Chapter 4

Materials and Methods

4.1. Materials

RSV and poly (D,L-lactide-co-glycolide) 75:25 (Resomer® RG 752S) were provided by Cayman Chemical Company (MI, USA) and Evonik Degussa India Pvt. Ltd., Mumbai, India, respectively. Tristearin, DSPE-PEG-2000, 4,5-(dimethylthiazol-2-yl) 2,5-diphenyl-tetrazolium bromide (MTT) and 2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI) were purchased from Sigma-Aldrich (St Louis, MO, USA). Phosphatidylcholine from soybean (LIPOID S 100) and TPGS were kind gift samples of Lipoid GmbH (Ludwigshafen, Germany) and Antares Health Products Inc. (St. Charles, Illinois, USA), respectively. Coumarin 6 was kindly provided by Parishi Chemicals (Surat, India). Analytical grade chloroform, ethanol, ethyl acetate, acetonitrile and methanol were purchased from SD Fine-Chem Ltd. (Mumbai, India). C6 glioma cells were obtained from National Centre for Cell Science (NCCS), Pune, India. Dulbecco's Modified Eagle's Medium (DMEM) with high glucose and foetal bovine serum albumin (FBS) were purchased from Himedia (Mumbai, India) and Thermo Fisher Scientific-Gibco (Waltham, MA, USA). Buffer salts and other chemicals used in this study were of analytical grade.

4.2. HPLC analytical method

A reverse-phase high-performance liquid chromatography (HPLC) was used for RSV quantifications as reported earlier [174]. The HPLC system consisted of inline degasser, 515 HPLC binary pump (Waters, USA), rheodyne 7725i manual injector (Waters, USA), C18 reverse-phase (250 × 4.6 mm, 5 µm) ODS2 column protected with a guard column (12 × 4.6 mm, 5 µm) of the same material (Waters Corp., Milford, MA, USA), photo diode array (PDA) detector (Waters, USA). The mobile phase was methanol: phosphate buffer pH 6.8 (pH adjusted with 0.5% v/v orthophosphoric acid solution in Milli-Q water) (63:37% v/v). The flow rate of mobile phase was set at 1.0 ml/min. The column was maintained at 25±2 °C using column oven (Waters, USA) and the effluent was detected at 306 nm. HPLC peak area and retention time measurements were determined using the operating software Empower Node 2054. Standard curves were plotted in plasma in the range of 10-5000 ng/mL. Liquid-liquid extraction (protein precipitation) method was used for separation of RSV from plasma. The plasma was deproteinized by adding ethyl acetate and centrifuged at 15,000 rpm for 15 min. The supernatant was transferred to a fresh tube and evaporated to dryness at 45 °C in nitrogen gas atmosphere. The dried residue was reconstituted with mobile phase and centrifuged at 15,000 rpm for 10 min. Supernatant (20 µL) was injected in to HPLC for quantification of RSV. Precision and accuracy were estimated by analyzing RSV at three different levels, i.e., 10, 2500 and 5000 ng/mL. Calibration curves were also plotted in mobile phase, brain, lungs, liver, spleen and kidney homogenates for entrapment efficiency, *in vitro* drug release and biodistribution studies. Different known concentration of RSV was spiked in

tissue homogenates, incubated at 37 °C for 1 hour, extracted using ethyl acetate and analyzed for plotting HPLC calibration in the respective organs.

4.3. Preparation methods

4.3.1. Solid lipid nanoparticles

The proposed structure of RSV loaded TPGS or DSPE PEG 2000 coated SLN (RSV-TPGS-SLN and RSV-PEG-SLN, respectively) are shown in Figure 4.1 (a) and (b), respectively. RSV-TPGS-SLN and RSV-PEG-SLN were prepared by solvent emulsification evaporation method as reported elsewhere with slight modifications [175]. Briefly, RSV, tristearin, oyaphosphotidyl choline (S-100) and TPGS or DSPE PEG 2000 were dissolved in 2 mL mixture of chloroform and methanol (2:1) by warming in a water bath. The warm organic phase was emulsified with 20 mL of aqueous phase (triple distilled water) maintained at 75 °C using ultraturrax (IKA, Germany) using a probe of 1 cm diameter, operated at 18,000 rpm for 10 min. The dispersion was sonicated at a frequency of 0.5 cycles and 60% amplitude using a probe type ultrasonicator (Heilscher, Germany) and was stirred using magnetic stirrer for 24 h for evaporation of organic solvent. Drug loading was 0.05% to the volume of formulation. The concentration of soya phosphatidyl choline and TPGS were 0.25% each to the volume of formulation (20 mL). The resultant nanoparticles were washed twice with HPLC grade water by centrifugation method using Amicon Ultra-4 Centrifugal Filter Unit with Ultracel-10 membrane; Molecular-weight cut-off = 10,000 (Millipore, Billerica, MA, USA) at 4000 rpm for 20 min (C-24BL, Remi Centrifuge, India) to remove un-entrapped RSV. The

filtered nanoparticles were re-suspended in required volume of 0.9% w/v of sodium chloride (normal saline). The drug to lipid ratio and sonication time (varied to get lower particle size and higher entrapment efficiency) of various batches of RSV-TPGS-SLN are presented in Table 4.1. The formulation composition and operating parameters of optimized formulation of RSV-TPGS-SLN was used to formulate RSV-PEG-SLN (TPGS was replaced by DSPE PEG 2000). Placebo-TPGS-SLN and Placebo-PEG-SLN were also prepared without drug. Similarly, coumarin-6 loaded fluorescent SLNs (COU-TPGS-SLN and COU-PEG-SLN) for cellular internalization studies were prepared using the same procedure by replacing RSV with coumarin-6 (0.05% w/v).

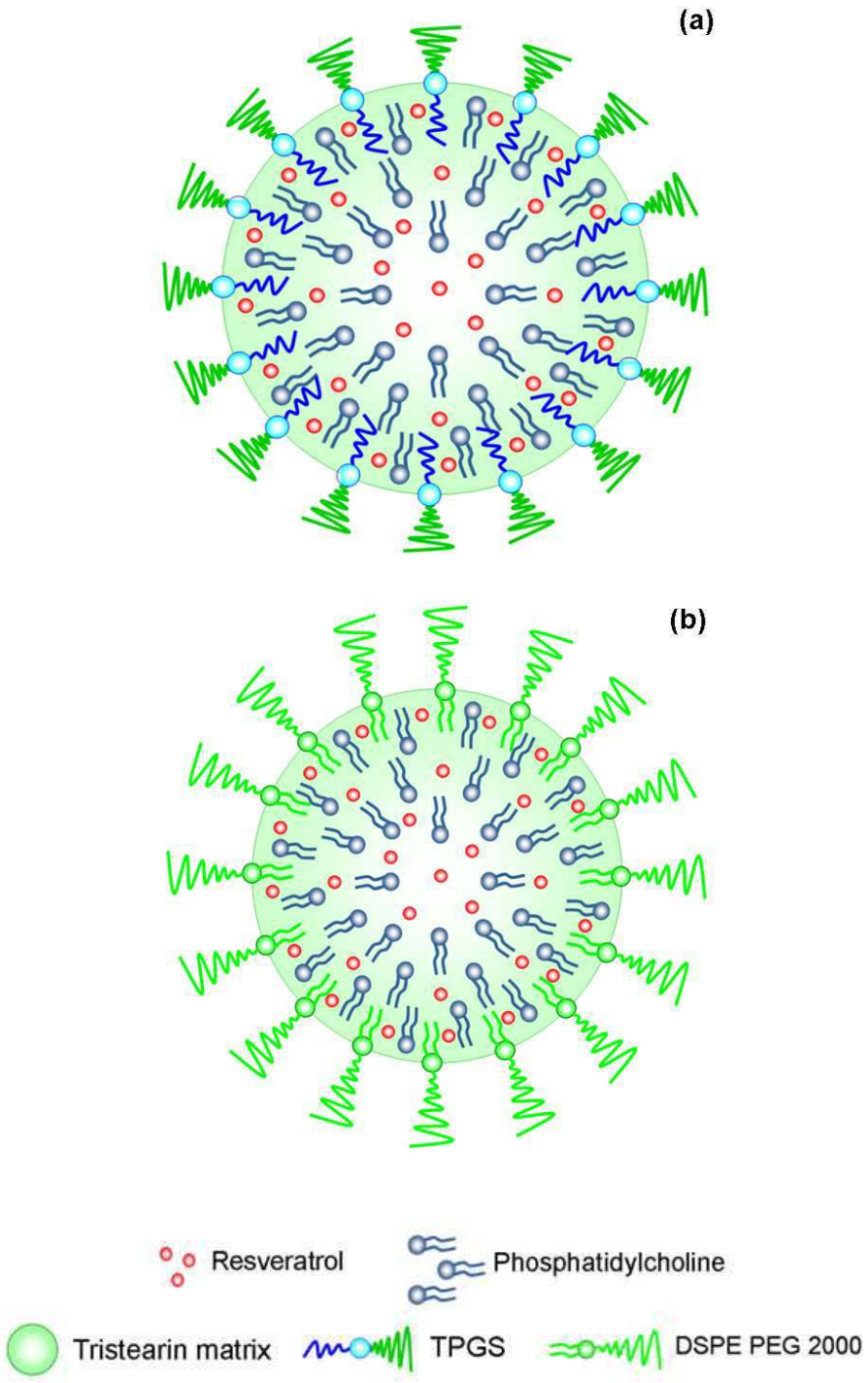

Figure 4.1. Schematic diagram of RSV-TPGS-SLN and RSV-PEG-SLN showing molecular arrangement of drug and other SLN components.

Table 4.1. Drug to lipid ratio and sonication time of various batches of RSV-TPGS-SLN.

Formulation code	Drug: Tristearin ratio (mg)	Sonication time (min)
RSV-TPGS-SLN 1	1:5	1
RSV-TPGS-SLN 2	1:5	3
RSV-TPGS-SLN 3	1:5	5
RSV-TPGS-SLN 4	1:10	1
RSV-TPGS-SLN 5	1:10	3
RSV-TPGS-SLN 6	1:10	5
RSV-TPGS-SLN 7	1:15	1
RSV-TPGS-SLN 8	1:15	3
RSV-TPGS-SLN 9	1:15	5

4.3.2. Blend nanoparticles

The proposed structure of RSV loaded PLGA:TPGS blend nanoparticles (RSV-PLGA-BNPs) is shown in Figure 4.2. RSV-PLGA-BNPs were prepared by single-emulsion solvent-evaporation technique with slight modifications [30, 176]. Briefly, RSV (5 mg), PLGA (50 mg) and TPGS at various blend ratios were dissolved in ethyl acetate at room temperature. Formulation variables such as PLGA: TPGS blend ratio and organic to aqueous ratio were varied to obtain lower particle size and higher entrapment efficiency. The composition of various batches of RSV-PLGA-BNPs is shown in Table 4.2. The organic phase containing RSV, PLGA and TPGS was poured rapidly into 25 mL of

aqueous phase (HPLC grade water) at room temperature and emulsified using probe type sonicator at a frequency of 0.5 cycles and 60% amplitude (Heilscher, Germany). The resulting o/w emulsion was stirred using magnetic stirrer at 600 rpm for 24 h at room temperature to evaporate of organic solvent. During this process, organic phase droplets were solidified in the aqueous system up on evaporation of organic solvent. The obtained nanosuspension was centrifuged at 15,000×g for 10 min (C-24BL, Remi Centrifuge, India) and supernatant was discarded. The pellet was washed two times with HPLC grade water. The resulting RSV-PLGA-BNPs were resuspended in normal saline solution. Similarly, coumarin-6 loaded PLGA: TPGS blend nanoparticles (COU-PLGA-BNPs) for cell internalization studies were prepared using the same procedure by replacing RSV with coumarin-6 (0.05% w/v). Placebo-PLGA-BNPs were also prepared without RSV to assess *in vitro* cytotixicity in cancer cells. PLGA: DSPE PEG 2000 blend nanoparticles were not included in our study because of its unstable nature (agglomeration and precipitation after preparation).

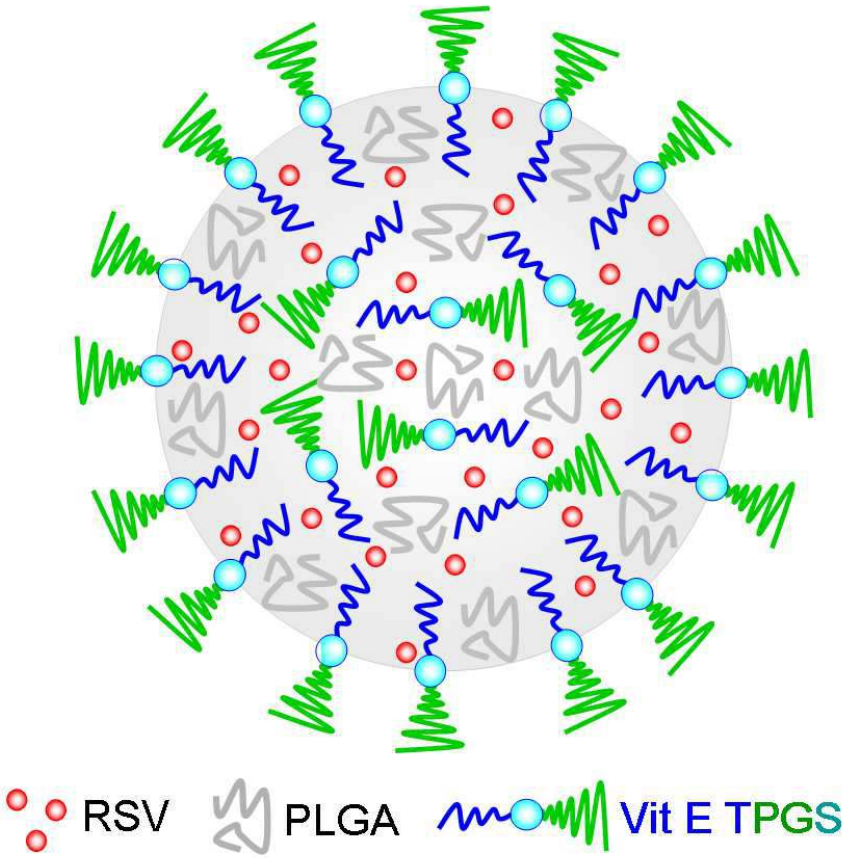

Figure 4.2. Schematic diagram of RSV-PLGA-BNPs showing molecular arrangement of RSV, PLGA and TPGS

Table 4.2. PLGA: TPGS blend ratio and organic to aqueous ratio of various batches of RSV-PLGA-BNPs

Formulation code	PLGA: TPGS blend ratio (mg)	Organic to aqueous ratio
RSV-PLGA-BNPs 1	1:0.5	1:10
RSV-PLGA-BNPs 2	1:0.75	1:10
RSV-PLGA-BNPs 3	1:1	1:10
RSV-PLGA-BNPs 4	1:0.5	2:10
RSV-PLGA-BNPs 5	1:0.75	2:10
RSV-PLGA-BNPs 6	1:1	2:10
RSV-PLGA-BNPs 7	1:0.5	3:10
RSV-PLGA-BNPs 8	1:0.75	3:10
RSV-PLGA-BNPs 9	1:1	3:10

4.3.3. Core-shell polymer-lipid hybrid nanoparticles

The proposed structure of TPGS coated HNPs (RSV-TPGS-HNPs) and DSPE PEG 2000 coated HNPs (RSV-PEG-HNPs) are shown in Figure 4.3 (a) and (b), respectively. RSV-TPGS-HNPs and RSV-PEG-HNPs were prepared by nanoprecipitation method as reported elsewhere [177-179]. Briefly, 3 mg of RSV and 30 mg of PLGA were dissolved in acetonitrile to form organic solution. Calculated amounts of phosphatidylcholine (PC) and TPGS were dissolved in 10 mL of 5% ethanol aqueous solution at 65 °C. The composition of various batches of RSV-TPGS-HNPs is shown in Table 4.3. The organic

phase containing RSV and PLGA was dropped into preheated aqueous ethanolic solution of phospholipid and TPGS with magnetic stirring. The resultant mixture was subjected to vortex vigorously to disperse the organic droplets evenly for 3 minutes at 65 °C and slow magnetic stirring for 12 h at room temperature to evaporate the organic solvents. Resultant HNPs were washed twice with HPLC grade water using Amicon Ultra-4 Centrifugal Filter Unit with Ultracel-10 membrane; Molecular-weight cut-off = 10 000 (Millipore, Billerica, MA, USA) by centrifugation method at 4000 rpm for 20 min for each wash. The filtered HNPs were resuspended in 0.9% w/v of sodium chloride (normal saline) and stored at 4 °C until further use. The formulation composition and operating parameters of optimized formulation of RSV-TPGS-HNPs was used to formulate RSV-PEG-HNPs (TPGS was replaced by DSPE PEG 2000). Similarly, coumarin-6 loaded TPGS coated COU-TPGS-HNPs and DSPE PEG 2000 coated COU-PEG-HNPs were prepared using the same procedure by replacing RSV with coumarin-6 (0.05 % w/v) for cellular internalization studies. Placebo HNPs coated with TPGS (Placebo-TPGS-HNPs) or DSPE PEG 2000 (Placebo-PEG-HNPs) were also prepared without RSV for comparative cytotoxic evaluations.

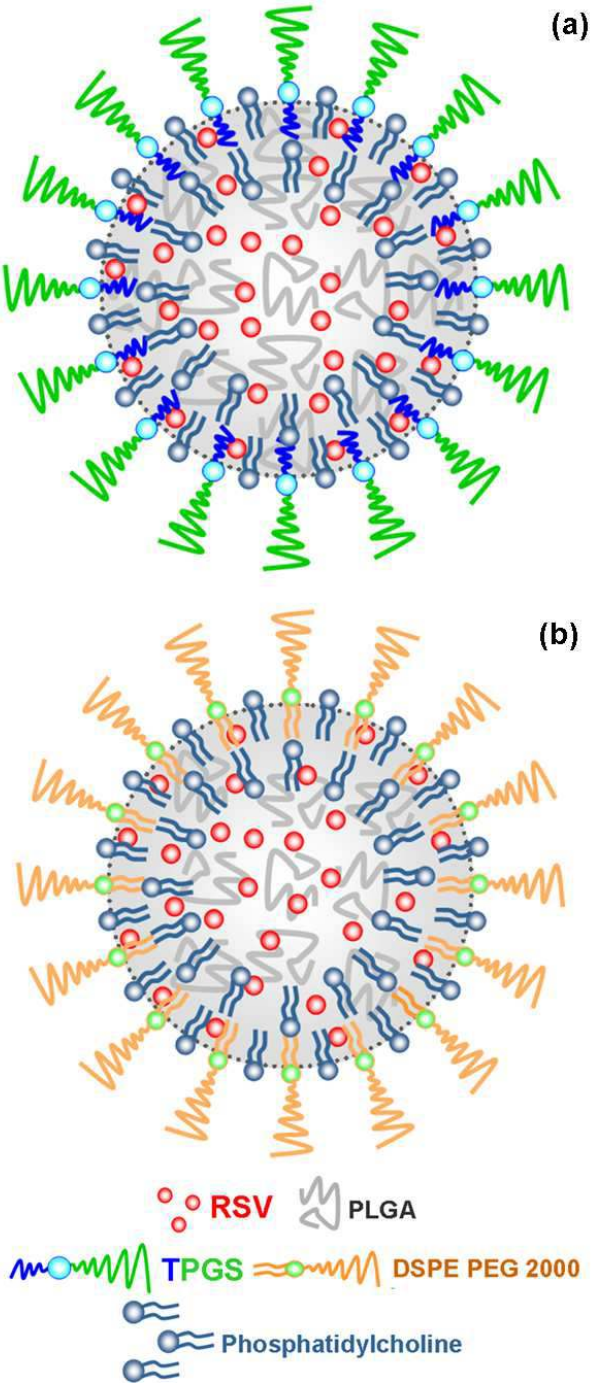

Figure 4.3. Schematic diagram of RSV-TPGS-HNPs and RSV-PEG-HNPs showing molecular arrangement of drug and other nanoparticle components

Table 4.3. TPGS+Phospholipid (phosphotidyl choline; PC) concentration and volume of organic solvent of various batches of RSV-TPGS-HNPs

Formulation code	TPGS+PC (mg)	Volume of organic phase (mL)
RSV-TPGS-HNPs 1	15+15	1
RSV-TPGS-HNPs 2	15+15	3
RSV-TPGS-HNPs 3	15+15	5
RSV-TPGS-HNPs 4	30+30	1
RSV-TPGS-HNPs 5	30+30	3
RSV-TPGS-HNPs 6	30+30	5
RSV-TPGS-HNPs 7	45+45	1
RSV-TPGS-HNPs 8	45+45	3
RSV-TPGS-HNPs 9	45+45	5

4.3.4. Liposomes

The proposed structure of RSV loaded TPGS and DSPE PEG 2000 coated liposomes (RSV-TPGS-Lipo and RSV-PEG-Lipo, respectively) are shown in Figure 4.4. RSV loaded RSV-Lipo (uncoated liposomes), RSV-TPGS-Lipo and RSV-PEG-Lipo were prepared by thin film hydration method as reported elsewhere [180]. Briefly, RSV, phosphatidylcholine and cholesterol with or without TPGS or DSPE PEG 2000 were dissolved in chloroform: methanol (2:1 v/v) mixture in a clean and dry round bottom flask. TPGS coated liposomes was used for optimization process. The molar ratio of

phosphatidylcholine: cholesterol: TPGS used in the preparation of RSV-TPGS-Lipo batches was 10:5:1. Organic solvents were removed completely by rotary flash evaporator (IKA® RV 10) above the lipid transition temperature (51 °C) at 75 rpm for 3 h to obtain a uniform thin lipid film on the wall of the flask. The vacuum was initially set at 500 mbar and slowly reduced to 25 mbar to prevent eviction of organic solution. The deposited thin lipid film was hydrated with appropriate volume of 0.9 % sodium chloride for 1 h at 51 °C to form multilamellar vesicles (MLV) at a final liposomal components concentration of 10 mg/mL. Small unilamellar vesicles (SUV) were obtained by sonicating the dispersion by a probe type sonicator (Ultrasonic Processor, UP200S, Hielscher Ultrasound Technology) using 6 mm ultrasonic probe at 60 % amplitude and 0.5 cycles per second [20, 58]. The liposomal formulations were optimized to get lower vesicular size, narrow polydispersity index, higher zeta potential and larger entrapment efficiency. The molar ratio between RSV/all other liposomal components and increment in sonication times are shown in Table 4.4. The liposomal formulations were kept at room temperature for 24 h to anneal any structural defects. Finally, liposomes were centrifuged at 12,000 rpm for 15 min to remove free drug and stored at 4 ^{o}C [20, 58]. The formulation composition and operating parameters of optimized formulation of RSV-TPGS-Lipo was used to formulate RSV-PEG-Lipo (TPGS was replaced by DSPE PEG 2000). Similarly, Placebo-Lipo, Placebo-TPGS-Lipo and Placebo-PEG-Lipo were also prepared without RSV. Coumarin-6 loaded fluorescent liposomes coated with TPGS or DSPE PEG 2000 (COU-TPGS-Lipo and COU-PEG-Lipo, respectively) were also prepared for cellular internalization studies using the same procedure by replacing RSV with 0.05 % w/v of coumarin-6 in the formulation.

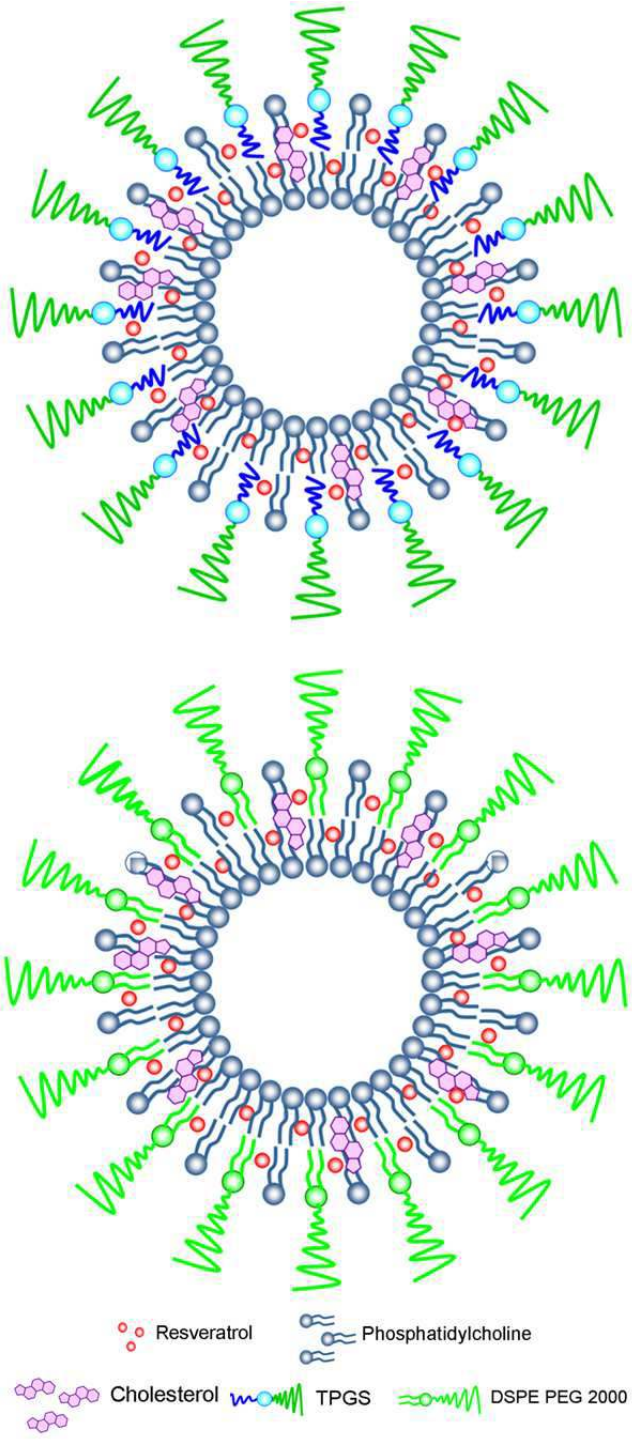

Figure 4.4. Schematic diagram of RSV-TPGS-Lipo and RSV-PEG-Lipo showing molecular arrangement of RSV and other liposomal components

Table 4.4. Drug to liposomal components ratio and sonication time of various batches of RSV-TPGS-Lipo.

Formulation code	RSV:Liposomal components ratio	Sonication time (min)
RSV-TPGS-Lipo 1	0.5:10	1
RSV-TPGS-Lipo 2	0.5:10	3
RSV-TPGS-Lipo 3	0.5:10	5
RSV-TPGS-Lipo 4	1:10	1
RSV-TPGS-Lipo 5	1:10	3
RSV-TPGS-Lipo 6	1:10	5
RSV-TPGS-Lipo 7	2:10	1
RSV-TPGS-Lipo 8	2:10	3
RSV-TPGS-Lipo 9	2:10	5

4.4. Particle size, polydispersity index and zeta potential

Nanoformulations were filled in sample cuvette made up of polystyrene and the particle size/polydispersity index were measured using Delsa^{TM} Nano C particle analyzer (Beckman, USA). The mean particle size and polydispersity index were determined from triplicate measurement of 50 accumulation time of each sample. Similarly, the samples were filled in capillary tube of zeta cell and the zeta potential was measured in triplicate.

4.5. Entrapment efficiency

The entrapment efficiency of nanoformulations was determined after 24 h of preparation by direct method using HPLC. Briefly, 10 mg of lyophilized samples was dissolved in 1 mL of methanol and further diluted suitably with mobile phase. In case of liposomes, 100 µL of liposomal suspension was mixed with 900 µL of methanol for disruption of bilayer structure and diluted suitably with mobile phase. The diluted solutions were filtered through 0.45 µm syringe filter and injected in to HPLC for quantification.

$$\text{Entrapment efficiency} = \frac{W_N}{W_T} \times 100$$

Where, W_N is the amount of RSV encapsulated in nanoformulation, and W_T is the total amount of RSV used for the preparation of nanoformulation.

4.6. Shape of nanoformulations

The shape of nanoformulations was analyzed by transmission electron microscope (TEM, JEM 2010F, JOEL, Japan). Samples for TEM was prepared by placing 10 µL of nanosuspension on a copper grid and dried under vacuum. The dried samples were stained with 1% phosphotungstic acid for 30 s and examined.

4.7. *In vitro* drug release

In vitro drug release of nanoformulations was carried out using dialysis membrane (12000-14000 Dalton molecular weights) in phosphate buffer saline pH 7.4 containing 0.1% w/v of tween 80. Nanoformulation equivalent to 2 mg of RSV was filled in dialysis

tube and placed in 50 mL of release medium stirred at 100 rpm using a magnetic stirrer at 37 °C. Aliquot of samples (1 mL) were withdrawn at pre-determined time intervals (0, 0.083, 0.25, 0.5, 0.75, 1, 2, 3, 4, 6, 8, 10, 12, 24, 36 and 48 hours). The sink condition was maintained by replacing equal volume of fresh release medium. The samples were diluted with mobile phase and analyzed using HPLC for quantification. The percentage of drug release was plotted against time to assess the RSV release pattern. The *in vitro* release data were also plotted for zero order, first order, Higuchi model and Korse-Meyer Peppas model to assess the kinetics and mechanism of drug release.

4.8. Drug excipient interaction analysis

The chemical interaction of RSV with other excipients was assessed by fourier transform infrared (FTIR) spectroscopy. FTIR spectra of pristine drug and lyophilized nanoformulations were obtained by conventional KBr disk/pellet method (SHIMADZU, Model 8400S, Tokyo, Japan). Samples (5 mg) were grounded gently with anhydrous KBr (50 mg) and compressed to form a thin film in a FTIR sample holder. FTIR spectrum was obtained between the wave number of 400 to 4000 cm^{-1}.

4.9. Differential scanning calorimetric (DSC) analysis

DSC thermograms of RSV, lyophilized nanoformulations and its individual components (as mentioned below) were obtained to study the drug-excipient interaction and crystalline nature of RSV using DSC (DSC Q1000, TA instrument, USA). Samples (2 mg) were sealed in aluminium pans and scanned at a heating rate of 10 °C min^{-1} over the temperature range of 4-300 ^{o}C, under nitrogen flow of 50 mL min^{-1}.

Formulation		**Samples**
SLNs	:	RSV, tristearin, phosphotidylcholine, TPGS, lyophilized RSV-TPGS-SLN and RSV-PEG-SLN
BNPs	:	RSV, PLGA, TPGS and lyophilized RSV-PLGA-BNPs
HNPs	:	RSV, PLGA, TPGS, DSPE PEG 2000, lyophilized RSV-TPGS-HNPs and RSV-PEG-HNPs
Liposomes	:	RSV, phosphatidylcholine, cholesterol, TPGS, DSPE PEG 2000, lyophilized RSV-TPGS-Lipo and RSV-PEG-Lipo

4.10. X-Ray diffraction analysis

X-Ray diffraction pattern of RSV and lyophilized nanoformulations of each category were obtained by X-ray diffractometer (Bruker-D8 Discover), using Ni-filtered Cu-K radiation at 45 kV voltage and 40 mA. The scattered radiation in crystalline regions of the sample was measured by vertical goniometer. The diffraction patterns were obtained between 5° and 80° angle using step size of 0.045 °C with detector resolution in 2θ (diffraction angle) at 25 ^{o}C.

4.11. Cytotoxicity against C6 glioma cells

In vitro cytotoxicity of RSV, nanoformulations and respective placebo formulations in C6 glioma cells was evaluated by MTT assay. Briefly, C6 glioma cells were seeded onto 96-well micro titre plates at 1×10^4 cells/well in complete Dulbecco's Modified Eagle's Medium (DMEM) and incubated at 37 °C in humidified CO_2 (5%) incubator (Galaxy® 170 S, Eppendorf, Germany) environment for 24 h. The cells were exposed to fresh

DMEM culture medium containing different concentration of test samples for 72 h at 37 °C in a humidified CO_2 (5%) incubator. After incubation, the medium was replaced with 20 µL of 4,5-(dimethylthiazol-2-yl) 2,5-diphenyl-tetrazolium bromide (MTT; 5 mg/mL in PBS) solution and the cells were incubated for 4 h at 37 °C in humidified CO_2 (5%) incubator. Culture medium and MTT were removed completely. The formed insoluble formazan crystals that are proportional to the number of viable cells were dissolved in 100 µL dimethyl sulfoxide (DMSO). The plate was agitated for 10 min and absorption was measured at 570 nm using a multimode reader (Synergy H1 hybrid, Biotek, USA). The absorbance of control cells treated with equivalent quantity of nanoparticulate dispersion medium (0.9% w/v of sodium chloride, normal saline) was used to calculate the cytotoxicity. The percentage of cytotoxicity was calculated by the following equation:

$$\% \text{ cytotoxicity } = 100 - [\frac{\text{Absorbance of test}}{\text{Absorbance of control}} \times 100]$$

Data are expressed as mean±SD (n = 3).

4.12. Cellular uptake of Coumarin-6 loaded nanoformulations

C6 glioma cells in complete DMEM were seeded on a cover glass placed in 35 mm Petri dishes at 1×10^4 viable cells/cover glass. The cells were incubated overnight at 37 °C in a humidified CO_2 (5%) incubator and subsequently incubated with coumarin-6 loaded nanoformulations (100 µL/cover glass), separately. After 2 h, the cells were washed thrice with cold phosphate buffer saline (PBS pH 7.4) and fixed using paraformaldehyde for 20 min. The cells were washed thrice with cold PBS. The nuclei were stained by incubating with 0.1 µg/mL of DAPI for 10 min. Further, the cell monolayer was washed

thrice with PBS, cover glass was taken from Petri dishes, mounted on microscope slides and observed by confocal laser scanning microscope (CLSM) (Zeiss LSM510 Meta LSM with Plan-Apo 40X (1.3-NA) and Zeiss LSM Meta 510 software)[20].

4.13. Evaluation of haemolysis

As the prepared nanoformulations were intended for intravenous administration, the evaluation of haemolytic property was essential to prove the haemocompatibility. Haemolytic analysis of RSV, nanoformulations and respective placebo formulations were performed based on earlier publications [36, 181]. Human blood samples were purchased from an authorized blood bank. Blood sample (2 mL) was centrifuged at 1344×g for 10 min at room temperature in a sterile graduated centrifuge tube. Plasma layer was removed carefully using a micropipette. Equal volume of normal saline solution was added to erythrocyte pellet and the tube was mixed gently. The erythrocyte suspension was centrifuged again at 1344×g for 10 min at room temperature. This washing protocol was repeated 3 times. Finally, the erythrocytes were diluted up to 10 mL using normal saline solution and resuspended gently. RSV (at 10, 50 and 100 μg/mL), nanoformulations (equivalent to 10, 50 and 100 μg/mL of RSV) and equivalent volume of respective placebo formulations were mixed with 2 mL of erythrocyte suspension separately in a sterilized eppendorf tubes. Positive control (100% lysed erythrocytes) and spontaneous negative control samples were prepared by diluting equal volume of erythrocyte suspension with 1% Triton X-100 and normal saline, respectively. All samples were incubated at 37 °C and mixed gently in every 15 min. Aliquots (200 µL) were collected at predetermined time intervals (0.5, 1, 2, 4 and 8 h) and centrifuged at 1344 ×g for 10 min.

Supernatants (100 μL) were incubated for 30 min at room temperature for oxidation of haemoglobin in to oxyhaemoglobin. The absorbance was measured spectrophotometrically at 540 nm. The percentage of haemolysis was calculated using the following formula:

$$\% \, Haemolysis = \frac{A_{Sample} - A_{Spontaneuous\ Control}}{A_{Positive\ Control}} \times 100$$

Where A_{sample} is the absorbance of supernatants of erythrocytes incubated with RSV/nanoformulations, $A_{Spontaneous\ Control}$ is the absorbance of supernatants of erythrocytes incubated with normal saline equivalent to the volume of samples and $A_{Positive\ Control}$ is the absorbance of supernatants of erythrocytes incubated with 1% Triton X-100 solution in normal saline. Triplicate of experiments were performed and the data is expressed as mean±SD (n=3).

4.14. Evaluation of erythrocyte membrane integrity

The LDH enzyme released from erythrocytes by particle treatment was assessed photometrically using the LDH commercial kit (Coral Clinical Systems, Goa, India) as reported earlier [182]. Erythrocyte suspension was treated with RSV (at 10, 50 and 100 μg/mL), nanoformulations (equivalent to 10, 50 and 100 μg/mL of RSV), equivalent volume of respective placebo formulations and normal saline (spontaneous negative control) as described in haemolytic evaluations. In addition, LDH standard was also included by treating 150 UL^{-1} of lactate dehydrogenase enzyme with the erythrocyte suspension. All the samples were incubated at 37 ^{o}C. Aliquots (200 µL) of the

erythrocyte suspensions were collected at predetermined time intervals (1, 4 and 8 h) and centrifuged at 1344 ×g for 10 min. Supernatants (100 μL) were treated with LDH assay ready to use solution at 1:50 ratio and the LDH release was detected at 500 nm. The concentration of LDH enzyme was calculated using the following formula:

$$Lactate\ dehydrogenase(U/L) = \frac{A_{Sample} - A_{Negative\ Control}}{A_{Standard}} \times 150$$

where A_{sample} is the absorbance of supernatant of erythrocytes incubated with nanoformulations, $A_{Negative\ Control}$ is the absorbance of supernatant of erythrocytes incubated with normal saline and $A_{Standard}$ is the absorbance of supernatant of the erythrocytes incubated with 150 UL^{-1} of lactate dehydrogenase enzyme. Triplicate of experiments were performed and the data is expressed as mean±SD (n=3).

4.15. Platelet Aggregation

Quantitative evaluation of platelet aggregation was carried out by counting the platelets using haematological counter (Multisizer 4, Beckmann Coulter, USA) after incubation with test samples [36, 181]. Briefly, RSV (at 10, 50 and 100 μg/mL), nanoformulations (equivalent to 10, 50 and 100 μg/mL of RSV) and equivalent volume of respective placebo formulations were incubated with 1 mL of blood for 2 h at 37 °C. Similarly, spontaneous control was prepared by incubating whole blood samples with PBS (equivalent to the volume of nanoformulations) at same experimental conditions. After incubation, samples were diluted and analysed by haematological counter (Multisizer 4,

Beckmann Coulter, USA) after mixing. All samples were analyzed in triplicate and the values are expressed as mean±SD (n=3).

Qualitative analysis of platelet aggregation was carried out by microscopic observation of stained peripheral blood smears after incubating heparinised whole blood with test samples (like quantitative analysis). After incubation, peripheral blood smears were prepared on a clean glass slide. The slides were air dried for 2 min and stained by Leishman's stain for 5 min (Span Diagnostics, India). After rinsing the slides with distilled water, cover glass was placed on it and analyzed by optical microscope in immersion objective and images were captured using digital camera.

4.16. Pharmacokinetic studies

The protocol for pharmacokinetic study was approved by Central Animal Ethical Committee of the University (CPCSEA). Healthy Charles Foster rats (150–200 g) of either sex were obtained from Central Animal House, Institute of Medical Science, Banaras Hindu University, Varanasi, India. The rats were housed in poly propylene cages over dust free husk for one week before experiment with 12 h light/dark cycle at 25±2 °C and 40–70% relative humidity. The animals were fed with rat chow and water ad libitum. The animals were divided into required number of groups of 6 rats in each group (as mentioned below). Free RSV solubilised in 0.3 M β-cyclodextrin in sterile water for injection and nanoformulations (equivalent to 2 mg/kg of RSV) were administered via *i.v.* route through tail vein.

Formulation		**Samples**
SLNs	:	RSV, RSV-TPGS-SLN and RSV-PEG-SLN (3 groups)
BNPs	:	RSV, RSV-PLGA-BNPs (2 groups)
HNPs	:	RSV, RSV-TPGS-HNPs and RSV-PEG-HNPs (3 groups)
Liposomes	:	RSV, RSV-Lipo, RSV-TPGS-Lipo and RSV-PEG-Lipo (4 groups)

Blood samples (200 µL) were collected through retro orbital vein at predetermined time intervals (0.25, 0.5, 1, 2, 4, 8, 12, 24, 36 and 48 h) under ether anaesthesia. Blood samples were collected in heparinised eppendorf tubes. The collected blood was centrifuged at 4000 rpm (C-24BL, Remi Centrifuge, India) for 15 min at room temperature. Plasma was separated and stored at −20 °C until analysis. Liquid-liquid extraction (protein precipitation) method was used for separation of RSV from plasma. The plasma was deproteinized by adding ethyl acetate and centrifuged at 15,000 rpm for 15 min. The supernatant was transferred to a fresh tube and evaporated to dryness at 45 ^{o}C in nitrogen gas atmosphere. The residue was reconstituted with mobile phase and centrifuged at 15,000 rpm for 10 min. Supernatant (20 µL) was injected in to HPLC for quantification of RSV. Peak area of chromatogram of samples was calculated using Empower Pro® HPLC software and respective plasma concentration was calculated using linearity equation of calibration curve. Pharmacokinetic analysis was carried out using WinNonlin 6.1 professional software (Pharsight Corporation, NC, USA). Noncompartmental intravascular analysis was employed for calculation of pharmacokinetic parameters.

4.17. Tissue distribution studies

The complete protocol for tissue distribution studies was approved by the Central Animal Ethical Committee of the University (CPCSEA). Healthy Charles Foster rats (150–200 g) of either sex, obtained from Central Animal House were randomly divided into required number of groups consisting of 3 rats in each group (as mentioned in pharmacokinetics studies). RSV solubilised in 0.3 M β-cyclodextrin in sterile water for injection and nanoformulations (equivalent to 2 mg/kg of RSV) were administered via *i.v.* route through tail vein in overnight fasted animals. The animals were sacrificed at 90 min after injection by cervical dislocation. Brain, liver, kidney, lungs and spleen were rapidly excised and washed with sterile physiological saline solution. The tissue samples were wiped with filter paper, weighed and immediately stored in frozen condition until analysis. The tissue samples were finely minced with scissors and homogenised with 2 mL of ethyl acetate. Homogenized samples were centrifuged at 15,000 rpm for 15 min. The supernatant was collected in a clean tube. The residue was extracted again with 2 mL of ethyl acetate and combined with first portion of supernatant. The supernatant was evaporated to dryness under nitrogen gas stream. The residue was reconstituted with mobile phase and analyzed by HPLC. The results are reported as the amount of RSV per gram of tissue.

4.18. Statistical analysis

Statistical analysis was carried out by one-way ANOVA followed by Bonferroni test to compare formulation optimization data. Cytotoxicity results were analysed by two-way ANOVA followed by Bonferroni test. Pharmacokinetic and tissue distribution results

were analysed using student t test for BNPs and one-way ANOVA for SLN, HNPs and liposomes. All statistical analysis was performed using GraphPad Prism statistical software (GraphPad Software Inc., La Jolla, CA). $P<0.05$ was considered statistically significant.