4 Materials and methods

4.1 Materials

The following chemicals and instruments/equipment were used for the formulation and evaluation of vinorelbine bitartrate loaded nanocarriers.

Table 4.1: List of chemicals

CHEMICALS	SOURCE
Vinorelbine bitartrate	Sipra Life Sciences, Hyderabad
Resveratrol	Sami Labs, Banglore
Glycerylmonooleate, GMO	Sigma Aldrich Chemicals Pvt. Ltd, INDIA
Glycerylmonostearate, GMS	Sigma Aldrich Chemicals Pvt. Ltd, INDIA
Poly(lactic-co-glycolic acid), PLGA	Evonik, Mumbai
TPGS	Antares Health Products inc., USA
Ethanol	Merck Pvt. Limited, Mumbai
Poloxamer-188	BASF India Ltd., Mumbai
Chloroform	Merck Pvt. Limited, Mumbai
Tween 80	Merck Pvt. Limited, Mumbai
Span 80	Merck Pvt. Limited, Mumbai
Disodium Hydrogen orthophosphate	Qualigens fine chemicals, Mumbai
Potassium Dihydrogen orthophosphate	Merck Pvt. Limited, Mumbai
Triethylamine	Merck Pvt. Limited, Mumbai
Ortho Phosphoric acid	Spectrochem Pvt. Limited, Mumbai
Sodium Chloride	Merck Pvt. Limited, Mumbai
Acetonitrile	Sigma Aldrich Chemicals Private Limited,
Dialysis Bag, MWCO 10-12 K	Sigma- Aldrich Chemicals Private Limited,

	Bangalore
MTT	Sigma- Aldrich Chemicals Private Limited, Bangalore
FBS	Himedia, Mumbai
Dulbecco's modified Eagle's medium (DMEM)	Himedia, Mumbai
Coumarin 6	Parishi chemicals, Surat, Gujrat
Leishman's stain	Span Diagnostics, India

Table 4.2: List of equipments used

EQUIPMENTS	**SOURCE**
Particle size analyzer	Beckman Coulter , USA
Digital Electronic Balance	Schimadzu A×200, Electronic Balance
pH meter	Eutech Instruments,Germany
Magnetic Stirrer	Eltek Elecrocraft , Mumbai, India
Ultraturrex	T25 IKA, Germany
FT-IR spectrophotometer	SHIMADZU, Model 8400S, Tokyo, Japan
U.V/Visible spectrophotometer	SHIMANDZU Double Beam U.V, Japan
Scanning electron microscope	Hitachi High Technology, Pleasanton
TEM microscope	Tenai G2 20 Twin, FEI Company, USA
Differential scanning calorimeter	DSC 6000, SV 11.0.0.0449, Perkin Elmer, Switzerland
Microplate reader	(Biorad, Germany)
Haematological counter	Multisizer 4, Beckmann Coulter, USA

Syringe filter	Millipore, 0.45 µm and 0.22 µm
Microlitre syringe	Pall corporation, Banglore
HPLC grade water system	Millipore
Cooling centrifuge	Remi, India
Micropipette	Eppendorf
Refrigerator	Kelvinator, India
Lyophilizer	Decibel Electronic Technology
HPLC	Waters 515
Rotatory evaporator	IKA, Mumbai
Vernier calipers	Mitutoyo JAPAN
Ultracentrifuge	Hitachi Microultracentrifuge CSI50NX

4.2 HPLC analytical method development for simultaneous estimation of VRL and RES

4.2.1 HPLC conditions

The HPLC instrument comprised of an inline degasser, binary pump (515 Waters, USA), manual injector (rheodyne 7725i, Waters, USA), reverse-phase C18 (250×4.6 mm, 5 µm) ODS2 column with a guard column (12×4.6 mm, 5 mm) of the same material (Waters Corp., Milford, MA, USA), and a PDA detector (Waters, USA).

4.2.2 Selection of mobile phase

The solvent media for preparation of calibration curve was selected on the basis of solubility of VRL and RES at room temperature. By understanding the requirements for simultaneous quantification of VRL and RES during various studies, acetonitrile (60 %) and 50 mM phosphate buffer (40 %, with 1 % triethyamine), pH 3.5, (pH adjusted with orthophosphoric acid) was selected as mobile phase for elution and for preparation of calibration curve (Li et al., 2012).

4.2.3 Standard stock solution preparation

4.2.3.1 In mobile phase

Accurately weighed 5 mg of VRL was transferred to a 5 mL volumetric flask and dissolved completely in 3 mL of mobile phase. The resultant solution was sonicated and the volume was made up to the mark (5 ml) with mobile phase to give stock solution containing 1mg mL^{-1} of VRL. Similarly stock solution of RES was prepared in mobile phase.

4.2.3.2 In Phosphate buffer Saline (PBS) pH 7.4

PBS was prepared by dissolving potassium dihydrogen phosphate (0.19g), di-sodium hydrogen phosphate (2.38g) and sodium chloride (8g) in 1000ml of distilled water. Accurately weighed 5 mg of VRL was transferred to a 5 mL volumetric flask and dissolved completely in 3 mL of PBS. The resultant solution was sonicated and the volume was made up to the mark (5 ml) with PBS to give stock solution containing 1mg mL^{-1} of VRL. As RES was insoluble in PBS, pH 7.4, firstly it was dissolved in 1 mL of acetonitrile and then diluted up-to the mark with PBS, pH 7.4.

4.2.4 Identification of λmax for VRL and RES

The stock solution was further successively diluted with respective solution, in order to obtain a final concentration of 10 µg mL^{-1}. The resultant solution was scanned between 200 - 400 nm using U.V –Visible spectrophotometer (SHIMANDZU Double Beam U.V, Japan) and λmax for VRL and RES was determined.

4.2.5 Sample preparation

From the stock solution, a working standard solution of 100 µg mL^{-1} was prepared. Aliquots were pipette out from this working stock solution and transferred into a series of volumetric flasks of 5 mL capacity. The volume was adjusted up to the mark with respective solvents to obtain final concentrations in the range of 0.5 -100 µg mL^{-1} for VRL and 10 - 5000 ng mL^{-1} for RES.

4.2.6 Calibration curve preparation

20 µL of the samples were injected into the HPLC and corresponding peak areas were recorded. The peak areas at λ max were then plotted against concentration of each standard

solution to prepare calibration curve. The calibration curves were generated on three consecutive days using freshly prepared samples.

4.2.7 Method validation

The suitability of developed method for quantitative estimation of VRL and RES was confirmed by evaluating the different validation parameters as per ICH Q2B guideline (Guideline). The developed method was validated for the typical validation parameters, i.e., accuracy, precision, linearity, range, specificity, selectivity, limit of detection (LOD) and limit of quantification (LOQ).

4.2.7.1 Accuracy

The accuracy associated with an analytical procedure can be understood as the proximity of the test results to the true value. It was determined by performing recovery study using standard addition method. Recovery can be calculated by taking the ratio of observed result to the predicted result and usually expressed as percentage. Three concentrations (low, medium and high) of VRL and RES were selected. Each sample was scanned in HPLC at same day (n=3) and over three consecutive days (n=3) to access intra-day and inter-day variation, respectively. The accuracy was also expressed as % bias and the acceptable values should be within ± 15% at all concentration levels. The % bias was calculated as follows

$$\% \text{ bias} = \frac{\text{Observed concentration} - \text{Predicted concentration}}{\text{Predicted concentration}} \times 100 \qquad \text{Equation 4.1}$$

4.2.7.2 Precision

The precision is the degree of closeness among a series of measurements obtained after numerous sampling of the same sample under the prescribed conditions. The precision was

determined by measuring the relative standard deviations (RSD) of a series of measurements. Three different drug concentrations were scanned in HPLC at same day (n=3) and over three consecutive days (n=3) in order to measure intra-day and inter-day precision, respectively. The acceptable values for % RSD should be within ≤ 15% at all concentration levels. The % RSD was calculated as follows

$$\% \text{ RSD} = \frac{\text{Standard Deviation}}{\text{Mean observed concentration}} \times 100 \qquad \text{Equation 4.2}$$

4.2.7.3 Linearity and range

The linearity of an analytical procedure is its ability to obtain test results which are directly proportional to the concentration of analyte in the samples. The range is the interval between the upper and lower concentration of analyte for which the analytical method demonstrated a suitable level of precision, accuracy and linearity. Linearity and range was determined by measuring the peak areas of the series of samples over the concentration range in triplicate over three consecutive days.

4.2.7.4 Specificity and selectivity

Specificity is the ability of analytical procedure to assess unequivocally the analyte in the presence of other components which may be present. Selectivity is the procedure to detect qualitatively the analyte, in presence of other components in the solvent media. Specificity and selectivity of the method were determined by scanning known concentration of analyte in HPLC.

4.2.7.5 Limit of detection (LOD)

The limit of detection for an analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantified with accuracy and precision. The LOD was calculated by using following formula

$$\mathrm{LOD} = \frac{3.3 \times \sigma}{S} \qquad \text{Equation 4.3}$$

Where, σ is the standard deviation of y-intercept of the regression equation and S is the slope of the calibration curve.

4.2.7.6 Limit of quantification (LOQ)

The limit of quantitation for an individual analytical procedure is the lowest amount of analyte in a sample which can be quantified with suitable precision and accuracy under the given conditions. The LOQ was calculated using following formula

$$\mathrm{LOQ} = \frac{10 \times \sigma}{S} \qquad \text{Equation 4.4}$$

Where, σ is the standard deviation of y-intercept of the regression equation and S is the slope of the calibration curve.

4.3 Formulation development

4.3.1 Preliminary screening of polymers and lipid

Before preparing formulations polymers (PLGA & PCL) and lipids (GMO and GMS) were selected for preparation of nanoformulations. Drug loading and EE (%) was calculated for all polymers and lipids. Briefly, organic phase was prepared by dissolving VRL and polymer/lipid in 6 mL of acetone. This organic phase was added drop wise to aqueous surfactant solution while stirring at 15000 rpm. The nanosuspension was left for stirring at room temperature to evaporate acetone. The prepared formulations were evaluated for particle size and entrapment efficiency.

Drug loading= Wd/Wnp x 100 Equation 4.4

Wd= amount (mg) of drug found in nanoparticles

Wnp= amount (mg) of nanoparticles sample

4.3.2 Solid Lipid Nanoparticles

4.3.2.1 Experimental design

The 3-level, 4 factors, Taguchi orthogonal design was employed to optimize and investigate the influence of independent variables on critical quality attributes namely particle size and entrapment efficiency. Lipid concentration (X_1), surfactant concentration (X_2), homogenization speed (X_3) and homogenization time (X_4) were chosen as variables at three different levels, higher, middle and lower, of each variable. The respected properties of

SLNs such as particle size (Y_1) and entrapment efficiency (Y_2) were taken as response variables (Table 4.3). Experimental design matrix was constructed and evaluated with the help of Design expert (7.0.0) software. Total 9 batches were generated and the experimental bias was minimized by randomizing the experimental runs. The correlation between independent variables and responses was investigated by three dimensional response surface plots and quantified with the help of best fit model (Mishra et al., 2017, Patel et al., 2016, Vardhan et al., 2017). The effect of factors and their interactions on response variables was assessed through multiple linear regressions employing ANOVA. Desirability approach was utilized to optimize SLNs.

Table 4.3: Independent variables with their levels and dependent variables in Taguchi orthogonal optimization design

Variables	Coded levels		
	Low (1)	Medium (2)	High (3)
Independent variables			
X_1 = Lipid concentration (mg)	100	200	300
X_2 = Surfactant concentration (% w/v)	0.05 [a]/ 0.5 [b]	0.1 [a] / 1 [b]	0.15 [a] / 1.5 [b]
X_3= Homogenization speed (rpm)	5000	10000	15000
X_4 = Homogenization time (min)	5	10	15
Dependent variables		**Constraints**	
Y_1/Y_1^* = Particle size		minimize	
Y_2/Y_2^* = Entrapment efficiency		maximize	

[a] for TPGS, [b] for Poloxamer 188; Y_1 and Y_2 are particle size and entrapment efficiency of TPGS-VRL-SLNs; and Y_1^* and Y_2^* represents particle size and entrapment efficiency of PL-VRL-SLNs, respectively

4.3.2.2 Preparation of solid lipid nanoparticles (SLNs)

A solvent diffusion method, at the temperature above the melting point of the lipid, was used for the preparation of TPGS and poloxamer emulsified SLN (TPGS-VRL-SLNs and PL-VRL-SLNs, respectively). GMO was selected as a solid lipid, due to its low meting point in order to minimize any degradation of thermolabile VRL. Briefly, VRL and GMO were dispersed in 2 mL of ethanol which would serve as the organic phase. In another beaker required amount of TPGS/ PL-188 was dispersed in 25 mL of double distilled water and stirred well to obtain a clear solution. The organic phase was injected into the aqueous phase under homogenization using ultraturrax (IKA, Germany) with a probe of 1 cm diameter at 15,000 rpm for 15 min at 50 ºC. Then the suspension was allowed to cool at room temperature. The prepared SLN formulations were stored at 5 ºC. The formulations containing TPGS and PL-188 were designated as TPGS-VRL-SLNs and PL-VRL-SLNs, respectively (Maurya et al., 2018a).

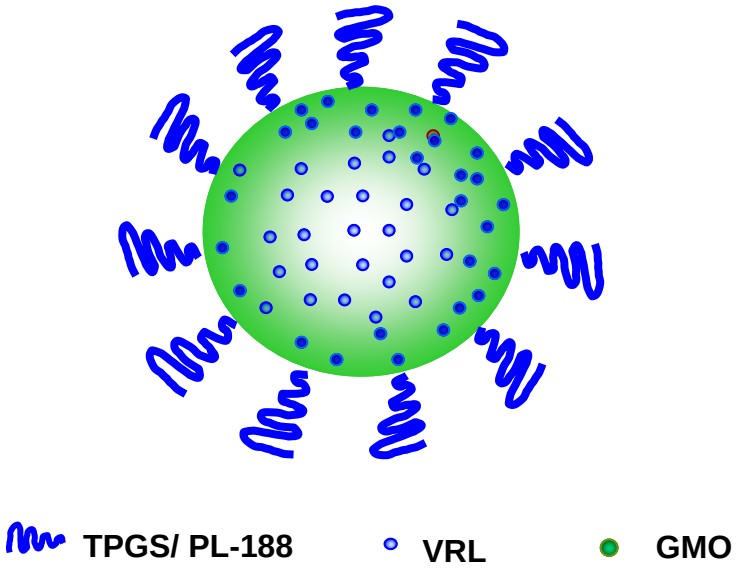

Figure 4.1: Schematic representation of Solid Lipid Nanoparticles (SLNs)

4.3.3 Aqueous core nanocapsules (ACNs)

4.3.3.1 Experimental Design

A 3 factor, 3 level Box- Behnken design (BBD) was employed to optimize and investigate the influence of independent variables on dependent variables or responses. Polymer concentration (X_1), surfactant concentration (X_2) and homogenization speed (X_3) were chosen as independent variables at three different levels higher, middle and lower levels of each variable (Table 4.4). The respected properties of ACNs such as particle size and entrapment efficiency were taken as response variables. Design expert (7.0.0) software was utilized for construction and evaluation of experimental design matrix. Total 13 batches were generated and experimental runs were randomized in order to minimize the experimental bias. Three dimensional response surface plots were generated and the correlation between independent variables and responses was investigated. The quantification of the same was done with the help of best fit model (Mishra et al., 2017, Patel et al., 2016, Vardhan et al., 2017). Analysis of variance, ANOVA was performed to assess the influence of factors along with their interactions on the response variables and formulation optimization was achieved utilizing desirability approach.

Table 4.4: Independent variables with their levels and dependent variables in Box Behnken design

Variables	Coded levels		
	Low (1)	Medium (2)	High (3)
ndependent variables			
X_1 = Polymer concentration (mg)	15	20	25
X_2 = Surfactant concentration (% w/v)	0.3	0.4	0.5
X_3= Homogenization speed (rpm)	5000	10000	15000
Dependent variables		**Constraints**	
Y_1 = Particle size		minimize	
Y_2= Entrapment efficiency		maximize	

4.3.3.2 Preparation of ACNs

A double emulsification solvent evaporation method was utilized to prepare VRL loaded ACNs. Briefly primary emulsion (W_1/O) was prepared by adding inner aqueous phase (W_1, 0.2 mL water) containing VRL to organic phase (GMS/PLGA dissolved in 4 mL chloroform) and sonicated at a frequency of 0.5 cycles and 60% amplitude using a probe-type ultrasonicator (Heilscher, Germany). The resultant primary emulsion (W_1/O) was further added drop-wise to outer aqueous phase (W_2, containing TPGS dissolved in 6ml of water) under homogenization using ultraturrax (IKA, Germany) with a probe of 1 cm diameter at desired rpm (5000rpm -15000rpm) for required time (5min to 15 min) to obtain a double emulsion (W_1/O/W_2). The resultant double emulsion was left for stirring for evaporation of chloroform which will result in formation of aqueous core nanocapsules (Singh et al., 2018). The prepared nanocapsules were separated by centrifugation, lyophilized and stored for further characterizations.

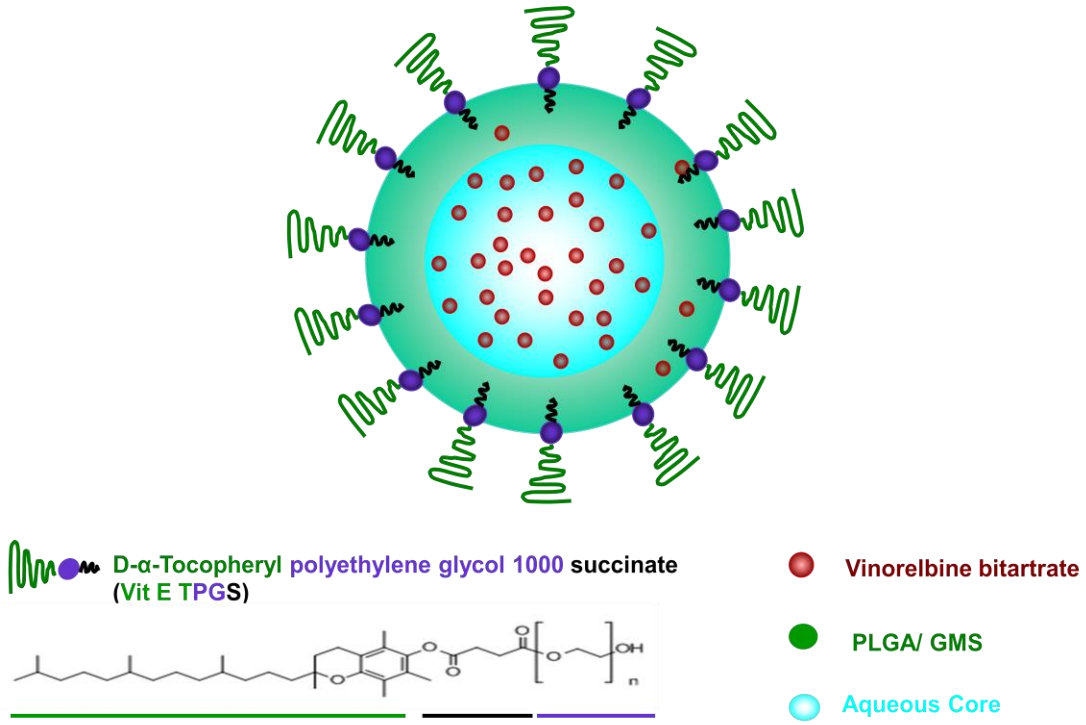

Figure 4.2: Schematic representation of aqueous core nanocapsules (ACNs)

4.3.4 Dual drug loaded aqueous core nanocapsules (dd-ACNs)

4.3.4.1 Determination of synergistic ratio of VRL and RES

VRL and RES were combined in different ratios (10:1, 5:1, 1:1, 1:5 and 1:10) and evaluated for cytotoxicity against MCF-7 cell lines. The cytototixity was measured at different f_a values. The combination index (CI) was determined with the help of CalcuSyn (Biosoft, Ferguson, MO, USA). According to Chou Talalay principle CI <1, CI=1 and CI>1 represented synergistic, additive and antagonistic activity of combination of two drugs (Mayer et al., 2006, Tardi et al., 2009, Wang et al., 2014). The CI was calculated based on the following formula:

$$CI = \frac{D1}{Dx1} + \frac{D2}{Dx2}$$ Equation 4.5

Where, Dx1 and Dx2 are the doses of drug 1 and drug 2 inhibiting 'x%' individually

D1 and D2 are the doses of drug1 and drug 2 in combination, respectively, inhibiting 'x%'

The most synergistic combination was selected for preparation of ACNs.

CI was calculated from several data points with Fa ≥0.6, which demonstrated the fraction of cells affected. Further the dose of individual drugs in combination were evaluated which is designated as dose reduction index, (DRI)

$$DRI = \frac{Dx1}{D1}$$ Equation 4.6

DRI < 1 depicted that combination might lead to reduced doses of both drugs when used in combination compared with the doses for each drug used individually eliciting similar response. Isobolograms were created by plotting VRL and RES concentrations (alone and in combination) inhibiting 75 % MCF-7 cells growth. Firstly, the concentrations of VRL and RES necessary to generate a defined effect, when used alone, were plotted on the x and y axes which corresponds to (cVRL, 0) and (0, cRES), respectively. A line was drawn which connects these two points. The connecting line was considered as the line of additivity. Further, the concentrations of these drugs in combination eliciting the same effect (cVRL, cRES), were marked in the same plot. Synergy was confirmed when (cVRL,cRES) is located below the line however additivity and antagonism are indicated when it is located on, or above the line of additivity (Wang et al., 2014).

4.3.4.2 Preparation of dd-ACNs

A double emulsification solvent evaporation method was utilized to prepare PLGA-VRL-RES-ACNs (Maurya et al., 2018b). Briefly primary emulsion (W_1/O) was prepared by adding inner aqueous phase (W_1, 0.2 mL water) containing VRL to organic phase (RES and GMS/PLGA dissolved in 4 mL chloroform with 2 % (v/v) span 80) and sonicated at a rate of 0.5 cycles per min and 60 % amplitude utilizing a probe-type ultrasonicator (Heilscher, Germany). The resultant primary emulsion (W_1/O) was further added drop-wise to outer aqueous phase (W_2, containing required amount of TPGS, dissolved in 6 mL of water) under homogenization using ultraturrax (IKA, Germany) with a probe of 1 cm diameter at desired rpm for required time to obtain a double emulsion (W_1/O/W_2). The resultant double emulsion was left for stirring for evaporation of chloroform which will result in formation of aqueous core nanocapsules. The ACNs encapsulating VRL+RES were designated as PLGA-VRL-ACNs, PLGA-VRL-RES-ACNs. The prepared nanocapsules were separated by centrifugation, lyophilized and stored for further characterizations. The schematic representation of prepared PLGA-VRL-RES-ACNs is given in Figure 4.3.

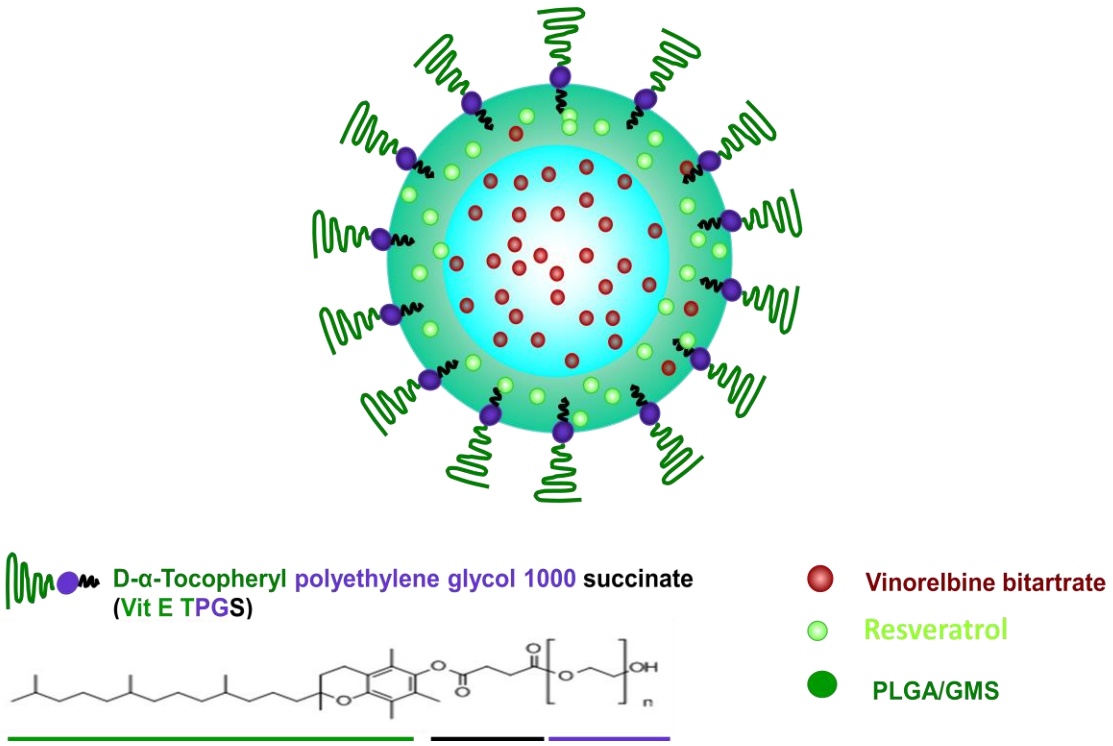

Figure 4.3: Schematic representation of dual drug loaded Aqueous Core Nanocapsules (dd-ACNs)

4.4 Characterization of nano-formulations

4.4.1 Particle size, polydispersity index (PDI) and zeta potential

Particle size, polydispersity index (PDI) and zeta potential of nano-formulations were determined using particle size analyzer, Delsanano C (Beckman Coulter, USA) which is based on dynamic light scattering technique. Briefly, nano-formulations (1 mL) was transferred in polystyrene cuvettes and particle size and PDI were recorded. Zeta potential was recorded by placing 10 times diluted ACNs in zeta cell.

4.4.2 Total drug content (TDC) and encapsulation efficiency (EE)

4.4.2.1 Total drug content

Nano-formulaions (100 µL) were incubated in 1 mL acetonitrile for 5 min to completely dissolve the nanoparticle components. 20 µL of resultant dilution was injected into HPLC to calculate total drug content (TDC).

4.4.2.2 Entrapment efficiency of SLNs

The encapsulation efficiency was determined by direct method using ultracentrifugation (Daneshmand et al., 2018). Briefly, 1 mL of TPGS-VRL-SLNs and PL-VRL-SLNs suspension were transferred to centrifuge tubes and centrifuged (Microultra Centrifuge: Hitachi) at 80,000 rpm for 30 min at 4 ºC. The amount of entrapped VRL present in the pellet was estimated by dissolving the pellet in 1 mL of acetonitrile and injecting 20 µL of the sample into HPLC system. The EE (%) was calculated using following equation.

$$\mathrm{EE}(\%) = \frac{\text{Total drug in the pellet}}{\mathrm{TDC}} \times 100 \qquad \text{Equation 4.7}$$

4.4.2.3 Encapsulation efficiency of ACNs

The encapsulation efficiency (EE) was determined by indirect method using Nanosep tubes. Briefly, VRL-ACNs (0.5 mL) were transferred to upper compartment of Nanosep tubes and centrifuged (Cooling Centrifuge, 24BL Remi) at 12,000 rpm for 30 min at room temperature. Filtrate (100 µL) was diluted to 1mL with acetonitrile and 20 µL was injected to the HPLC

system to calculate free drug. Encapsulation efficiency of ACNs was calculated using the following formula

$$\text{EE\%} = \frac{\text{Total drug} - \text{free drug}}{\text{Total drug}} \times 100 \qquad \text{Equation 4.8}$$

4.4.3 Morphology of nano-formulations

4.4.3.1 Scanning electron microscopy (SEM) for SLNs

Morphological evaluation of TPGS-VRL-SLNs and PL-VRL-SLNs was carried out by scanning electron microscope (Hitachi High Technology, Pleasanton). TPGS-VRL-SLNs and PL-VRL-SLNs (50 µL) were diluted with 1 mL of distilled water and a tiny drop of this dispersion was placed over a coverslip and air dried. The dried thin film of SLN was carbon coated and observed under scanning electron microscope.

4.4.3.2 Transmission electron microscopy (TEM) for ACNs

Freshly prepared PLGA-VRL-RES-ACNs (50 µL) were diluted with 1 mL of distilled water, sonicated for 5 min and a tiny drop was placed on a circular copper grid and air dried. The sample was negative stained with 2 % w/v phosphotungstic acid and visualized under TEM microscope (Tenai G2 20 Twin, FEI Company, USA) for internal morphology of ACNs.

4.4.4 FTIR

FT-IR spectra of drugs (VRL & RES), Eexcipients (GMO, GMS, PLGA, TPGS, PL-188) and lyophilized nano-formulations were obtained by the conventional KBr disk/pellet method using Infrared spectroscopy (SHIMADZU, Model 8400S, Tokyo, Japan). Briefly, the samples were grounded with anhydrous KBr and compressed to form pellet. The disc

preparation was done by Jasco MP-1 Mini Press (Jasco, Japan). The FT-IR spectrum was obtained between the wavelengths of 400 to 4000 cm^{-1}.

4.4.5 DSC analysis

DSC thermo grams drugs (VRL & RES), excipients (GMO, GMS, PLGA, TPGS, PL-188) and lyophilized nano-formulations were obtained to study the drug- excipients' compatibility and any physical change during preparation of ACNs using differential scanning calorimeter (DSC 6000, SV 11.0.0.0449, Perkin Elmer, Switzerland). Samples (5-10 mg) were sealed in aluminium pans and scanned at a heating rate of 10 $^{o}C\ min^{-1}$ over a temperature range of 0 - 300 ^{o}C, under nitrogen flow of 20 mL min^{-1}.

4.4.6 *In-vitro* drug release studies

Nano-formulations (5 mL) were filled in pre-hydrated dialysis membrane, sealed and placed in a beaker containing 250 mL of phosphate buffer saline (PBS), pH 7.4 ensuring that the dialysis bag was completely dipped in the PBS. The entire assembly was maintained at 37 ± 1 ^{o}C with continuous magnetic stirring at 200 rpm and order to prevent any loss of dissolution media it was covered with aluminium foil. For RES encapsulated formulations 5 % Tween 80 was added in order to accelerate the release of highly insoluble RES. Aliquots (1 mL) were withdrawn at predetermined time intervals (5, 10, 15, 30, 45, 60 min, 2, 4, 6, 12, 24, 48 and 72 h) and the medium was replenished with 1 mL of fresh PBS at every sample withdrawal. The amount of drug released at each time points was measured by injecting 20 µL of the sample in HPLC. The *in-vitro* drug release data was further fitted to various release kinetic models, including zero-order model, first-order model, Higuchi model and Korsemeyer–Peppas model, in order to ascertain the release kinetics and mechanism of drug release followed by nanoparticles. The correlation coefficient (R^2) was determined by

regression analysis to determine linearity and best fitted model. Release kinetic modelling was performed as per the following set of equations:

Zero order kinetics : $Q_t = Q_0 + Kt$ Equation 4.9

First order kinetics : $Q_t = Q_0 e^{-Kt}$ or $\log Q_t = \log Q_0 - Kt/2.303$ Equation 4.10

Higuchi model : $Q_t/Q_0 = Kt^{1/2}$ Equation 4.11

Korsemeyer–Peppas model : $Q_t/Q_0 = Kt^n$ Equation 4.12

Where, Q_0 is the initial amount of drug i the release media, Q_t is the amount of drug present in the media at time 't', K is the kinetic rate constant, t is time, and n is release exponent which characterizes the drug release mechanism as follows:

$n \leq 0.5$: Fickian diffusion (Case I)

$0.5 < n < 1$: non Fickian release (anomalous transport)

$n = 1$: Zero order release (Case II transport)

$n < 1$: Super case II transport or drug release that is erosion-controlled.

4.4.7 Stability Studies

The stability studies of optimized batches of nano-formulations were carried out at 30 ± 2 °C / 65 % ± 5 % RH for 90 days. Sealed vials containing ACNs were placed in stability chamber maintained at 30 ± 2 °C / 65 ± 5 % RH for three months as per ICH Q1A R(2) guideline (Guideline, 2003). The samples were withdrawn at different time intervals (0, 1, 2, and 3 months) and evaluated for particle size, PDI and EE.

4.5 Safety for intravenous administration

4.5.1 Evaluation of haemolysis.

The prepared nano-formulations were intended for intravenous administration and as per safety guidelines issued for safety evaluation of excipients it was essential to perform haemolytic activity to prove haemocompatibility (Health et al., 2005). Haemolytic analyses of VRL, placebo nano-formulations and VRL loaded nano-formulations were performed as reported by earlier publications (Vijayakumar et al., 2016a, Vijayakumar et al., 2016b, Vijayakumar et al., 2016c). Briefly, 4 mL of blood was centrifuged at 1344 g for 10 min at 4 ^{o}C and erythrocyte pellet was collected. The pellet was washed with equal volume of normal saline, centrifuged, diluted and re-suspended with normal saline up to 40 mL. VRL, placebo nano-formulations and VRL loaded nano-formulations (equivalent to 10, 50 and 100 µg mL^{-1}) were added to erythrocyte suspension and incubated at 37 ^{o}C. At the same time erythrocyte suspension was incubated with saline (spontaneous control) and 1 % triton X (100 % haemolysis, positive control). Samples (200 µL) were collected at 0.5, 1, 2, 4 and 8h after incubation, centrifuged and supernatants (100 µL) were collected. Supernatants were incubated for 30 min at room temperature for oxidation of haemoglobin in to oxyhaemoglobin. Samples (100 µL) were analyzed spectrophotometrically by microplate resder (Biorad, Germany) at 540 nm and the % haemolysis was calculated using the following formula:

$$\% \text{ Haemolysis} = \frac{\text{Abs(sample)} - \text{Abs (spontaneous control)}}{\text{Abs(positive control)}} \times 100 \qquad \text{Equation 4.13}$$

Where, Abs (sample) stands for absorbance of samples

Abs (spontaneous control) is the absorbance with normal saline equivalent to the volume of samples and Abs (positive control) is the absorbance with 1 % Triton X-100 solution in normal saline. All experiments were performed in triplicate and the results are expressed as mean ± SD (n=3).

4.5.2 Platelet aggregation

The whole blood (1mL) was collected in heparinised tubes to avoid coagulation. The blood was incubated with placebo nano-formulations and VRL loaded nano-formulations (equivalent to 10, 50 and 100 µg mL^{-1} of VRL) for 2 h and platelet aggregation was studied quantitatively and qualitatively (Vijayakumar et al., 2016a, Vijayakumar et al., 2016b, Vijayakumar et al., 2016c). The quantitative analysis was done by counting the number of platelets using a haematological counter (Multisizer 4, Beckmann Coulter, USA) and qualitative analysis was done by microscopic evaluation of the stained blood smears. The blood smears were prepared on clean glass slides after 2h. The slides were air dried and stained with Leishman's stain for 5 min (Span Diagnostics, India). The stained slides were then rinsed with distilled water. A cover glass was placed on each slide for visualization under optical microscope utilizing immersion objective and images were captured using a digital camera. Whole blood incubated with equivalent volume of PBS was used as spontaneous control. All test samples were analyzed in triplicate and the results are expressed as mean ± SD (n = 3).

4.6 *In-vitro* anticancer activity

The *in-vitro* cytotoxicity of placebo nano-formulations and VRL loaded nano-formulations was assessed in MCF-7 cells by MTT assay (Vijayakumar et al., 2016a, Vijayakumar et al., 2016b, Vijayakumar et al., 2016c, Van Meerloo et al., 2011). Briefly, MCF-7 cells were

cultured onto 96-well microtitre plates in complete DMEM medium and incubated at 37 ºC in humidified CO_2 (5%) incubator for 24 h. The cells were treated with fresh DMEM culture medium containing test samples (placebo nano-formulations and VRL loaded nano-formulations) for 48 h. After incubation, the medium was replaced with MTT (100 µL, 5 mg mL^{-1} in medium) solution and the cells were incubated for additional 2 h. Culture medium and MTT were then removed completely. The insoluble formazan crystals formed were dissolved in 100 µL dimethyl sulfoxide (DMSO). The plates were kept for 30 min at 37 ºC and absorption was calculated at 570 nm using a multimode reader (Microscan MS5608A). The absorbance of control cells treated with equivalent quantity of nanoparticulate dispersion medium (normal saline) was used to measure the cytotoxicity. The percentage viability and cytotoxicity was calculated by the following equation:

$$\% \text{ cyototoxicity} = 100 - \left(\frac{\text{Absorbance of test}}{\text{Absorbance of control}}\right) \times 100 \qquad \text{Equation 4.14}$$

4.7 Toxicity studies

4.7.1 Animals

Female Sprague dawley rats (150-200 g) were obtained from central animal house, Institute of medical science, Banaras Hindu University, Varanasi, India (Dean/2016/CEAC/331). The rats were housed in polypropylene cages over a dust free husk in an animal house for one week before commencement of experiment with 12 h light/dark cycle at 25 ºC. the animals were provided with feed and water ad libitum.

4.7.2 Experimental protocol:

The animals were divided into 4 groups of 6 rats in each group. Group I was control group treated with saline only, Group II was treated with VRL solution, Group III was treated with physical combination of VRL and RES. Group IV and V were treated with PLGA-VRL-ACNs and PLGA-VRL-RES-ACNs, respectively. Test samples (equivalent to 10 mg kg^{-1} VRL) were administered via *i.v.* route through tail vein to all groups. Blood was drawn after retro-orbital puncture at 2, 14 and 28 days after injection for hematology analysis. Whole blood was centrifuged twice at 3000 rpm for 15 min in order to separate serum. Serum biochemical analysis was carried out to determine the serum level of alkaline phosphatase (ALP), alanine transaminase (ALT) and total bilirubin (TBIL) in order to assess liver functions. Nephrotoxicity was evaluated by estimation of urea and creatinine (CREA) levels (Hajhashemi et al., 2001, Ivanov et al., 2012). During the experiment, relative body weight and percentage survival were also determined as an indicator of toxicity.

4.8 *In-vivo* Anticancer efficacy

4.8.1 Animals

In bred Balb/c mice were house in individually ventilated cage system and fed with standard sterilized diet and sterilized water ad libitum. The protocol was approved by CPCSEA vide 1410/c/11/CPCSEA. Animals were maintained in 12-12 light dark cycle, 25ºC air conditioned and controlled noise rooms.

4.8.2 Experimental protocol

Balb/c mice, 8-10 weeks, were used for the experiment. Briefly 4T1 cells (1×10^5) were injected on the back of the mice and allowed to form tumors. Tumors were minced and re

grafted in experimental animals. All animals were divided into four groups containing 6 animals each. Administration of test samples (PBS, VRL, VRL (10 mg kg^{-1}) plus RES (1 mg kg^{-1}) and PLGA-VRL-RES-ACNs (equivalent to 10 mg kg^{-1} VRL+ 1mg kg^{-1} RES)) was done after the tumor reached a palpable size (Thummar et al., 2016). Test samples were administered *i.v.* on 6^{th}, 12^{th}, 18^{th}, 24^{th}, 30^{th} day and the control (untreated) group received PBS.

Table 4.5: Treatment groups for *in-vivo* anticancer efficacy study

Groups	No. of animals	Treatment
Group I	6	Disease control (Untreated)
Group II	6	Vinorelbine (10 mg kg^{-1} i.v daily)
Group III	6	VRL (10 mg Kg^{-1})+ RES (1 mg Kg^{-1}) every 6^{th} day
Group IV	6	PLGA-VRL-RES-ACNs, equivalent to VRL (10 mg Kg^{-1}) every 6^{th} day

Tumor diameter was measured using digital vernier calipers (Mitutoyo JAPAN). Tumor volume was measured as a marker utilizing following formula:

$$\text{Volume} = (\text{width})^2 \times \text{length}/2 \qquad \text{Equation 4.15}$$

Relative tumor volume (RTV) was calculated based on the measured results using the following formula:

$$\text{RTV} = Vt/V0 \qquad \text{Equation 4.16}$$

(V0: the tumor volume at initial administration, Vt: the tumor volume at each time measurement).

Anti-tumor activity was evaluated by the relative tumor growth rate (T/C %)

$$T/C\ (\%) = TRTV/CRTV \times 100\ ,$$ Equation 4.17

(TRTV: treatment group RTV; CRTV: negative control group RTV). At the end of the experiment the animals were sacrificed by cervical dislocation. The animals were dissected and tumors were excised. The excised tumors were immediately imaged (Wang et al., 2014).

4.9 Statistical analysis

All the experiments were performed in triplicate (n=3) and the results were expressed as mean ± SD. All the statistical comparisons with control were performed using ANOVA taking $p < 0.05$ as statistically significant level. All statistical analyses were performed using GraphPad Prism statistical software (GraphPad Software Inc., La Jolla, CA)