step nanoprecipitation with little modification followed by extrusion was found to formulate PLHNCs of desired characteristics. Preliminary studies were performed to define the ranges of formulation as well as process parameters. Preliminary studies also played significant role in determination of amount of cationic lipid needed to achieve maximum entrapment efficiency of fulvestrant. It was confirmed that the 30:50:20 ratio of DOPE:SPC – 3: DSPE PEG2000 has enough capacity to form a lipid layer onto the polymeric surface. Further increase in lipid ratio doesn't necessarily increases the encapsulation efficiency. Total seven factors (polymer concentration (mg/mL), lipid/polymer percentage (%), drug input percentage (%), stirring speed (RPM), stirring time (h), sonication time (S) and extrusion cycle (Nos) were selected for Placket-Burman screening study. From those, three factors (i.e., concentration of polymer, lipid to polymer ratio and drug input) were selected to further optimize the design space using Box-Behnken design.

QbD enabled design expert software suggested an optimized batch having composition of polymer concentration (8mg/ml), lipid to polymer ratio (15%) and drug input percentage (11%) which possessed predicted size of 118 nm and 79.84% entrapment efficiency and the same batch was formulated to validate the results and particle size was found to be 122.2 ± 3.8 nm with the PDI of 0.045 ± 0.003 and Zeta potential was found to be 28.3 ± 1.28 mV which is due to presence of cationic lipid i.e., DOPE. The entrapment efficiency was determined using Ultracentrifuge to separate entrapped and unentrapped drug. The % Entrapment efficiency was found to be 82.13 ± 2.52 % (n =3) in the optimized formulation.

The transmission electron microscopy (TEM) was performed to characterize PLHNCs structure with negative staining by uranyl acetate. The thickness of the ring is less than 20 nm, which equals the thickness of DOPE monolayer plus a DSPE-PEG_{2000} shell. The PLGA core was found to be dense indicating its presence inside vesicles. The average size was found to be 133.4 nm through TEM. Surface visualization and shape of the vesicle was confirmed by SEM and PLHNCs were found to spherical in the shape with the size of 130 nm approximately.

In vitro drug release studies were performed in phosphate buffer pH 7.4, phosphate buffer pH 6.6 and acetate buffer pH 5.5 for fulvestrant loaded PLHNCs and it showed sustained release pattern. Fulvestrant portrayed pH dependent release at pH 5.5 it shows highest release as

compared to phosphate buffer pH 7.4. The order of release from fulvestrant PLHNCs at different pH media was pH 7.4 to pH 5.5: 8.84% < 11.24% < 15.62%. Estimation of residual solvent was checked by Gas chromatography. The USP guidelines suggests that acetonitrile is class II solvent and the limit for PDE (Permitted Daily Exposure) is 4.1 mg/day equivalent to 410 ppm. From the data of residual solvent, it was confirmed that acetonitrile present in the final optimised batch of PLHNCs was within the limits as per USP guidelines of residual solvents.

Cell line studies were performed to assess the cytotoxicity, cellular uptake of formulation on MDA MB 231 and MCF – 7 breast cancer cell lines. MTT assays of fulvestrant suspension, blank PLHNCs, F-PLHNC and FA FLV PLHNC were performed to assess the effect of lipid and polymer on cell cytotoxicity. All the blank formulations were found to be non-toxic to cell lines indicating safety of formulation components. From the images of confocal microscopy obtained for cellular uptake studies it can be concluded that the PLHNCs could successfully carry fulvestrant into cytosol. Successful engulfment into cell using PLHNCs depict that they are easily up taken by the cells through endocytosis due to their cationic characteristic. Chemosensitization of MDA MB 231 cells was confirmed by performing and comparing MTT assays of Fulvestrant suspension, F-PLHNC, and FA FLV PLHNC and it was found that FA FLV PLHNC was having IC_{50} value of 0.55 µM against 1.23 µM for FLV suspension after 72 hrs., indicating 2.23-fold reduction. From the cell migration assay it was concluded that both FLV PLHNC and FA FLV PLHNC have enhanced anti migratory effect of the fulvestrant, which could provide a great tool for the shrinkage of the tumors and regression of tumor metastasis.

The in vivo pharmacokinetic parameters were calculated and it was found that the maximum $t_{1/2}$ value of 12 days was observed with FA FLV PLHNC compared to 16.94 hours with fulvestrant suspension after intravenous administration. Eventually, there was an increment in the AUC for PLHNCs compared to the AUC of Fulvestrant suspension. FA FLV PLHNC showed 23.2 times higher AUC values compared with AUC values of fulvestrant suspension after intravenous administration. The Tmax values for FA F-PLHNC was 38 hours compared to fulvestrant suspension which has only 3 hours, thereby confirming the maintenance of effective drug concentration with F-PLHNC in blood for prolonged period compared to FLV suspension.

The in vivo anticancer activity of various formulations was checked against chemically induced tumor in Sprague Dawley rats and compared with control groups: Normal control (no treatment, no tumor), model control (no treatment but tumor), Standard control (tumor + tamoxifen), Drug control (tumor + fulvestrant suspension), and test controls with different formulation. A significant change ($p<0.001$) in the weight of different treatment group rats was observed as compared to model control. Drug control and Standard control were found to reduce the weight due to side effect, whereas FLV PLHNC and FA FLV PLHNC were found to maintain the initial weight. According to the Kaplan Meier survival curve, all the model control rats died after 12 weeks. The rats treated with standard control showed 50 % survival and treated with fulvestrant suspension showed 66.67% survival, those treated with FLV PLHNCs showed 83.33% survival whereas the animals treated with FA FLV PLHNCs showed 100% survival during treatment (6 weeks).

Stability studies were carried out for lyophilized PLHNCs at accelerated condition (25°C ± 2°C, 60% RH ± 5% RH) for three months and at long term conditions (5°C ± 3°C) up to 6 months. During stability monitoring, no significant differences ($p<0.05$) were observed in particle size, assay, and zeta potential of lyophilized PLHNCs stating the storage in the form of lyophilized formulation at refrigerated conditions.

EXEMESTANE PLHNCs

Analytical techniques were developed to estimate pure exemestane, exemestane in PLHNCs, Dissolution media (Phosphate buffer saline pH 7.4, phosphate buffer pH 6.6 and acetate buffer pH 5.5). Calibration curve of exemestane was prepared by direct UV estimation. The calibration was plotted by measuring the absorbance at 243 nm (λmax), calibration curve was prepared for exemestane in Acetonitrile, Methanol, Phosphate buffer pH 7.4, phosphate buffer and acetate buffer 5.5 and the methods were validated for accuracy, precision, LOD and LOQ. Phospholipid content in formulation was estimated by Stewart method, calibration of total phospholipid was prepared in chloroform. For estimation of exemestane in biological samples, calibration curve of exemestane was developed by HPLC method for plain drug, in rat plasma for pharmacokinetic studies.

Different methods were screened for preparation of PLHNCs such as two-step method, double emulsion solvent evaporation and single step nanoprecipitation method to formulate PLHNCs. From this, single step nanoprecipitation with little modification followed by extrusion was found to formulate PLHNCs of desired characteristics. Preliminary studies were performed to

define the ranges of formulation as well as process parameters. Preliminary studies also played significant role in determination of amount of cationic lipid needed to achieve maximum entrapment efficiency of exemestane. It was confirmed that the 30:40:30 ratio of DOPE:P90G: DSPE PEG_{2000} has enough capacity to form a lipid layer onto the polymeric surface. Further increase in lipid ratio doesn't necessarily increases the encapsulation efficiency. Total seven factors (polymer concentration (mg/mL), lipid/polymer percentage (%), drug input percentage (%), stirring speed (RPM), stirring time (h), sonication time (S) and extrusion cycle (Nos) were selected for Placket-Burman screening study. From those, three factors (i.e., concentration of polymer, lipid to polymer ratio and drug input) were selected to further optimize the design space using Box-Behnken design.

QbD enabled design expert software suggested an optimized batch having composition of polymer concentration (4mg/ml), lipid to polymer ratio (30%) and drug input percentage (18%) which possessed predicted size of 117.9 nm and 84.06 % entrapment efficiency and the same batch was formulated to validate the results and particle size was found to be 120.8 ± 2.38 nm with the PDI of 0.045 ± 0.003 and Zeta potential was found to be 6.89 ± 0.86 mV which was due to presence of cationic lipid i.e., DOPE. The entrapment efficiency was determined using Ultracentrifuge to separate entrapped and unentrapped drug. The % Entrapment efficiency was found to be 86.84 ± 3.57 % (n =3) in the optimized formulation.

The transmission electron microscopy (TEM) was performed to characterize PLHNCs structure with negative staining by uranyl acetate which stains DOPE and the lipids conjugated with PEG to enhance their electron density, resulting in dim ring surrounding the PLGA core. The thickness of the ring is less than 20 nm, which equals the thickness of DOPE monolayer plus a DSPE-PEG_{2000} shell. The PLGA core was found to be dense indicating its presence inside vesicles. The average size was found to be 129.6 nm through TEM. Surface visualization and shape of the vesicle was confirmed by SEM and PLHNCs were found to spherical in the shape with the size of 140 nm approximately.

In vitro drug release studies were performed in phosphate buffer pH 7.4, phosphate buffer pH 6.6 and acetate buffer pH 5.5 for exemestane loaded PLHNCs and it showed sustained release pattern. Exemestane portrayed pH dependent release at pH 5.5 it showed highest release as compared to phosphate buffer pH 7.4. The order of release from exemestane PLHNCs at different pH media was from pH 7.4 to pH 5.5 Estimation of residual solvent was checked by Gas chromatography. The USP guidelines suggest that acetonitrile is class II solvent and the

limit for PDE (Permitted Daily Exposure) is 4.1 mg/day equivalent to 410 ppm. From the data of residual solvent, it was confirmed that acetonitrile present in the final optimised batch of PLHNCs was within the limits as per USP guidelines of residual solvents.

Cell line studies were performed to assess the cytotoxicity, cellular uptake of formulation on MDA MB 231 and MCF – 7 breast cancer cell lines. MTT assays of exemestane suspension, blank PLHNCs, EXE PLHNC and FA EXE PLHNC were performed to assess the effect of lipid and polymer on cell cytotoxicity. All the blank formulations were found to be non-toxic to cell lines indicating safety of formulation components. From the images of confocal microscopy obtained for cellular uptake studies it can be concluded that the PLHNCs could successfully carry exemestane into cytosol. Successful engulfment into cell using PLHNCs depict that they are easily up taken by the cells through endocytosis due to their cationic characteristic. Chemosensitization of MCF – 7 cells was confirmed by performing and comparing MTT assays of Exemestane suspension, EXE PLHNC, and FA EXE PLHNC and it was found that FA EXE PLHNC was having IC_{50} value of 3.24 µM against 4.76 µM for EXE suspension after 72 hrs., indicating 1.47-fold reduction. From the cell migration assay it was concluded that both E-PLHNC and FA-E-PLHNC have enhanced ant-migratory effect of the exemestane, which could provide a great tool for the shrinkage of the tumors and regression of tumor metastasis.

The in vivo pharmacokinetic parameters were calculated and it was found that the maximum $t_{1/2}$ value of 7 days was observed with FA EXE PLHNC compared to 5.21 hours with exemestane suspension after intravenous administration. Eventually, there was an increment in the AUC for PLHNCs compared to the AUC of Exemestane suspension. FA EXE PLHNC showed 5.59 times higher AUC values compared with AUC values of exemestane suspension after intravenous administration. The Tmax values for FA EXE PLHNC was 31.87 hours compared to exemestane suspension which has only 2.24 hours, thereby confirming the maintenance of effective drug concentration with EXE PLHNC in blood for prolonged period compared to EXE suspension.

The in vivo anticancer activity of various formulations was checked against chemically induced tumor in Sprague Dawley rats and compared with control groups: Normal control (no treatment, no tumor), model control (no treatment but tumor), Standard control (tumor + tamoxifen), Drug control (tumor + exemestane suspension), and test controls with different formulation. A significant change ($p<0.001$) in the weight of different treatment group rats was

observed as compared to model control. Standard control and Drug control were found to reduce the weight due to side effect, whereas EXE PLHNC and FA EXE PLHNC were found to maintain the initial weight. According to the Kaplan Meier survival curve EXE PLHNCs showed 83.33% survival and FA EXE PLHNCs showed 100 % up to the course of treatment which suggested the improvement of efficacy of EXE with NPs compared EXE suspension.

Stability studies were carried out for lyophilized PLHNCs at accelerated condition (25°C ± 2°C, 60% RH ± 5% RH) for three months and at long term conditions (5°C ± 3°C) up to 6 months. During stability monitoring, no significant differences ($p<0.05$) were observed in particle size, assay, and zeta potential of lyophilized PLHNCs stating the storage in the form of lyophilized formulation at refrigerated conditions.

MESOPOROUS SILICA NANOPARTICLES (MSNs)

First, basic skeleton of Mesoporous silica nanoparticles was fabricated based on modified Stober template-based synthesis. Thereafter, the surfactant template was removed by acid solvent reaction method. The surfactant free mesoporous silica nanoparticles were further used for post synthetic surface modification. Amination was done on the MCM – 41 MSNs by using APTES. Functionalization offers advantages like sustained release. The amination of nanoparticles also served as strong platform for further functionalization with folic acid. The initial zeta potential before functionalization was -22.7 mV, on surface functionalization with amine group the zeta potential shifted towards positive i.e., +26.5 mV, further on folic acid conjugation the zeta shifted towards negative -17.1 mV. The particle size for blank mesoporous silica nanoparticles was found to be 54.5 nm.

The surface area and porosity estimation from the nitrogen sorption studies gave a clear trend of maximum surface are and pore size obtained for MCM-41 nanocarriers. On drug loading when compared to bare carriers there was reduction in surface area due to engulfment of drug in its pores. The BET and BJH surface areas were highest for bare MCM-41 with value of 1229.12 m^2/g and 1386.56 m^2/g respectively. The trend was same for BET and BJH both the surface areas. The SEM and TEM images of the fabricated MSNs were proof of spherical and uniform morphology and intact hexagonal structure of MSNs. After complete characterization of the synthesized MSNs, drug loading was performed by solvent immersion method. The solvent used was methanol which was easily evaporated giving a facile way of obtaining drug loaded MSNs.

The Plackett-Burman study design has been implemented for screening of various formulation and process related parameters i.e., surfactant concentration (%) (factor A), Silica source concentration (%) (factor B), TEA concentration (w/w) (factor C), Ethanol concentration (%) (factor D) stirring speed (RPM) (factor E), stirring time (h) (factor F) and stirring temperature (°C) (factor G) and its impact on particle size, %yield and surface area. These parameters were assessed to be of high importance in consideration with other factors based on different trials. Based on the results of the primary factor screening design, three variables (i.e., surfactant concentration, silica source concentration and stirring temperature) were selected for further optimization using Box-Behnken design.

FULVESTRANT LOADED MSNs

Fulvestrant loaded MSNs have followed the sustained release kinetics (Figure 6.24a). From the three pH conditions, the highest release curve was observed in pH 5.5, which suggested maximum release of the drug in cancer cells. Release of the fulvestrant from the MSNs in the different media was observed to be in decreasing order of pH 5.5 > pH 6.6 > pH 7.4, which indicates the least drug release in plasma and blood. The sustained release of fulvestrant was achieved owing to the presence of drug in MSN pores. The release of fulvestrant suspension was found to be completed within 24 hours, indicating the need for dose administration frequently. There was no significant difference in the drug release pattern in different pH conditions.

Cell line studies were performed to assess the cytotoxicity, cellular uptake of formulation on MDA MB 231 and MCF – 7 breast cancer cell lines. MTT assays of fulvestrant suspension, FMSN, QMSN, FQMSN were performed. From the images of confocal microscopy obtained for cellular uptake studies it can be concluded that the MSNs could successfully carry fulvestrant into cytosol. Successful engulfment into cell using MSNs depict that they are easily up taken by the cells through endocytosis due to their cationic characteristic. Chemosensitization of MDA MB 231 cells was confirmed by performing and comparing MTT assays of Fulvestrant suspension, FMSN, and FQMSN and it was found that FQMSN was having IC_{50} value of 0.59 µM against 2.07 µM for FLV suspension after 72 hrs., indicating 3.51-fold reduction. From the cell migration assay it was concluded that QMSN, FMSN and FQMSN have enhanced ant-migratory effect of the fulvestrant, which could provide a great tool for the shrinkage of the tumors and regression of tumor metastasis.

The in vivo pharmacokinetic parameters were calculated and it was found that the maximum $t_{1/2}$ value of 7 days was observed with FQMSN compared to 12.61 ± 0.54 hours with fulvestrant suspension after intravenous administration. Eventually, there was an increment in the AUC for MSNs compared to the AUC of Fulvestrant suspension. FQMSN showed 20.05 times higher AUC values compared with AUC values of fulvestrant suspension after intravenous administration. The Tmax values for FQMSN was 34.18 hours compared to fulvestrant suspension which had only 3.62 hours, thereby confirming the maintenance of effective drug concentration with FQMSN in blood for prolonged period compared to FLV suspension.

The survival time for different experimental rats inoculated with MNU were observed after inoculation. All the rats were monitored up to 12 weeks after first tumor palpitation and then sacrificed. The data of survival study postulated that the nanoparticles prolonged the survival of the animals, as the samples treated with standard and drug control showed death of 50 % animals (3 animals) within the course of treatment (6 weeks), whereas with FMSN there was death of only 13.33 % of animals (1 animal). The animals treated with FQMSN, showed no death, and had 100 % survival up to weeks, though 1 animal died after 2 weeks of discontinuing the treatment. So, it can be said that FQMSN showed 100 % survival rate during treatment as opposed to standard and drug control that had only 50 % survival rate.

EXEMESTANE LOADED MSNs

Exemestane loaded MSNs have followed the sustained release kinetics. From the three pH condition, the highest release was found in pH 5.5, which suggested maximum release of drug in cancer cells. Release of exemestane from the MSNs in the different media was observed to be in decreasing order of pH 5.5 > pH 6.6 > pH 7.4, which indicates least drug release in plasma and blood. From the kinetic model fitting analysis, it was concluded that for exemestane and quercetin co-loaded MSNs, the best fit model was Korsmeyer Peppas model with the R^2 value of 0.9926, with the n value of 0.892, which is consistent with the drug release by anomalous transport or non-Fickian diffusion that involves two phenomena: drug diffusion and relaxation of the polymer matrix.

Cell line studies were performed to assess the cytotoxicity, cellular uptake of formulation on MDA MB 231 and MCF – 7 breast cancer cell lines. MTT assays of exemestane suspension, EMSN, QMSN, EQMSN were performed. From the images of confocal microscopy obtained for cellular uptake studies it can be concluded that the MSNs could successfully carry exemestane into cytosol. Successful engulfment into cell using MSNs depict that they are easily

up taken by the cells through endocytosis due to their cationic characteristic. Chemosensitization of MDA MB 231 cells was confirmed by performing and comparing MTT assays of Exemestane suspension, EMSN, and EQMSN and it was found that EQMSN was having IC_{50} value of 2.08 µM against 5.68 µM for EXE suspension after 72 hrs., indicating 2.73-fold reduction. From the cell migration assay it was concluded that QMSN, EMSN and EQMSN have enhanced ant-migratory effect of the exemestane, which could provide a great tool for the shrinkage of the tumors and regression of tumor metastasis.

The in vivo pharmacokinetic parameters were calculated and it was found that the maximum $t_{1/2}$ value of 5 days was observed with EQMSN compared to 5.92 ± 0.24 hours with exemestane suspension after intravenous administration. Eventually, there was an increment in the AUC for MSNs compared to the AUC of Exemestane suspension. EQMSN showed 2.44 times higher AUC values compared with AUC values of exemestane suspension after intravenous administration. The Tmax values for EQMSN was 37.41 hours compared to exemestane suspension which has only 3.12 hours, thereby confirming the maintenance of effective drug concentration with EQMSN in blood for prolonged period compared to EXE suspension.

The survival time for different experimental rats inoculated with MNU were observed after inoculation. Rats treated with standard and drug control showed 50% survival. The samples treated with QMSN showed 66.67% survival which is 1.33 times more compared to its standard counterpart and standard drug sample. The sample with EMSN showed 83.33% survival which is 1.66 times more compared to standard and drug control. EQMSN showed 100% survival which was 1.20 times more than that of EMSN, whereas 2 times more than that of the standard and model control.

4. CONCLUSION

In current investigations, folic acid conjugated Polymer lipid hybrid nanocarriers and folate conjugated and dual drug loaded Mesoporous silica nanoparticles were developed for the delivery folate receptor targeted delivery of fulvestrant and exemestane respectively. The results suggested that the developed folate conjugated PLHNCs and folate conjugated quercetin co loaded mesoporous silica nanoparticles with fulvestrant and exemestane have potential to target the breast cancer cell and reduce their toxicity towards normal cells. The formulations showed sustained release of drug and the pharmacokinetic studies also supported the prolonged drug release action. The biodistribution studies for folate conjugated nanoparticles showed increased concentration of drugs within tumor cells. The in vivo anticancer activity carried out on chemical induced rat tumor model showed reduction in overall tumor burden and increased the survival rate of animals. Thus, based on the obtained results it can be said that the formulated nanoparticles could show ligand responsive intracellular drug release which may help to enhance the efficacy of anticancer treatment and reduce undesirable side effects.

5. References

1. Lei S, Zheng R, Zhang S, Wang S, Chen R, Sun K, et al. Global patterns of breast cancer incidence and mortality: A population-based cancer registry data analysis from 2000 to 2020. Cancer Communications. 2021;41(11):1183-94.
2. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, et al. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. CA: A Cancer Journal for Clinicians. 2021;71(3):209-49.
3. Sopik V. International variation in breast cancer incidence and mortality in young women. Breast Cancer Research and Treatment. 2021;186(2):497-507.
4. Abd-Elnaby M, Alfonse M, Roushdy M. Classification of breast cancer using microarray gene expression data: A survey. Journal of Biomedical Informatics. 2021;117:103764.
5. Gupta V, Vasudev M, Doegar A, Sambyal N. Breast cancer detection from histopathology images using modified residual neural networks. Biocybernetics and Biomedical Engineering. 2021;41(4):1272-87.
6. Britt KL, Cuzick J, Phillips K-A. Key steps for effective breast cancer prevention. Nature Reviews Cancer. 2020;20(8):417-36.
7. Hanker AB, Sudhan DR, Arteaga CL. Overcoming Endocrine Resistance in Breast Cancer. Cancer Cell. 2020;37(4):496-513.
8. Torre LA, Islami F, Siegel RL, Ward EM, Jemal A. Global Cancer in Women: Burden and Trends. Cancer Epidemiology, Biomarkers & Prevention. 2017;26(4):444-57.
9. Hemmatzadeh M, Mohammadi H, Jadidi-Niaragh F, Asghari F, Yousefi M. The role of oncomirs in the pathogenesis and treatment of breast cancer. Biomedicine & Pharmacotherapy. 2016;78:129-39.
10. Bosch A, Eroles P, Zaragoza R, Viña JR, Lluch A. Triple-negative breast cancer: Molecular features, pathogenesis, treatment and current lines of research. Cancer Treatment Reviews. 2010;36(3):206-15.
11. Russo J, Russo IH. Role of differentiation in the pathogenesis and prevention of breast cancer. Endocrine-related cancer. 1997;4(1):7-21.
12. Feng Y, Spezia M, Huang S, Yuan C, Zeng Z, Zhang L, et al. Breast cancer development and progression: Risk factors, cancer stem cells, signaling pathways, genomics, and molecular pathogenesis. Genes & Diseases. 2018;5(2):77-106.
13. Brook N, Brook E, Dharmarajan A, Dass CR, Chan A. Breast cancer bone metastases: pathogenesis and therapeutic targets. The International Journal of Biochemistry & Cell Biology. 2018;96:63-78.
14. King J, Mir H, Singh S. Chapter Four - Association of Cytokines and Chemokines in Pathogenesis of Breast Cancer. In: Lakshmanaswamy R, editor. Progress in Molecular Biology and Translational Science. 151: Academic Press; 2017. p. 113-36.
15. Thomas M, Kelly ED, Abraham J, Kruse M. Invasive lobular breast cancer: A review of pathogenesis, diagnosis, management, and future directions of early stage disease. Seminars in Oncology. 2019;46(2):121-32.

16. Caswell-Jin JL, Plevritis SK, Tian L, Cadham CJ, Xu C, Stout NK, et al. Change in Survival in Metastatic Breast Cancer with Treatment Advances: Meta-Analysis and Systematic Review. JNCI Cancer Spectrum. 2018;2(4):pky062.
17. Spiegel D, Kraemer H, Bloom J, Gottheil E. EFFECT OF PSYCHOSOCIAL TREATMENT ON SURVIVAL OF PATIENTS WITH METASTATIC BREAST CANCER. The Lancet. 1989;334(8668):888-91.
18. Gradishar WJ. Taxanes for the Treatment of Metastatic Breast Cancer. Breast Cancer: Basic and Clinical Research. 2012;6:BCBCR.S8205.
19. Bender CM, Sereika SM, Berga SL, Vogel VG, Brufsky AM, Paraska KK, et al. Cognitive impairment associated with adjuvant therapy in breast cancer. Psycho-Oncology. 2006;15(5):422-30.
20. Jabo B, Lin AC, Aljehani MA, Ji L, Morgan JW, Selleck MJ, et al. Impact of Breast Reconstruction on Time to Definitive Surgical Treatment, Adjuvant Therapy, and Breast Cancer Outcomes. Annals of Surgical Oncology. 2018;25(10):3096-105.
21. Jasra S, Anampa J. Anthracycline Use for Early Stage Breast Cancer in the Modern Era: a Review. Current Treatment Options in Oncology. 2018;19(6):30.
22. de Gregorio A, Janni W, Friedl TWP, Nitz U, Rack B, Schneeweiss A, et al. The impact of anthracyclines in intermediate and high-risk HER2-negative early breast cancer—a pooled analysis of the randomised clinical trials PlanB and SUCCESS C. British Journal of Cancer. 2022;126(12):1715-24.
23. Abotaleb M, Kubatka P, Caprnda M, Varghese E, Zolakova B, Zubor P, et al. Chemotherapeutic agents for the treatment of metastatic breast cancer: An update. Biomedicine & Pharmacotherapy. 2018;101:458-77.
24. Giuliano M, Schettini F, Rognoni C, Milani M, Jerusalem G, Bachelot T, et al. Endocrine treatment versus chemotherapy in postmenopausal women with hormone receptor-positive, HER2-negative, metastatic breast cancer: a systematic review and network meta-analysis. The Lancet Oncology. 2019;20(10):1360-9.
25. Bortolini Silveira A, Bidard F-C, Tanguy M-L, Girard E, Trédan O, Dubot C, et al. Multimodal liquid biopsy for early monitoring and outcome prediction of chemotherapy in metastatic breast cancer. npj Breast Cancer. 2021;7(1):115.
26. Mukohara T, Hosono A, Mimaki S, Nakayama A, Kusuhara S, Funasaka C, et al. Effects of Ado-Trastuzumab Emtansine and Fam-Trastuzumab Deruxtecan on Metastatic Breast Cancer Harboring HER2 Amplification and the L755S Mutation. The Oncologist. 2021;26(8):635-9.
27. Pegram MD, Bondarenko I, Zorzetto MMC, Hingmire S, Iwase H, Krivorotko PV, et al. PF-05280014 (a trastuzumab biosimilar) plus paclitaxel compared with reference trastuzumab plus paclitaxel for HER2-positive metastatic breast cancer: a randomised, double-blind study. British Journal of Cancer. 2019;120(2):172-82.
28. Bachelot T, Ciruelos E, Schneeweiss A, Puglisi F, Peretz-Yablonski T, Bondarenko I, et al. Preliminary safety and efficacy of first-line pertuzumab combined with trastuzumab and taxane therapy for HER2-positive locally recurrent or metastatic breast cancer (PERUSE). Annals of Oncology. 2019;30(5):766-73.

29. Francies FZ, Hull R, Khanyile R, Dlamini Z. Breast cancer in low-middle income countries: abnormality in splicing and lack of targeted treatment options. Am J Cancer Res. 2020;10(5):1568-91.
30. Noonan S, Pasa A, Fontana V, Caviglia S, Bonanni B, Costa A, et al. A Survey among Breast Cancer Specialists on the Low Uptake of Therapeutic Prevention with Tamoxifen or Raloxifene. Cancer Prevention Research. 2018;11(1):38-43.
31. Xu T, Ding W, Ji X, Ao X, Liu Y, Yu W, et al. Molecular mechanisms of ferroptosis and its role in cancer therapy. Journal of Cellular and Molecular Medicine. 2019;23(8):4900-12.
32. Yin L, Duan J-J, Bian X-W, Yu S-c. Triple-negative breast cancer molecular subtyping and treatment progress. Breast Cancer Research. 2020;22(1):61.
33. Dallavalle S, Dobričić V, Lazzarato L, Gazzano E, Machuqueiro M, Pajeva I, et al. Improvement of conventional anti-cancer drugs as new tools against multidrug resistant tumors. Drug Resistance Updates. 2020;50:100682.
34. Robey RW, Pluchino KM, Hall MD, Fojo AT, Bates SE, Gottesman MM. Revisiting the role of ABC transporters in multidrug-resistant cancer. Nature Reviews Cancer. 2018;18(7):452-64.
35. Liu R, Chen Y, Liu G, Li C, Song Y, Cao Z, et al. PI3K/AKT pathway as a key link modulates the multidrug resistance of cancers. Cell Death & Disease. 2020;11(9):797.
36. Rocca A, Maltoni R, Bravaccini S, Donati C, Andreis D. Clinical utility of fulvestrant in the treatment of breast cancer: a report on the emerging clinical evidence. Cancer Manag Res. 2018;10:3083-99.
37. Li J, Wang Z, Shao Z. Fulvestrant in the treatment of hormone receptor-positive/human epidermal growth factor receptor 2-negative advanced breast cancer: A review. Cancer Medicine. 2019;8(5):1943-57.
38. Perey L, Paridaens R, Hawle H, Zaman K, Nolé F, Wildiers H, et al. Clinical benefit of fulvestrant in postmenopausal women with advanced breast cancer and primary or acquired resistance to aromatase inhibitors: final results of phase II Swiss Group for Clinical Cancer Research Trial (SAKK 21/00). Annals of Oncology. 2007;18(1):64-9.
39. Zbieg JR, Liang J, Li J, Blake RA, Chang J, Friedman L, et al. Discovery of GNE-502 as an orally bioavailable and potent degrader for estrogen receptor positive breast cancer. Bioorganic & Medicinal Chemistry Letters. 2021;50:128335.
40. Ribas R, Pancholi S, Guest SK, Marangoni E, Gao Q, Thuleau A, et al. AKT Antagonist AZD5363 Influences Estrogen Receptor Function in Endocrine-Resistant Breast Cancer and Synergizes with Fulvestrant (ICI182780) In Vivo. Molecular Cancer Therapeutics. 2015;14(9):2035-48.
41. Spoerke JM, Gendreau S, Walter K, Qiu J, Wilson TR, Savage H, et al. Heterogeneity and clinical significance of ESR1 mutations in ER-positive metastatic breast cancer patients receiving fulvestrant. Nature Communications. 2016;7(1):11579.
42. Alves CL, Elias D, Lyng M, Bak M, Kirkegaard T, Lykkesfeldt AE, et al. High CDK6 Protects Cells from Fulvestrant-Mediated Apoptosis and is a Predictor of Resistance to Fulvestrant in Estrogen Receptor–Positive Metastatic Breast Cancer. Clinical Cancer Research. 2016;22(22):5514-26.

43. Hascicek C, Sengel-Turk CT, Gumustas M, Ozkan AS, Bakar F, Das-Evcimen N, et al. Fulvestrant-loaded polymer-based nanoparticles for local drug delivery: Preparation and in vitro characterization. Journal of Drug Delivery Science and Technology. 2017;40:73-82.
44. Purohit P, Brahmkshatriya P, Goswami V. Development of orally bioavailable prodrugs of fulvestrant for the treatment of metastatic/advanced breast cancer. Journal of Clinical Oncology. 2021;39(15_suppl):e13027-e.
45. TECHNOLOGY XALMSa, inventor; WIPO, assignee. Method of manufacturing fulvestrant sustained-release microspheres CHINA2007 03-08-2007.
46. Park J, Thomas S, Zhong AY, Wolfe AR, Krings G, Terranova-Barberio M, et al. Local delivery of hormonal therapy with silastic tubing for prevention and treatment of breast cancer. Scientific Reports. 2018;8(1):92.
47. Nabholtz J-M, Mouret-Reynier M-A, Durando X, Van Praagh I, Al-Sukhun S, Ferriere J-P, et al. Comparative review of anastrozole, letrozole and exemestane in the management of early breast cancer. Expert Opinion on Pharmacotherapy. 2009;10(9):1435-47.
48. Lønning PE, Paridaens R, Thürlimann B, Piscitelli G, di Salle E. Exemestane experience in breast cancer treatment. The Journal of Steroid Biochemistry and Molecular Biology. 1997;61(3):151-5.
49. Sobral AF, Amaral C, Correia-da-Silva G, Teixeira N. Unravelling exemestane: From biology to clinical prospects. The Journal of Steroid Biochemistry and Molecular Biology. 2016;163:1-11.
50. Amaral C, Augusto TV, Tavares-da-Silva E, Roleira FMF, Correia-da-Silva G, Teixeira N. Hormone-dependent breast cancer: Targeting autophagy and PI3K overcomes Exemestane-acquired resistance. The Journal of Steroid Biochemistry and Molecular Biology. 2018;183:51-61.
51. Chalasani P, Stopeck A, Clarke K, Livingston R. A Pilot Study of Estradiol Followed by Exemestane for Reversing Endocrine Resistance in Postmenopausal Women With Hormone Receptor-Positive Metastatic Breast Cancer. The Oncologist. 2014;19(11):1127-8.
52. Singh AK, Chaurasiya A, Singh M, Upadhyay SC, Mukherjee R, Khar RK. Exemestane Loaded Self-Microemulsifying Drug Delivery System (SMEDDS): Development and Optimization. AAPS PharmSciTech. 2008;9(2):628-34.
53. Hiremath PS, Soppimath KS, Betageri GV. Proliposomes of exemestane for improved oral delivery: Formulation and in vitro evaluation using PAMPA, Caco-2 and rat intestine. International Journal of Pharmaceutics. 2009;380(1):96-104.
54. Jukanti R, Sheela S, Bandari S, Veerareddy PR. Enhanced Bioavailability of Exemestane Via Proliposomes based Transdermal Delivery. Journal of Pharmaceutical Sciences. 2011;100(8):3208-22.
55. Rizwanullah M, Perwez A, Mir SR, Alam Rizvi MM, Amin S. Exemestane encapsulated polymer-lipid hybrid nanoparticles for improved efficacy against breast cancer: optimization, in vitro characterization and cell culture studies. Nanotechnology. 2021;32(41):415101.
56. Kaur S, Jena SK, Samal SK, Saini V, Sangamwar AT. Freeze dried solid dispersion of exemestane: A way to negate an aqueous solubility and oral bioavailability problems. European Journal of Pharmaceutical Sciences. 2017;107:54-61.

57. Eedara BB, Bandari S. Lipid-based dispersions of exemestane for improved dissolution rate and intestinal permeability: in vitro and ex vivo characterization. Artificial Cells, Nanomedicine, and Biotechnology. 2017;45(5):917-27.
58. Yavuz B, Bilensoy E, Vural İ, Şumnu M. Alternative oral exemestane formulation: Improved dissolution and permeation. International Journal of Pharmaceutics. 2010;398(1):137-45.
59. Musa MN, David SA-O, Zulkipli IA-O, Mahadi AH, Chakravarthi S, Rajabalaya RA-O. Development and evaluation of exemestane-loaded lyotropic liquid crystalline gel formulations. (2228-5652 (Print)).
60. Jayapal JJ, Dhanaraj S. Exemestane loaded alginate nanoparticles for cancer treatment: Formulation and in vitro evaluation. International Journal of Biological Macromolecules. 2017;105:416-21.
61. Li Z, Liu K, Sun P, Mei L, Hao T, Tian Y, et al. Poly(D, L-lactide-co-glycolide)/montmorillonite nanoparticles for improved oral delivery of exemestane. Journal of Microencapsulation. 2013;30(5):432-40.
62. Timur B, Yilmaz Usta D, Teksin ZS. Investigation of the Effect of Colloidal Structures Formed During Lipolysis of Lipid-Based Formulation on Exemestane Permeability Using the In Vitro Lipolysis-Permeation Model. JDDST-D-22-01412.
63. Ballout F, Habli Z, Rahal ON, Fatfat M, Gali-Muhtasib H. Thymoquinone-based nanotechnology for cancer therapy: promises and challenges. Drug Discovery Today. 2018;23(5):1089-98.
64. Qin J, Gong N, Liao Z, Zhang S, Timashev P, Huo S, et al. Recent progress in mitochondria-targeting-based nanotechnology for cancer treatment. Nanoscale. 2021;13(15):7108-18.
65. Tran P, Nguyen TN, Lee Y, Tran PN, Park J-SJTKJoP, Society POJotKP, et al. Docetaxel-loaded PLGA nanoparticles to increase pharmacological sensitivity in MDA-MB-231 and MCF-7 breast cancer cells. 2021;25(5):479-88.