Development and Characterization of Nanostructured Lipid Carrier System(S) against Photoinduced Tumorigenicity

SUMMARY
SUBMITTED TO
Babasaheb Bhimrao Ambedkar University
(A Central University)
Lucknow

For the Degree of

DOCTOR OF PHILOSOPHY

In

Pharmaceutics

By

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(Enrolment No. 487/12)

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2017

Nanotechnology promises superior treatment and management of chronic diseases such as cancer. Several advantages, such as low skin irritation, increased protection of encapsulated drug and increased penetrability through the skin are offered. Solid Lipid Nanoparticles (SLN) was developed as an alternative colloidal carrier system to emulsion, liposomes and polymeric nanoparticles. SLNs had the drawback of drug leakage after long term storage, and drug expulsion due to an on going crystallization process of the lipid towards a perfect crystal. Nanostructured lipoidal carriers (NLC) were developed to overcome potential limitations associated with SLN and are the second generation nanoparticles composed of solid and liquid lipid matrix. NLC composed of diverse oils with solid lipid, can produce imperfections in their lattice structure and create separation in the fatty acid chain packing, making more space for the drug. Liquid lipids are better solubilizer for drugs as compared to solid lipids.

There are numerous methods to produce lipid nanoparticles such as micro emulsion method, solvent evaporation in o/w emulsion, solvent displacement technique, solvent diffusion technique and Hot High Pressure Homogenization (HHPH) technique. However, HHPH possesses several advantages over other methods such as scale up possibility, avoidance of organic solvents and short production time.

NLC was prepared by **Hot High Pressure Homogenization (HHPH) technique**, which showed several advantages over other methods, like scale up possibility, avoidance of organic solvents and short production time. In addition, HHPH allows the regulation of control drug release by modifying the production parameters such as temperature, number of cycles and homogenization pressure. At the same time, this technique uses lesser concentration of surfactant in contrast to other methods.

Silymarin was selected as drug of choicedue to its potential activity in prevention of various types of cancer. Silymarin has high anti-radical and anti-oxidant activity on skin cells. It is able to decrease cellular peroxidation and shield the skin from photo-induced tumours. Furthermore, silymarin effectively causes inhibition of proliferation, and growth arrest in G0-G1 and G2-M phases of the cell cycle. However, despite these interesting features, the utility of silymarin is limited due to several limitations like skin irritation, low water solubility and instability in the presence of air and light. The low water solubility of Silymarin (0.383mg/ml) may limit its incorporation in a suitable vehicle, while its poor photostability

may render the topically applied drug ineffective. **Hence, it was thought worthwhile to investigate its anticancer efficacy in the form of silymarin NLC in SK-MEL 2 cell line**.

In the present study factorial design was implemented to optimize process parameters of homogenization and **effect of homogenization pressure, number of cycles and stirring speed on the mean particle size (Z-Ave), polydispersity index (PI) and zeta potential (ZP) of NLC dispersions were observed.**

Factorial design allows extraction of large amount of information from the collected data, which results in limiting the number of experiments. In the present work, preparation of a lipoidal delivery system of NLC was done by HHPH using Compritol 888 ATO as solid lipid and Miglyol 812 as a liquid lipid and its anticancer activity was investigated by cell line studies. Suitability of the topical dosage form was proven by skin irritation and permeability studies. *In vivo* efficacy was proven by UV induced skin edema studies.

Following objectives were set to achieve the answer of the following questions

- Screening of **different lipids and surfactants** on the basis of solubility and compatibility studies
- Selection of **best suitable method for preparation** of Silymarin-NLC based on Physicochemical characterization
- **Optimization of Process parameters** for the preparation of Silymarin-NLC by factorial design
- **Optimization of Product parameters** for preparation of Silymarin-NLC by factorial design
- Conversion of Optimised formulation in **to Silymarin-NLC Topical gel** and its further characterization
- Analysis of protectant action against UV light, by using **Cell Line (SK-MEL-2) studies and SRB assay** method
- Suitability to skin was tested by **Skin irritation test and Skin Permeation te**st
- ***In vivo*** activity was evaluated by **UV induces edema study**
- Activity on molecular level were studied by **Western blot and RT-PCR** studies

The conclusion drawn on the basis of above studies is as follows:

- Based on highest solubility and compatibility studies of silymarin in lipids Miglyol 812, Oleic acid, Glycerol monostearate and Compritol 888 ATO were selectedfor the preparation of Silymarin-NLC.Glycerol monostearate also acts as a carrier and emulsifier in nanoformulation.
- For selection of method silymarin-NLC was prepared by Microemulsion technique, Melt- emulsification and low temperature solidification method, Ultra sonication method and Hot High Pressure Homogenization.
- Based on obtained desired particle size and less amount of surfactant used; hot high pressure homogenization method was selected for preparation of NLC.
- Optimization of process parameters by 2^3 factorial design was done. Homogenization pressure, speed and number of cycles were selected as independent variables and their effect on mean particle size; Polydispersity index and Zeta potential were evaluated. Data was analysed by using Design of expert.
- Optimal NLC formulation was produced at 1000 bars homogenization pressure, 3 cycles and at 15000 rpm. Based on the physicochemical parameters and % entrapment efficiency values of NLC dispersion, **NLC 3 formulation was selected.**
- Optimised formulation was converted in to NLC gel by using Carbopol; viscosity, pH, and drug content were checked. Physical appearance of silymarin-loaded NLC gel was found to be **creamy and smooth in texture**, and translucent in appearance. The pH of the final formulation was found to be in range of 6.7±0.2 and the viscosity was in the range of 2645.67±19.79 centipoise. This was found to be **suitable for topical application on skin**, without irritation. Drug content of the developed silymarin-NLC 3 gel was found to be well above 97.05±0.3 and it also possessed good chemical stability.
- **Spreadibilty** was found to be in the range of 5 to 8 cm (7±1), which indicates that it is suitable for topical application.
- Rheological data showed that the viscosity of the optimized formulation was sufficient for bio-adhesion and would increase the residence time, enhance penetration across the skin.
- Morphology was observed through **Transmission electron microscopy** and found to be uniform, smooth and particles without any aggregation.

- Drug release behaviourfor NLC-3 was found to be biphasic, with the initial burst effect followed by gradual release. Silymarin encapsulated within the core of solid lipid is not released completely at the end of 10 hr and a drug release of only 78% was obtained in the NLC-gel, while it increased to 98% at the end of 24 hr.

- **After optimization of process parameters for homogenization, product was optimised by taking fixed formulation parameters.**
- Three independent variables were the **lipid amount, surfactant concentration** and homogenization pressure, were fixed at lower and higher values on the basis of initial studies. **Mean particle size (Z-ave) and entrapment efficiency (EE %) were taken as response variables.**
- The factors that displayed a significant effect (p value < 0.05) on Z-Ave were lipid amount and homogenization pressure.
- The results propose that an **optimum concentration of 3% w/v sufficient** was sufficient to cover the surface of nanoparticles efficiently and avoid collection during the homogenization process while there was statistically difference among the results of 0.5 - 3 % w/v, adequate concentration of surfactant stabilized surfaces well and reduced aggregation of particles.
- Thus, from the obtained results, an optimal NLC formulation was prepared **at 1000 bars homogenization pressure, 3%w/v of surfactant concentration and 100 mg of total lipid.** NLC 9 was thus selected as the optimized formulation on the basis of high entrapment efficiency and desired particle size below 150 nm.
- The optimized batch was prepared and evaluated for particle size, zeta potential, entrapment efficiency, surface morphology, *in vitro* drug release, *ex vivo* permeation, and stability studies. **Cell proliferation activity was evaluated by sulfo rhodamine-B (SRB) assay on cell line studies (SK-MEL-2).** Acceptability to skin was tested by skin irritation test on wistar rats. Permeation parameters and cell proliferation activity was compared with marketed Phytosome formulation.
- Silymarin-**NLC 3 gel showed better inhibition in cell viability, because IC_{50} value was lower for silymarin-NLC as compared to marketed formulation**. Several other methods have been used in order to encapsulate poor soluble natural actives for topical delivery, all of which cause biochemical or metabolic changes in skin layer. However, through formation of lipoidal drug carrier system, it was possible to enhanced silymarin delivery into the skin surface without altering the skin barrier.

- In **Photostability studies** light exposure caused a sharp degradation of silymarin in the first few minutes. The rate of silymarin photo degradation slowed down after 20 min but the photo degradation process continued, leaving a small residual silymarin concentration of 20% at the end of 180 min, while silymarin concentrations in all irradiated NLC was significantly higher than unencapsulated Silymarin.Results obtained from this photostability study led us to the conclusion **that better photo protection** is awarded to silymarin in NLC as compared to silymarin in methanolic solution.
- To study **combination drug effect, silymarin and 5-fluorouracil NLC** were prepared by HHPH.
- Silymrin-5-FU NLC as a topical carrier was successfully prepared in the nanometer range using hot high pressure homogenization. Silymrin-5-FU nanoparticles showed an average particle size **within range of 160 nm to 190 nm**. Nanoparticles with combinations were spherical, with a narrow size distribution and low polydispersity index. In this formulation, the zeta potential values obtained for silymarin and 5-fluorouracil was within range of -20mV to – 30 mV. No significant change in size, polydispersity, and surface charge was seen on entrapping the nanoparticles with combination of drug.
- From SRB assay, we obtained IC 50 value (11 µg/mL) for silymarin-5-Fluorouracil-NLC, proved that they act as protectant and cause inhibition in progression. LC_{50} of positive control (48.6 µg/ml), whereas silymarin-5-Fluorouracil did not show any sign of activity. Based on this observation (LC_{50}> 1000 µg/ml), we can conclude that silymarin-NLC and **silymarin-5-Fluorouracil NLC, did not show any cytotoxicity to cancerous cell as similar to silymarin-NLC.**
- Silymarin-5-Fluorouracil-NLC gel showed better inhibition activity when used in combination. **Lethal activity was not enhanced significantly, in combination**, so*In vivo* studies were not conducted for Silymarin-5-Fluorouracil-NLC gel, but it is possible that entrapment of 5-fluorouracil with silymarin may result in reduced toxicity and in turn reduced side effect profile.
- The skin irritation effect of the gel was graded A, which implies no reaction at 3, 5 and 7 days interval. The results of **the skin irritation study showed that, by application of silymarin NLC 9 gel, there was no reaction on the skin**.
- The anti-proliferative activity of silymarin NLC was studied in 7,12-dimethylbenz[a]anthracene(DMBA)induced cellular progression/ differentiation in

albino mice model using **western blot and reverse transcription polymerase chain reaction (RT-PCR)** analysis.

- In this research we evaluated the expression of cyclin D1 in DMBA-induced mice and the relationship between the antitumor effect of silymarin-NLC and the expression of cyclin D1. We investigated this relationship because research had proved that **COX-2 and cyclin D1** were both up-regulated in Photoinduced tumour.
- In conclusion, our research clearly indicates that topical application of silymarin inhibits DMBA-induced cellular progression/ differentiation and decreases cell proliferative and inflammatory responses involving COX-2, Cyclin D1, ODC and PCNA.
- In conclusion, **Miglyol 812 and Compritol 888 ATO based stable Silymarin-NLC gel of desired particle size was prepared successfully by using HHPH technique** with limited use of surfactant. This topical Nano lipoidal carrier system serves as a solubilisation matrix for poorly soluble drug silymarin and acts as a local depot for sustained release of silymarin, with enhanced permeation, faster and prolonged action against the studied marketed formulation.
- This work documents **for the first time** that silymarin can be formulated into nanostructured lipoidal carrier system for enhanced permeation, greater stability as well act as protectant for **Photoinduced tumourgenicity**. Silymarin-NLC gel exert**suppression of cellular proliferation**, progression and differentiation for DMBA induced deregulation of COX-2, ODC, Cyclin-D1 and PCNA in mice.

- **The novelty of this work is the possible synergistic effect of drug and excipients, Formulation Optimization, and its*in vivo* studies in 7,12-dimethylbenz[a]anthracene (DMBA) mice model, at enzyme level.**

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