# 4 Material and Methods

## 4.1 Materials, Equipment, Software used

### 4.1.1 Materials

| Sr.No. | Material | Company |
|---|---|---|
| 1. | Lapatinib | Xi'an Kerui Biotechnology Co. Ltd (Xi'an, China) |
| 2. | Soluplus® | BASF India Limited (Navi Mumbai, India) |
| 3. | Solutol®HS15 | BASF India Limited (Navi Mumbai, India) |
| 4. | Pluronics® F127 | BASF India Limited (Navi Mumbai, India) |
| 5. | Methanol | Sigma Aldrich, India |
| 6. | Tween 80 | Sigma Aldrich, India |
| 7. | SKBr3 cancer cell line | Nation Centre for Cell Sciences, Pune |
| 9. | Essential antibiotics | Sigma Aldrich, India |
| 10. | Leibovitz Medium | Himedia, India |
| 11. | McCoy's 5A Medium | Himedia, India |
| 12. | Fetal Bovine Serum | Himedia, India |
| 13. | Triton-X100 | HImedia, India |
| 14. | Required buffers | SD Fine chemicals, India |

### 4.1.2 Equipments

| Sr. No. | Instruments | Source |
|---|---|---|
| 1. | Magnetic Stirrer | IKA Germany |
| 2. | Electronic Balance | Dewinter, India |
| 3. | pH meter | IKON Instruments, India |
| 4. | Digital Optical Microscope | Dewinter, India |
| 5. | Cooling centrifuge | ELTEK, 4100 RCF, India |
| 6. | Particle size analyzer | Beckmen Coulter, USA |
| 7. | Microplate absorbance reader | Biorad, USA |
| 8. | Ultra probe sonicator | UP50H, Hielscher, USA |

| | | |
|---|---|---|
| 9. | Bath sonicator | Fisher Scientific, India |
| 10. | FTIR | Shimadzu, Japan |
| 11. | $CO_2$ incubator | Sanyo, Japan |
| 12. | HPLC | Waters, USA |
| 13. | Deep freezer | Thermoscientific, USA |
| 14. | Lyophilizer | Labconco, India |
| 15. | Rotary Evaporator | Ika, India |
| 16. | SPM | NT MDT, Russia |
| 17. | Powder XRD | Rigaku, Japan |
| 18. | Vortex mixer | Fisher Scientific, India |
| 19. | Filtration assembly | Millipore, USA |
| 20. | UV Spectrophotometer | Shimadzu, Japan |

### 4.1.3 Software

| Sr. No. | Software | Source |
|---|---|---|
| 1. | Minitab 17 | Coventry, UK |
| 2. | Design Expert 10 | MN, USA |
| 3. | Graph Pad Prism 5 | CA, USA |
| 4. | Origin Pro 8 | MA, USA |

## 4.2 Pre-formulation studies

### 4.2.1 HPLC analytical method development

The HPLC analytical method was used for the determination of amount of LP in plasma obtained from rats during pharmacokinetic studies. The components of HPLC system (Waters, USA) were rheodyne 7725i manual injector (Waters, USA), 1525 binary HPLC pump (Waters, USA), C18 reverse-phase (4.6x75 mm; 3.5 µm) Symmetry® C18 column and waters 2998 photodiode array detector (Waters, USA). The mobile phase consisted of Methanol:Milli-Q water (80:20) and was run at a rate of 1.0 ml/min.

Column was maintained at a temperature of 30±1˚C during the process. The estimation of LP was performed at 262 nm. Breeze2 software was used to integrate peak area and determine retention time and was further used for drug content determination. Standard calibration curves in desired media were constructed in range of 500-3000 ng/mL of LP for *in vivo* samples. Typical validation characteristics (specificity, precision, accuracy, linearity range, limit of detection, and limit of quantification) were calculated in method validation to meet the acceptance criteria described in ICH Q2 (R1) guidelines.

### 4.2.2 Determination of critical micelle concentration (CMC)

The concentration of surfactant at which they tend to self-assemble into a micellar structure is reffered to as critical micelle concentration. The CMC of SOL, mixture of SOL + PF127 and SOL + SHS was estimated by iodine hydrophobic probe method, using a UV Spectrophotometer [Zhao et al. 2017]. Briefly, a series of aqueous SOL micellar dilutions ranging from 0.1 mg/mL to 0.0001 mg/mL were prepared in the de-ionized water. The iodine standard solution (ISS) was then prepared by dissolving potassium iodide (2%) and iodine (1%) in 50 mL de-ionized (DI) water. The fixed amount of ISS (25 μL) was added to (10 mL) each of the already prepared series of dilutions. After incubation of these mixtures in the dark for 12 hours at room temperature, UV absorbance at 366 nm was measured. The experiments were run in triplicates. The point of drastic rise in the plot between absorption intensity and the log soluplus concentration is considered as CMC of soluplus.

### 4.2.3 Physicochemical characterization of LP and excipients

#### *4.2.3.1 Fourier Transform Infra-red (FTIR) study*

The IR spectra recording of drug and surfactants were done on FTIR (SHIMADZU 8400S, Japan). KBr press method was followed for sample preparation where test sample and potassium bromide was mixed (Sample:KBr Ratio of 1:50) by trituration in a mortar. Finally, the triturated mix was compressed on a press to form pellet which was kept on the sample holder for spectra measurement. The % transmittance from 4000 $cm^{-1}$ to 400 $cm^{-1}$ was recorded for all the samples.

#### *4.2.3.2 X-ray Powder Diffraction (XRPD) study*

The crystallinity and physical state of drug and excipients was investigated via XRPD studies. X-ray diffractometer (Rigaku, Japan) with Copper-Kα source and nickel filter was used for recording the XRPD patterns. X-ray tube operation was done at 40kV potential and 25 mA and scanning rate was fixed at 2°/min with step size of 0.02°, over a 2θ range of 5–70°.

## 4.3 Formulation Development and optimization

### 4.3.1 Screening of components for the formulation of micelles

Different product and process parameters should be optimized for the improvement of the quality of the drug product which was carried out by risk assessment analysis. The elements of the QbD are:

- Quality target product profile (QTPP) consisting of critical quality attributes (CQAs) of the drug products.
- Identification of critical material attributes (CMAs).
- Identification of critical process parameters (CPPs).

- Process capability and continual improvement

Risk assessment studies govern the identification of CMAs and CPPs which might affect the product quality. Further, failure mode effect analysis (FMEA) was employed to provide ranks to CTQs based on relative effectiveness to prioritize the independent variables to be selected for further employment of DoE strategies for optimization of formulation.

### 4.3.2 Optimization of micelles by Box-Behnken Design

The experimental model was designed by employing the Box-Behnken design (BBD) having 3 levels, 3 factors, and 3 Centre points. The effect of individual parameter was assessed by applying both statistical as well as mathematical techniques in response surface methodology (RSM). The design was developed to ensure optimization of independent variables and to organize the experiments in a manner to achieve the best outcome. The variable (dependent and independent) selection was based on the preliminary trial experiments as well as the previous studies by other researchers. The independent factors were the ratio of drug: SOL (A), volume of methanol required (B), and lastly surfactant (PF-127) concentration (C) while particle size (PS) ($Y_1$), poly dispersity index (PDI) ($Y_2$) and entrapment efficiency (EE) ($Y_3$) were the responses.Variations were carried out at 3 levels; lower level variation (-1), medium level variation (0) and higher-level variation (+1). **Table** summarizes the independent variable details along with their coded levels and constraints. Design Expert (STAT-EASE, 7.0.0, Minneapolis, MN) software was used for evaluation of experimental design while the statistical significance was determined by application of ANOVA. All the experiments were randomized to exclude the chances of bias.

On the basis of the best-applicable model, the independent variables' effects were studied by the use of quadratic equations; illustrated as follows:

$$Y = X_0 + X_1A + X_2B + X_3C + X_4AB + X_5AC + X_6BC + X_7A^2 + X_8B^2 + X_9C^2$$

Where, Y = response, $X_0$ = constant, $X_1$, $X_2$, $X_3$ = linear coefficients, $X_4$, $X_5$ and $X_6$ = interaction coefficients, $X_7$, $X_8$ and $X_9$ = quadratic coefficients. Based on the Fisher's (F) value, regression co-efficient ($R^2$), p-value and lack of fit value generated by ANOVA, a suitable model was selected.

Desirability approach based numerical optimization was also done. The batch of formulation with lower particle size and PDI but high EE and was characterised for its physicochemical properties, *in vitro* and *in vivo* performance. By applying concept of design space, the deviations in responses were minimized under constrains and percentage biasness of the optimized formulation was calculated. Validation of mathematical models was done by calculating % bias from the comparison of predicted and experimental responses [Yadav et al. 2018].

### 4.3.3 Formulation of micelles

Preparation of LP loaded micelles was carried out by 'thin film hydration' method with minor changes [Dehghan Kelishady et al. 2015]. Clear methanolic solution of definite concentration of drug and SOL was prepared by dissolving the same with constant stirring. The prepared solution was transferred to round bottom flask for evaporation under vacuum at 35 °C and 60 rpm in a rotary evaporator. Thus, the thin film so formed after the solvent evaporation was dried in vacuum overnight to make sure that the solvent is completely removed. Later on, the film was hydrated with warm de-ionized water (10 mL) consisting of calculated quantity of PF 127. The obtained dispersion was further rotated at 80 rpm for 30 mins to ensure complete hydration of dried film.

Aggregated and unincorporated drug was removed by filtering the micellar dispersion through 0.45 µm filter. The placebo micelles were prepared by same method except using drug. The dispersion then was lyophilized using 5 % w/v mannitol as a cryoprotectant.

### 4.3.4 Physicochemical characterization

#### *4.3.4.1 Particle size and polydispersity index analysis*

Beckman Coulter Particle Analyzer (Delsa$^{TM}$ Nano C) operating on dynamic laser scanning technique was used for particle size analysis and PDI calculations. He-Ne laser was used as light source while scattering angle was fixed at 165°. The particles were first dispersed in deionized water for measurements.

#### *4.3.4.2 Zeta potential*

Stability of nanoparticles is governed by Zeta potential, which represent the net charge possessed by the particle surface. The particles with opposite charges tend to aggregate and increase the particle size while causing sedimentation and a uniform dispersion of particles. Hence, by measuring zeta potential, the causes of dispersion, aggregation and/or flocculation can be studied in detail and stability of the nanoformulations (emulsions, suspensions) can be improved. Measurements were done by using Particle size analyzer (Delsa Nano C BeckmanCoutler).

#### *4.3.4.3 High Resolution-Scanning Electron Microscopy*

Further, the morphology of another dried film, prepared as discussed above, was studied with the aid of HR-SEM (FEI-Nova NanoSEM 450). The sample was required to be sputter coated under vacuum with gold-palladium in an inert environment to provide conducting nature to the film surface. Then, film coated glass stub was mounted by

double sided tape on the stage for analyzing particle size and observe its morphology. Low energy (15kV) was used for analysis.

#### *4.3.4.4 Scanning Probe Microscopy*

For morphological evaluations via SPM, the sample was prepared by spin-coating a drop of diluted optimized formulation on a glass slide which was then dried to form a uniform film. The film was subjected to surface roughness examination by SPM (NT-MDT, NTGRA PRIMA, Russia) in semi-contact scanning mode at the scanning rate of 0.5 Hz.

#### *4.3.4.5 Fourier Transform Infra-red (FT-IR) study*

The spectra of drug, pharmaceutical excipients, their physical mixture and prepared formulation was recorded on FTIR (SHIMADZU 8400S, Japan) to determine the polymer-drug interactions. The evaluation was conducted as discussed in 4.2.3.1.

#### *4.3.4.6 X-ray Powder Diffraction (XRPD) study*

XRPD analysis reveals the changes in crystalline structure of drug/polymers during preparation of formulation. For the purpose, physical mixture of LP with all the excipients, LP-loaded micelles and lyophilized micelles were subjected to XRPD analysis (as discussed previously in 3.2.3.2). The obtained diffractograms were then compared with those of individual components of the formulation..

#### *4.3.4.7 Energy dispersive X-ray Analysis (EDX)*

EDX analysis gives information about the elemental composition of substances and thus the optimized formulation was subjected to analysis in EDX instrument (Ametek, NJ). The X-ray radiation was focused on the solid region of micelle at a fixed angular position for measuring elemental composition of the micelle.

#### *4.3.4.8 Effect of dilution*

Dilution studies of the LP-PMs were performed for assessing the integrity of LP-PMs following their dilution to higher extent upon per-oral/intravenous administration. The study was carried out by diluting a fixed quantity of LP-PMs (10x, 100x, 200x, 400x and 500x times dilution) with de-ionized water, as body fluids are aqueous in nature. The particle size and PDI of series of diluted samples were then measured by Beckman Coulter Particle Analyzer (Delsa$^{TM}$Nano C).

### 4.3.5 *In-vitro* Evaluations

#### *4.3.5.1 Entrapment Efficiency and Drug Loading*

The encapsulation efficiency (EE) and drug loading (DL) measurements of LP-PM were carried out using UV-Spectrophotometer (Shimadzu UV-1800). Around 200 µL of the dispersed formulation was added to methanol and vortexed to aid in complete dissolution. This was diluted to 1 mL and vortexed for a while. The obtained solution was subjected to centrifugation at 15000 RPM for 15mins (at 4 °C). Supernatant of centrifuged mixture was diluted to 10 mL with methanol and absorbance was read at 261.5 nm. Standard curve of drug in methanol, which was plotted earlier was used to determine the drug concentration for determining EE and LC based on following equations.

$$\text{EE}\ (\%) = \frac{\text{Amount of LP entrapped in PM}}{\text{Initial amount of LP}} \times\ 100$$

$$\text{DL}(\%) = \frac{\text{Amount of LP entrapped in PM}}{\text{Total dry weight of LP} - \text{PMs}} \times\ 100$$

#### *4.3.5.2 In-vitro drug release studies*

Prepared micelles were assessed for drug release by modifying previously reported dialysis bag method [Wei et al. 2015b]. Briefly, a pre-soaked dialysis membrane bag (MWCO: 14 kDa, Fisherbrand) was filled with 1 ml of optimized LP-PMs dispersion (equivalent to 2 mg of LP) and the two ends were sealed. For lyophilized form, the powder was re-dispersed in 1 mL of deionized water before sealing in dialysis bag. The dialysis bag was then introduced into 50 mL of release media (PBS pH 7.4, PBS pH 5.0), maintained at 37±0.5°C with 100 RPM stirring speed. The aliquot was withdrawn at appropriate time intervals and replenished with an equal volume of the fresh release media. The sink condition was maintained by adding 0.5% Tween 80 to ensure a drug release. Quantification of LP released from formulation was done using UV-Spectrophotometer as mentioned above and CPR (Cumulative percent release) with respect to time was calculated and plotted.

#### *4.3.5.3 Hemocompatibility study*

The hemolysis index is generally considered as the quantitative measure of material induced hemolysis to evaluate the effect of pure drug suspension and formulations on blood components. The blood from human volunteers was collected in heparinized tubes and erythrocytes were separated from plasma by centrifugation for 10 min at 5000 rpm and washed with physiological saline thrice. A fixed amount (900 µL) of isolated erythrocytes was mixed to the series of various dilutions (900 µL) of LP-PMs and pure drug suspensions. The samples were mixed gently and incubated for 1 h at room temperature. The mixture was added to 4 mL of physiological saline and subjected to centrifugation at 10000 rpm for 15 min. Saline/blood was taken negative control (NC) while hemolyzed blood sample (with 0.5% Triton-X100) served as positive control (PC). The absorbance of separated supernatant was measured at 540 nm

using UV-VIS Spectrophotometer, and following equation was used for calculation of % hemolysis [Mourtas et al. 2009]:

$$\text{Hemolysis (\%)} = \frac{OD_{test} - OD_{NC}}{OD_{PC} - OD_{NC}} \times 100 \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad 4$$

Where, $OD_{test}$, $OD_{NC}$ and $OD_{PC}$ represents the optical density of test solution, negative control and positive control.

#### *4.3.5.4 Platelet aggregation studies*

Platelet aggregation studies were done based on the previously reported method[Bender et al. 2012]. LP-PMs and whole blood (citrated) were incubated at 37°C for a period of 30 min with frequent agitation and PBS (pH7.4) was used as negative control. The incubated mixture was smeared on glass slide and air-dried followed by staining of cells with Leishman's stain (Span Diagnostic, India). Rinsing of slides was carried out to remove the excess reagent and the slides were then dried in air prior to visualization on an optical microscope (Dewinter Trinocular Microscopic Unit, Dewinter Technologies).

### 4.3.6 Stability Studies

The shelf-life of optimized LP-PMs were estimated. Accordingly, the LP-PMs were being stored at different storage conditions as per the Q1A R(2) ICH guideline i.e., Long term at room temperature (30±2°C/65 ± 5% RH) and in refrigerated condition (5±3°C) for a time period of 6 months. Periodical sampling at fixed time points of 0, 3 and 6 months was done. Visual inspection for any aggregation and EE of the fresh and stored formulation samples were carried out. The shelf life was calculated using Minitab® ver.17 [Mittal et al. 2018].

### 4.3.7 Screening of anticancer efficacy

#### *4.3.7.1 Cell Culture*

McCoy's 5A medium, supplemented with 100 U/mL penicillin, 10% fetal bovine serum, and 100 mg/mL streptomycin was utilized to culture SKBr3 cells. The cells were incubated at 37°C in the presence of 5% $CO_2$. For *in-vitro* screening, the cells were seeded appropriately as needed after trypsinization.

#### *4.3.7.2 Cytotoxicity assay*

MTT assay of LP-PMs was performed, to assess its cytotoxicity. 96-well culture plates were used for seeding the cells (density of $2\times10^4$ cells/well) and cultured for a period of 24 h. Next day, serial dilutions of LP-PMs and LS were added to the cultured cells; those were previously in range of 0.05 to 100 µg/mL. MTT assay was then completed as per protocol (details are given in supplementary materials). Obtained data was analyzed to calculate $IC_{50}$ values and percent cell viability by using GraphPad Prism software [Wan et al. 2015a, Wei et al. 2015b].

#### *4.3.7.3 In-vitro anti-cancer efficacy studies*

The changes in the morphology of SKBr3 cells before and after treatment were observed using inverted microscope. The cells ($10^4$cells/well) were inoculated in a 24-cell culture plate and treated with placebo micelle, LP solution (LS), LP-PMs (10 µg of LP/mL) and LP-BMs (10 µg of LP/mL) for 24 h. The cells were then carefully washed with PBS three times and fixed by 4% paraformaldehyde in PBS (pH 7.4) for 15 min at room temperature. Fixed cells were washed with PBS again and mounted on glass slides. The slides were then observed under inverted light microscope

### 4.3.8 *In-vivo* evaluations

#### *4.3.8.1 Pharmacokinetic studies*

*Wistar Rats* (female; 8–10 weeks, weight: 190-220 g) were procured from the Central Animal Facility, Banaras Hindu University, Varanasi (India) and acclimatized for 1 week before initiation of the experiment in well-ventilated plastic cages under standard laboratory conditions in the animal room with 12 h dark and 12 h light cycle, and fed with standard diet ab libitum. All experimental procedures were conducted according to CPCSEA guidelines and were duly approved by the Institutional Animal Ethics Committee (IAEC) of Banaras Hindu University, Varanasi (India) (vide approval no. Dean/2019/IAEC/1638).

For the pharmacokinetics study, the female *wistar rats* were further divided into five groups (n=6). Animals were intravenously administered with normal saline, LS and LP-PMs and LP-BMs at an equivalent dose of at 10 mg/kg whereas, marketed formulation equivalent dose of 100 mg/kg was administered by oral route. Blood samples were withdrawn from retro-orbital plexus into disodium EDTA containing microcentrifuge tubes at predetermined time points, the blood samples were collected and then centrifuged at 6500 rpm at 4°C for 15 min to collect plasma that was subsequently stored at -80°C until further analysis. For analysis, the plasma was de-proteinized and the drug was extracted with methanol. The amount of LP in plasma samples were quantified by HPLC. The hardware control and data handling were performed using Breeze 2 software version. Plasma concentration-time profiles of drug were plotted and analyzed for pharmacokinetic parameters by non-compartmental model approach using utilizing PK solver add in of the Microsoft excel.

#### *4.3.8.2 Pharmacodynamic studies*

Balb/c nude mice were caged in well ventilated and air-conditioned animal room and fed with the standard diet and supplied with water ad libitum. Animals were acclimatized in 12-12 light-dark cycle. Balb/c mice of 8-10 weeks and 18-25 g weight were used for the experiment. The xenograft ectopic mice models were developed as previously discussed in the literature [Wang et al. 2014] and experimental procedures were carried out in accordance with the protocol approved (vide approval no. Dean/2018/CAEC/639) by Central Animal Ethical Committee, Banaras Hindu University, Varanasi (India) following CPCSEA guidelines on animals and principles of laboratory animal care (National Institute of Health). Briefly, SKBr3 cells were harvested and injected subcutaneously to the nude mice after re-suspending cells in phosphate buffer saline (PBS). The tumor volume was calculated using the following formula:

$$V = \frac{W^2 \times L}{2}$$

Where, V= Volume of tumor; W = shortest diameter; L = longest diameters

On the formation of tumors of approximately 700-800 $mm^3$ size, different treatment groups (n=6 per group) were formed by randomly dividing the mice. Different mice groups received treatment by intravenous injection in the tail vein as follows: Group 1, PBS (control); Group 2, LS (10 mg/kg/3d); Group 3 LP-PMs (10 mg/kg/3d); Group 4 Tykerb®, a marketed tablet dosage form of LP (Oral administration 100 mg/kg/1d). On the $28^{th}$ day, mice were euthanized, and tumors were immediately harvested and their volume was noted.

#### *4.3.8.3 Histopathological studies*

The lapatinib was reported to show hepatotoxicity and thus, histochemical and pathological studies were performed. Briefly, the liver tissues excised from each group were fixed in 10% (v/v) formalin saline and processed for routine histopathological procedures. Paraffin embedded specimen were cut into 5 mm sections and stained with hematoxylin and eosin (H&E) for histopathological evaluations. The histological examination was performed under microscope after H&E staining.

## 4.4 Statistical Analysis

All the results are represented in the form of mean ± standard deviation (SD). By using the GraphPad Prism (GraphPad Prism Software, USA), student (unpaired) t-test and one-way/two-way ANOVA were applied for statistical comparisons. The disparity was supposed to be statistically significant when $p<0.05$ (95% confidence Interval).