Summary & Conclusions

SUMMARY AND CONCLUSIONS

SUMMARY

INTRODUCTION

Cancer, particularly **Breast Cancer**, is a significant global health challenge. This research focused on addressing the global burden of breast cancer, a leading cause of cancer-related mortality among women. Breast cancer has seen a rise in incidence and mortality rates over the past decades, with projections suggesting alarming increases by 2040. Despite advancements in oncology, conventional treatment strategies such as chemotherapy face numerous limitations, including systemic toxicity, poor bioavailability of drugs, and the emergence of resistance mechanisms. This study emphasizes the importance of innovative therapeutic approaches to improve treatment efficacy and patient outcomes in breast cancer management.

The motivation for this research stemmed from the pressing need to overcome the limitations of current cancer therapies. Breast cancer, particularly in advanced stages, exhibits high tumor heterogeneity and resistance to standard chemotherapeutic agents. Furthermore, disparities in survival rates between high-income and low-income regions underscore the inequities in access to effective treatments. The study seeks to bridge these gaps by developing advanced nanocarrier systems capable of enhancing drug delivery, reducing systemic toxicity, and targeting tumors more effectively. This work also aligns with the global emphasis on personalized medicine, aiming to tailor treatments to the specific needs of patients.

Traditional chemotherapy, while effective in certain cases, is fraught with challenges that hinder its success. Many chemotherapeutic drugs, including Paclitaxel (PAC) and Cyclophosphamide (CYC), have toxic effects on healthy cells, leading to severe side effects. PAC’s hydrophobic nature limits its bioavailability, requiring the use of solvents like Cremophor EL, which can cause hypersensitivity reactions. Tumor cells often develop resistance mechanisms, such as upregulation of efflux pumps and alterations in drug targets, reducing the efficacy of repeated chemotherapy cycles. The presence of diverse cellular subpopulations within a tumor complicates treatment, as different cells may respond variably to the same therapy. Rapid clearance of drugs from the bloodstream results in suboptimal

drug concentrations at the tumor site. These limitations necessitate the development of innovative delivery systems to enhance therapeutic efficacy while minimizing side effects.

The study introduced nanostructured lipid carriers (NLCs) and microemulsions as advanced drug delivery platforms for the co-delivery of PAC and CYC. These nanocarrier systems aim to address the challenges of conventional therapies by enhancing drug solubility, targeting tumor tissues, and overcoming drug resistance. NLCs and microemulsions were chosen for their unique properties. NLCs are composed of a blend of solid and liquid lipids, creating an unstructured matrix that allows for high drug-loading capacity and controlled release. Microemulsions are thermodynamically stable mixtures of oil, water, and surfactants that provide excellent solubilization and ease of formulation. By leveraging these platforms, the research aimed to improve the therapeutic index of PAC and CYC, offering a more efficient and patient-friendly approach to breast cancer treatment.

The **aim of present research** focused on the development of nanostructured lipid carriers and microemulsion for the simultaneous delivery of PAC and CYC for the treatment of Breast Cancer.

The **present research hypothesised** that Nanostructured Lipid Carriers (NLCs) and Microemulsion (MEs) containing PAC and CYC will improve drug solubility, stability, targeted delivery, and therapeutic efficacy while overcoming drug resistance mechanisms in breast cancer treatment.

ANALYTICAL TECHNIQUES

High performance liquid chromatography technique was used for the determination and estimation of PAC and CYC in NLCs and Microemulsion. These methods were adopted from USP monograph of PAC and CYC and were found to be suitable for the estimation of both the drugs in developed formulations.

For **Paclitaxel**, HPLC method from USP 43 NF 38 was adopted wherein Shimadzu Chromatograph, Prominence-I LC2030C plus was used. The quantitation of PAC was done using (250 x 4.6) mm, 5µm column with flow rate of 1.5mL/minute, and Injection volume of 100µL in isocratic mode with column oven temperature of 5°C and detection wavelength of

195nm. Water and Acetonitrile in the ratio of 30:70 were used as mobile phase with run time of 15minutes. The method for the estimation of PAC was found to accurate and precise.

For **Cyclophosphamide**, HPLC method from USP 43 NF 38 was adopted wherein Shimadzu Chromatograph, Prominence-I LC2030C plus was used. The quantitation of Cyclophosphamide was done using (250 x 4.6) mm, 5µm, L43 column with flow rate of 2.0mL/minute, Injection volume of 100µL in isocratic mode with column oven temperature of 25°C and detection wavelength of 227nm. Water and Acetonitrile in the ratio of 5.5:4.5 were used as mobile phase with run time of 15minutes. The technique utilized for the estimation of CYC was accurate and precise.

PREFORMULATION STUDIES

As a part of preformulation studies, both active ingredients were authenticated by melting point determination, FTIR spectroscopy, and determination of wavelength maxima. Screening of solid lipids and liquid lipids/ oils followed by solid lipid-liquid lipid compatibility studies was performed. PEG-100 stearate was screened as suitable solid lipid for PAC and CYC as they had highest solubility in PEG-100 stearate. PEG-8 Caprylic/Capric Glycerides was selected as liquid lipid/ oil for the manufacturing of nanostructured lipid carriers and microemulsion as it was able to solubilize highest amount of PAC and CYC. Also, PEG-100 Stearate and PEG-8 Caprylic/Capric Glycerides mixtures were observed to be compatible with each other at 1:1, 1:2, and 1:3 ratio. PEG-400 was selected as a cosurfactant for manufacturing of microemulsion as PAC was found to have highest solubility of more than 100mg/gram of PEG-400 and Cyclophosphamide had solubility of more than 250mg/gram of PEG-400. Acetone was chosen as a co-solvent due to its lower boiling point (56°C) leading to faster evaporation during compounding for NLCs. Also, PAC and CYC have solubility in acetone making it a preferred co-solvent for the formulation of NLCs. Drug-Drug and Drug-Excipient compatibility study demonstrated that physical mixtures had physical stability which was further confirmed through FTIR and HPLC as a part of isothermal testing method.

FORMULATION DEVELOPMENT: PAC-CYC NLCs

As a part of formulation development studies, quality by design (QbD) and OFAT approach were adopted for the development of PAC and CYC loaded NLCs. Critical quality attributes

were identified and qualitative risk assessment was done using Ishikawa diagram. Based on Ishikawa diagram and Initial risk assessment, formulation parameters and process parameters that were assessed to have high risk were optimized using OFAT (One Factor At a Time) technique. Upon evaluation of surfactants, Cremophor EL and Soluplus demonstrated the highest entrapment efficiency and filterability with lowest Z-average and desired PDI. It was further demonstrated that combination of 1% Soluplus and Cremophor EL 4% exhibited lowest particle size and PDI with maximum entrapment efficiency and filterability. Further, it was established that total lipid concentration of 2% with solid lipid to liquid lipid ratio of 1:1 and drug concentration for PAC as 1mg/mL and Cyclophosphamide as 8.75mg/mL was required to achieve quality target product profile. Process parameters were optimized at mixing speed of 500RPM, mixing time of 15minutes and phase temperature of 50±2°C to achieve the target critical quality attributes.

Further, Mannitol was selected as cryoprotectant at 4%w/v level for optimized NLCs based on the freeze thaw studies. Based on the Freeze Drying Microscopy (FDM), PAC-CYC NLCs containing 4% Mannitol were observed to be completely frozen at -19.5°C, Onset of collapse was observed at 0.5°C and complete collapse was observed at 1.5°C. Therefore, 0.5°C at which the collapse initiation was observed in FDM was the collapse temperature (Tc) considered for SMART^{TM} lyophilization cycle. Lyophilized NLCs were further compared with NLCs before lyophilization which confirmed that Mannitol was well suitable as a cryoprotectant for protecting the NLCs during lyophilization.

Lyophilized NLCs with optimized process and formulation parameters were further characterized. The lyophilized NLCs were found to be readily redispersible in sterile water for injection and phosphate buffer pH 7.4 with reconstitution time of 69 seconds and 74 seconds respectively. The product description after reconstitution was observed to be clear translucent liquid with blue tint and free from any visible particulate matter. The Z-average was achieved as 53.9 ± 2.0nm with PDI of 0.125 ± 0.018 and zeta potential was observed to be -5.3 ± 2.7 mV. The entrapment efficiency of PAC was obtained as 100.0±0.4 % whereas for Cyclophosphamide it was obtained as 99.0±0.8 %. Assay of PAC in NLCs was determined as 99.5 ± 0.6 % and assay of Cyclophosphamide in NLCs was reported as 100.2 ± 0.1 %. The water content of lyophilized NLCs was observed to be 0.5± 0.1 %. The FTIR spectra, DSC thermogram, and XRD of pure drugs versus PAC-CYC NLCs and Placebo

NLCs confirmed the entrapment and encapsulation of PAC and CYC within the lipid matrix. TEM also confirmed the particle size to be as 45.95 ± 7.98nm. Cumulative drug release of 98.27 ± 0.75 % and 96.87 ± 0.50 % was observed for both the INTAXEL and ENDOXAN within 3 hours respectively whereas, PAC and CYC loaded in NLCs required 24 hours for cumulative drug release of 96.07 ± 0.70 % and 98.37 ± 0.35 % respectively. PAC-CYC loaded NLCs demonstrated stability and sterility when stored at 2 to 8°C for 12 months. Based on the optimization of various factors impacting the formulation development and characterization of PAC-CYC NLCs, the updated risk assessment confirmed that risk was now updated as LOW.

FORMULATION DEVELOPMENT: PAC-CYC MICROEMULSION

As a part of formulation development studies, quality by design (QbD) and OFAT approach were adopted for the development of PAC and CYC loaded Microemulsion. Critical quality attributes were identified and qualitative risk assessment was done using Ishikawa diagram. Based on Ishikawa diagram and Initial risk assessment, formulation parameters and process parameters were assessed to have high risk and hence were optimized using OFAT (One Factor At a Time) technique. Upon evaluation of surfactants, Cremophor EL and Soluplus demonstrated the highest entrapment efficiency with lowest Z-average and desired PDI. It was further demonstrated that combination of 1% Soluplus and Cremophor EL 4% exhibited lowest particle size and PDI with maximum entrapment efficiency. Further, pseudo ternary diagrams were constructed to optimize the Surfactant: Cosurfactant ratio wherein 10% of S_{mix} ratio 5:1 could accommodate 15% of oil which is the highest as compared to other two ratios (2:1 and 3:1). Drug concentration for PAC as 3mg/mL (0.3%) and Cyclophosphamide as 26.25mg/mL (2.625%) was optimized to achieve quality target product profile. Additionally, concentration of oil and Smix was optimized as Oil at 15% and Smix at 10% as the microemulsion remained stable upon 100 times dilution. Process parameters were optimized at mixing speed of 500RPM, and mixing time of 15minutes to achieve the target critical quality attributes.

Optimized PAC-CYC microemulsion was further characterized wherein dilution with water at 1:10, 1:100 and 1:250 ratios produced clear and stable microemulsion without any separation or precipitation proving that the emulsion was oil-in-water type. The viscosity of optimized formulation was reported as 1.09 ± 0.06 cps as the system exhibited characteristics

akin to a homogeneous solution, indicating that the viscosity remained low and comparable to that of water. The zeta potential of microemulsion was reported as -2.6 ± 0.2 mV whereas the globule size distribution of optimized microemulsion revealed Z-average as 62.8 ± 1.0nm with PDI of 0.142 ± 0.031, indicating that size distribution is narrow. TEM revealed that optimized microemulsion were discrete and spherical oil globules dispersed in continuous phase of microemulsion with droplet size ~ 100 nm. The entrapment efficiency of PAC was obtained as 99.7±0.2 % whereas for Cyclophosphamide it was obtained as 100.6±0.8 %. Assay of PAC in microemulsion was determined as 99.0 ± 0.4 % and assay of Cyclophosphamide in microemulsion was reported as 100.8 ± 0.3 %. Cumulative drug release of 98.27 ± 0.75 % and 96.87 ± 0.50 % was observed for both the PAC and CYC marketed formulations within 3 hours respectively whereas, PAC and CYC loaded in microemulsion required 6hours for cumulative drug release of 87.89 ± 2.49 % and 96.10 ± 0.75 %respectively. PAC-CYC loaded microemulsion demonstrated stability and sterility when stored at 2 to 8°C for 12 months. Based on the optimization of various factors impacting the formulation development and characterization of PAC-CYC microemulsion, the updated risk assessment confirmed that risk was now updated as LOW.

IN-VITRO CELL LINE STUDIES

Synergism was determined for PAC and CYC at 1: 8.75 ratio as Literature review suggested that 200mg/m^2dose of PAC and 1750mg/m^2dose of CYC was suitable for the treatment of breast cancer. The Response Additivity approach confirmed the synergism between PAC and CYC where the observed effect was way too high as compared to the expected effect which is the sum of Effect of PAC and Effect of CYC.

In vitro cell line studies were performed using MCF-7 breast cancer cell lines to assess the cytotoxicity and therapeutic efficacy of the drug-loaded NLCs and microemulsions. For in-vitro cell viability assay, at 72 hours, pure PAC exhibited IC_{50} value of 9.10µg/mL whereas PAC in PAC-CYC NLCs exhibited IC_{50} value of 2.92µg/mL and PAC in PAC-CYC Microemulsion exhibited IC_{50} value of 2.86µg/mL. At 72 hours, pure CYC exhibited IC_{50} value of 39.80µg/mL whereas CYC in PAC-CYC NLCs exhibited IC_{50} value of 25.56µg/mL and CYC in PAC-CYC Microemulsion exhibited IC_{50} value of 24.99µg/mL. Cellular uptake and cell Apoptosis by Fluorescence Microscopy studies demonstrated that blebbing, aggregation, and cluster formation, a distinct morphological characteristic associated with

apoptosis in cell cycle, was more in PAC-CYC loaded NLCs, and PAC-CYC loaded Microemulsion as compared to pure PAC and pure CYC. The cellular uptake studies and apoptosis assays thus showed that the NLC and ME formulations induced significantly higher levels of apoptosis compared to free drugs or conventional formulations. In cell cycle analysis by flow cytometry, PAC and CYC loaded in NLCs and microemulsion could demonstrate better cell arrest in comparison to pure drugs. Both NLCs and MEs induced cell cycle arrest at the G2/M phase, a critical checkpoint for cell division, preventing tumor proliferation. The combination of PAC and CYC in a single nanocarrier disrupted multiple cancer signaling pathways, reducing the likelihood of resistance development.

IN-VIVO EFFICACY STUDIES

In vivo evaluations further validated the therapeutic potential of the developed formulations. The in-vivo efficacy of PAC and CYC loaded Nanostructured Lipid Carriers (NLCs) and Microemulsion (ME) was evaluated using tumor regression studies in xenograft model in female Sprague Dawley rats to mimic breast cancer conditions, providing a robust framework for assessing the potential of these nanocarrier systems in combating tumor growth and improving treatment outcomes. The disease control group showed significant tumor progression, with an average tumor volume increase to 180 mm^3 by the end of the study. In the groups treated with INTAXEL® and ENDOXAN™, tumor growth was noticeably slower compared to the control group. The groups treated with PAC-CYC NLCs and PAC-CYC MEs demonstrated the most significant tumor growth inhibition. Tumors showed either minimal growth or complete stasis, indicating the superior efficacy of the developed nanocarrier systems.

Tumors in the disease control group exhibited extensive infiltration of tumor cells into adipose and stromal tissues, accompanied by increased lymphocyte activity and calcification. Samples from the PAC-CYC NLC and ME groups revealed a marked reduction in tumor cell infiltration. The tissues in these groups retained more normal architecture, with fewer signs of calcification and lymphocyte abundance, indicating a reduction in cancer progression and inflammatory response. The PAC-CYC NLC and ME formulations showed superiority when compared to the conventional INTAXEL® and ENDOXAN™ treatments in both tumor suppression and histological outcomes. The in vivo studies conclusively demonstrated the advantages of using NLCs and MEs for the co-delivery of PAC and CYC. These advanced

formulations effectively suppressed tumor growth, and minimized cancer progression at the histological level.

CONCLUSIONS

Breast cancer continues to pose a significant global health burden, with increasing incidence and mortality rates necessitating the development of novel therapeutic strategies. Conventional chemotherapy, despite its efficacy in certain cases, is hindered by several limitations, including poor bioavailability, systemic toxicity, and the emergence of resistance mechanisms. Paclitaxel (PAC) and Cyclophosphamide (CYC) are commonly used chemotherapeutic agents for breast cancer treatment, yet their clinical potential is compromised by their physicochemical properties, rapid clearance, and adverse effects. This research aimed to overcome these limitations through the development of Nanostructured Lipid Carriers (NLCs) and Microemulsions (MEs) for the co-delivery of PAC and CYC, with the objective of improving solubility, providing targeted drug delivery, and enhancing therapeutic efficacy.

The nanostructured lipid carriers and microemulsion were successfully manufactured and optimized using a systematic Quality by Design (QbD) approach to have maximum entrapment efficiency, minimum size, and stability. The optimized nanostructured lipid carriers and microemulsion showed longer in-vitro release as compared to marketed formulations of INTAXEL (Paclitaxel Injection) and CYTOXAN (Cyclophosphamide Injection).

In-vitro cytotoxicity studies demonstrated that PAC-CYC NLCs and MEs exhibited significantly lower IC_{50} values compared to free drugs, indicating enhanced cancer cell killing efficiency. Cellular uptake and apoptosis studies revealed that the nanocarrier formulations induced greater cell death and disrupted tumor cell division by causing cell cycle arrest at the G2/M phase. The synergistic effect of PAC and CYC in these nanocarriers led to higher apoptosis rates, possibly improved drug retention within tumor cells, and reduced likelihood of resistance development.

Further validation through in-vivo studies in xenograft models confirmed the superior therapeutic efficacy of the PAC-CYC NLCs and MEs over conventional formulations (INTAXEL® and ENDOXAN™). Tumor regression studies indicated that nanocarrier-based

drug delivery significantly reduced tumor growth, while histological analyses showed reduced tumor cell infiltration, decreased inflammatory response, and preserved normal tissue architecture. Additionally, the nanocarrier formulations demonstrated excellent long-term stability and sterility, ensuring their feasibility for clinical translation.

Overall, this research established NLCs and MEs as viable nanocarrier platforms for the co-delivery of PAC and CYC, addressing key challenges associated with conventional chemotherapy. These formulations exhibited improved drug solubility, longer in-vitro release, increased tumor targeting, and therapeutic efficacy, thereby improving the therapeutic index of PAC and CYC in breast cancer treatment.