# Development and Characterization of Targeted Nanoparticulate System for Tumor Metastasis Inhibition

**SUMMARY**

**SUBMITTED IN FULFILMENT FOR THE AWARD OF THE DEGREE OF**

**Doctor of Philosophy**

**IN**

**PHARMACEUTICAL SCIENCES**

BABASAHEB BHIMRAO AMBEDKAR UNIVERSITY
LUCKNOW
प्रज्ञा शील करुणा
ESTABLISHED 1996


**Submitted By**

**Chandra Bhushan Tripathi**

**Enrollment no. 564/13**

**Supervisor**

**Prof. (Dr.) Shubhini A. Saraf**

**DEPARTMENT OF PHARMACEUTICAL SCIENCES**
**SCHOOL FOR BIOSCIENCES AND BIOTECHNOLOGY**
**BABASAHEB BHIMRAO AMBEDKAR UNIVERSITY**
**(A CENTRAL UNIVERSITY)**
**VIDYA VIHAR, RAIBARELI ROAD, LUCKNOW-226025, U.P., INDIA**


**(2018)**

## Summary and Conclusion

The current study aims at development and characterization of lipidic nanocarrier system for targeted drug delivery to breast cancer. The targeted delivery approach was employed for improvement of the potency of such systems to deliver the drug molecules specifically to the cancer cells thereby avoiding dose related toxicity. Naturally occurring agents with antioxidant and tumor inhibitory potential (α-linolenic acid (ALA) and *Perilla frutescence* oil in current study) was incorporated in lipidic systems potentiated the effect of anticancer molecules extensively utilized in breast cancer chemotherapy, ***i.e.,*** doxorubicin. Also, antioxidant property of these lipids helped in reducing the drug induced toxic effects. This approach maximized the therapeutic benefits of anticancer agents in comparison to conventional formulations.

The anticancer drug selected for current study was doxorubicin as it is utilized as first line therapy of benign as well as metastatic breast carcinoma. Further, doxorubicin therapy is restricted due to the development of cardiotoxicity and drug resistance. Doxorubicin was characterized for identification and purity by FTIR, NMR and UV spectroscopy.

Drug solubility of the drug was performed in various lipids, emulgents and co-emulgents. The excipients with highest solubility for drug were selected for development of nanoemulsion system. For NLCs, solid lipid, liquid lipids and surfactant was selected on the basis of stability, particle size, solubility and water absorption capacities.

Based upon solubility, stability and water absorption capacities, the α-linolenic acid, lecithin, Tween 80, and cholesterol were selected for the development of nanoemulsion. Similarly for development of NLCs, stearic acid, perilla oil, and lecithin were selected based on stability and water absorption capacities.

Pseudo-ternary phase diagram was constructed for selection of ratio of emulgents, i.e., by titrating different ratios of emulgents with aqueous phase. Lecithin and Tween 80 in 1:1 ratio showed highest nanoemulsion region with ALA as lipid phase. Water absorption capacity suggested that cholesterol among different co-emulgents produces nanoemulsion with highest stability and lowest globule sizes. For NLCs, solid lipid and liquid lipid in ratio of 70:30 yielded NLCs with lowest particle size and stability.

For the optimization purpose of nanoemulsion various factors selected include α-linolenic acid as lipid phase, lecithin and Tween 80 (1:1) as surfactant mixture and cholesterol as co-emulgent. 3-factor 3-level Box Behnken design (BBD) was selected for optimization of nanoemulsion which suggested 17 experimental trails with different combination of factors. Similarly, stearic acid and perilla oil (70:30) as lipid phase, lecithin as surfactant and sonication time were selected as factors for optimization using central composite design (CCD) (α=1) with 15 experimental trials. All the trials for nanoemulsion and NLCs were separately formulated and characterized for response variables like, particle size, dug release, entrapment efficiency and drug loading/drug content.

The observed data for both the systems were analyzed mathematically for model development to fit the data using quadratic equations that generated best fitted equations. Response surface methodology (RSM) best demonstrated the relationships among selected factors and responses along with interactions among factors, if any and was therefore selected. The RSM suggested significant effect of lipids surfactants and co-surfactant on different responses i.e., particle size, dug release, entrapment efficiency and drug loading/drug content of nanoemulsion and NLCs. Triethanolamine was added to NLCs that enhanced the entrapment of amphiphilic doxorubicin to the system by hydrophobic ion interactions best known as hydrophobic ion pairing (HIP) phenomenon.

Further, the design space was created and optimized formulation was identified by putting some criteria for different response factors. The criteria applied for the nanoemulsion optimization were globule size less than 100 nm, entrapment efficiency more that 90%, drug release more that 80% and drug loading more that 10% fixed. The criteria for optimization of the NLCs were applied like, particle size should be lower than 150 nm, drug release should be more than 90%, EE more than 95% and drug content should be more than 15 mg/g of NLCs.

The formulation composition optimized for nanoemulsion (Dox-NE) was ALA-400 mg), Lecithin-350 mg, Tween 80 (350 mg) and cholesterol-150 mg), where as the composition of NLCs (Dox-NLCs) was lipid-2% w/w (composed of 7:3 ratio of stearic acid to perilla oil), surfactant-3% w/w (lecithin) along with optimum

concentration of sonication time as 20 min. Good correlation was observed between observed values and predicted values of response variables of both the systems.

The folate was successfully anchored on the surface of nanoemulsions with help of NHS (N-hydroxysuccinimide) and DCC (N,N'-Dicyclohexylcarbodiimide) as observed by characteristic peaks for different groups and structural compositions in FTIR and NMR spectrum. Folate content analysis supported the anchoring of folate to the surface and the amount of folate attached was found to be 10.33 $\mu mol.g^{-1}$ of NE formulation (f-Dox-NE). The physicochemical characterization of f-Dox-NE revealed the globule size of 55.2±3.3 nm, zeta potential -31±2 mV, entrapment efficiency of 92.51 ± 3.62% and percent drug loading of 0.47±.03%, respectively.

The biotin was successfully attached on the surface of NLCs with help of NHS and DCC as represented by characteristic peaks in FTIR and NMR spectrum. Biotin content analysis supported the anchoring of biotin to the surface of NLCs and folate content was found to be 5.85±0.64 $\mu g.g^{-1}$of NLCs (b-Dox-NLCs). The physicochemical characterization of b-Dox-NLCs revealed the globule size of 105.2±3.5 nm, zeta potential -35±2 mV, entrapment efficiency of 99.15±1.71% and drug content of 19.67±2.6 $mg.g^{-1}$ of NLCs, respectively.

There was significant improvement in doxorubicin release from the optimized nanoemulsion when compared with the standard drug and marketed formulation. f-Dox-NE exhibited sustained release behaviour with 94.86±1.87% drug release in 72 h. b-Dox-NLCs showed sustained and pH dependent drug release with faster drug release as the pH was lowered, ***i.e.,*** 55.39±3.27% at pH 7.4, 81.63±4.5% at pH 6.8 and 98.66±3.43% at pH 5.4 in 72 h. No significant change in the doxorubicin release profiles was observed before and after surface decoration in both the formulations.

The f-Dox-NE and b-Dox-NLCs were found to be stable and robust as observed from different stability indicating studies like, dilution stability, thermodynamic stability as well as stability in plasma and intravenous infusion solutions. The accelerated stability studies as per ICH guidelines also exhibited stable formulations.

*In vitro* cell lines studies performed in the MCF-7 cell lines showed superiority of f-Dox-NE and b-Dox-NLCs as observed from cell cytotoxicity assay, cell cycle

analysis, cellular proliferation studies, mitochondrial membrane potential and reactive oxygen species studies.

MTT assay showed that cell cytotoxicity potential for f-Dox-NE and b-Dox-NLCs were 54.98% and 63.64%, respectively, higher as compared to marketed formulation. Also, the placebo formulation without having drug showed significant cell cytotoxicity potential as exhibited by $IC_{50}$ values for ALA-NE and p-NLCs, i.e., 4865±448 and 4465±267 $\mu g.mL^{-1}$, respectively.

Cell cycle studies through Fluorescence-activated cell sorting (FACS) demonstrated that ALA-NE, Dox solution and f-Dox-NE arrested the MCF-7 cells in G2 phase, G1phase and G1&G2/M phase respectively. While, p-NLCs, Dox solution and b-Dox-NLCs arrested the MCF-7 cells preferentially in G1-phase, G1-phase and G1&G2/M phase, respectively.

Cell proliferation studies exhibited the inhibition of cellular proliferation for nanoemulsion was in order of ALA-NE < Standard < marketed < Dox-NE < f-Dox-NE. While, the order for inhibition of cellular proliferation of different NLCs was found as p-NLCs < Standard < marketed < Dox-NLCs < b-Dox-NLCs. The data suggested that modified formulations exhibited highest cellular mortality as compared to marketed and standard formulations. Similarly, reactive oxygen species (ROS) and mitochondrial membrane potential (MMP) data suggested the superiority of the biotinylated NLCs and folate anchored nanoemulsions as compared to marketed and standard formulations. Cellular uptake studies demonstrated higher uptake of modified formulation in comparison to marketed formulation.

The superiority of modified formulations as revealed through *in vitro* cell lines data indicated that surface modifications helped these formulations in better cellular internalization through overexpressed receptors and better interaction between lipidic formulations and lipidic cell membrane leading to higher concentration of cytotoxic drug inside cell and hence higher cell mortality. Also, the tumor inhibitory potential of incorporated lipid phase (ALA and perilla oil) further potentiated the effects of anticancer agent resulting in better efficacy of such formulations.

*In vivo* studies were performed in female albino wistar rats, weighing 120-150 g. The experiment was performed according to the CPCSEA guidelines for laboratory

animals and ethics, Department of animal welfare, Government of India Animals were randomized and divided into seven groups of 10 animals each. Mammary gland carcinoma in each group (except control group) was induced by single tail vein injection of 7,12-Dimethylbenz[a]anthracene (DMBA) (8 mg/kg i.v.) on day 1. The drug/formulations were administered (10 mg/kg e.q. Dox) thrice/weeks in last six weeks of total 16 weeks of *in vivo* studies. A gap of 10 weeks was provided to develop the cancer in animals. Tumor incidence and size were monitored by measuring the diameter of mammary glands of rats, weight and survival of animals during the study.

Toxic group evidenced highest tumor volume, tumor incidence, reduction in animal weight and mortality in animals followed by standard group, marketed group, Dox-NE and f-Dox-NE. In case of nanoemulsion formulation reduction in animal weight reduction was found to be 44% for toxic group, 29.68% reduction for ALA-NE, 17.35% for standard group, 23.38% for marketed formulation, 14.96% for Dox-NE and 10.15% for b-Dox-NE group. Similarly for NLCs formulation, toxic group showed mean reduction in animal weight of 48%, p-NLCs treated group showed 36.71% reduction, standard group 10.74% reduction, marketed formulation 15.32% reduction, Dox-NE 13.38% reduction and b-Dox-NE group showed 5.47% reduction.

In case of animal survival highest animal survived the study for f-Dox-NE groups followed by Dox-NE, marketed and standard treated groups. Toxic group showed lowest animal survival. Similar results were also observed in case of NLC formulation. Parallel trends were observed for tumor incidence and tumor volumes in both the formulations.

Treatment of animals with DMBA resulted in significant disturbance in biochemical machineries of cells indicated by marked rise in the levels of biochemical markers like Thiobarbituric acid reactive substances (TBARS), protein carbonyl, glutathione (GSH), Superoxide dismutase (SOD) and catalase. Treatment with different formulation curtailed the levels of these markers and order of activity observed was f-Dox-NE>Dox-NE> marketed~ standard>ALA-NE. Likewise, surface modified NLCs showed highest efficacy in reinstatement of the levels of these biomarkers.

Western blotting data suggested that DMBA administration to the animals caused the elevation of antiapoptotic proteins like bcl-2, and MMP-9 and down-regulation of

pro-apoptotic markers such as bax and caspase-9. The treatment of animal with Dox and f-Dox-NE restored the level of these proteins towards normal but the effect was more profound in case of f-Dox-NE. Bcl-2 and MMP-9 have been shown to promote the breast tumor migration, invasion and metastasis. The level of these proteins were restored by treatment of animal with f-Dox-NE indicating that the formulation was able to inhibit tumor metastasis in DMBA administered animals. Treatment of DMBA treated group with b-Dox-NLCs resulted in downregulation of anti-apoptotic proteins bcl-2 and MMP-9 while upregulated the level of expression of pro-apoptotic proteins like BAX, caspase-9 and p16 indicating tumor inhibitory potential of the formulation. Further, the MMP-9, p16 and members of the bcl-2 family protein, including bcl-2, BAX *etc.* exhibited an important role in breast cancer migration, invasion and metastasis. The results demonstrated that the biotin decorated NLCs can effectively reduce the mammary tumor cell metastasis in the disease induced rats.

Angiogenesis and cellular proliferation are distinctive characteristics for the cancer growth and progression represented through Alveolar buds/terminal end buds (AB/TEB) count and cellular architecture of mammary gland tissue. The DMBA administration resulted in significant increase in the number of AB/TEB and significant changes in ducts, adipocytes, Loose connective tissue, Dense connective tissue and tissue architecture in mammary gland tissues as depicted by carmine staining and H&E stain. Dox-NE and f-Dox-NE was more efficient in diminishing the count of AB/TEBs as compared to the standard and the marketed groups. Similarly, b-Dox-NLCs treatment resulted in restoration of cellular architecture and lowering in the AB/TEBs count.

Doxorubicin is known to exhibit cardiotoxicity due to dose related cardiac tissue damage and necrosis. Doxorubicin treatment resulted in significantly alterations in levels of MDA and GSH in cardiac tissue. Surface modified drug delivery treated groups showed no significant changes in the MDA and GSH levels of cardiac tissues. The results suggested preferential delivery by molecularly guided systems through the overexpressed receptor cancer site.

The Dox levels attained in the mammary carcinoma was highest in the case of the f-Dox-NE (45.23±2.51 µg/g organ in 4 h) followed by the Dox-NE (35.43±2.91 µg/g organ in 4 h) indicating the specific accumulation of the folate decorated

nanoemulsion in FR over-expressed mammary tumor cells. Further, significant higher Dox levels were maintained in the mammary cancer cells for a period of upto 48 h (25.78±1.42 µg/g organ) in the f-Dox-NE ($p<0.001$); upto 12 h (25.55±2.22 µg/g organ) in the Dox-NE ($p<0.001$) treated groups *vis-à-vis* the pure Dox (15.55±2.25 µg/g in 12 h, 1.78±0.42 µg/g in 48 h) and the marketed formulation (14.54±2.21 µg/g in 12 h, 1.79±0.38 µg/g in 48 h) treated groups. Among different organs, highest Dox concentration was observed in the liver followed by the spleen, the heart and the kidney. Overall, the Dox levels in different organs of the folate decorated NE were relatively lower than that of other animal groups. Similar, results were observed in case biotinylated NLCs with highest concentration was maintained by b-Dox-NLCs in cancer tissue followed by Dox-NE, standard and marketed drug. The biodistribution data further affirmed the selective accumulation of the biotinylated NLCs to the biotin receptor which is over-expressed in the condition of mammary gland carcinoma.

As summarized above, ligand guided drug delivery system was safe and effective in regulating the cancer proliferation, invasion and migration in mammary gland carcinoma. The mammalian cells are incapable of themselves synthesizing the some of the biomolecules and therefore it is taken up through some transport systems like high-affinity biotin transporter or sodium-dependent multivitamin transporter (SMVT) in case of biotin and high affinity folate receptor for folate uptake. The overexpression of these receptors and selective uptake capacity for these molecules in the cancer cells may be a possible explanation for selective uptake of molecularly decorated NLCs for mammary cancer. Attachment of targeting ligands through a relatively long PEG chain linker resulted in effective ligand-receptor association and internalization of the complex in cancer cells. Further, it has been demonstrated that ALA and perilla oil has cancer ameliorative and inhibitory effect, also, act as antioxidants for normal cells, thereby preventing the drug induced toxicities. Further prolonged drug release maintained higher concentration of anticancer molecules that resulted in higher cell cytotoxicity. The combined effects of the above would effectively address mammary gland carcinoma. Therefore, it can be concluded that being safe and effective, these formulations can be used as a better alternative to the conventional formulations in management of mammary gland carcinoma. Further, the translational potential of this drug delivery system could prove to be beneficial for patients after suitable clinical evaluations, as future scope.