AAPS PharmSciTech, Vol. 19, No. 8, November 2018 (© 2018)
DOI: 10.1208/s12249-018-1156-3

Research Article

Theme: Lipid-Based Drug Delivery Strategies for Oral Drug Delivery
Guest Editor: Sanyog Jain

Lipid Architectonics for Superior Oral Bioavailability of Nelfinavir Mesylate: Comparative *in vitro* and *in vivo* Assessment

Tejashree Belubbi,[1] Sukhada Shevade,[1] Vivek Dhawan,[1] Vinay Sridhar,[2] Anuradha Majumdar,[2] Rute Nunes,[3,4,5,6] Francisca Araújo,[3,4,5,6] Bruno Sarmento,[3,4,6] Kalpa Nagarsenker,[7] Frank Steiniger,[8] Alfred Fahr,[7] Aniket Magarkar,[9,10] Alex Bunker,[9] and Mangal Nagarsenker[1,11]

Received 4 April 2018; accepted 18 August 2018; published online 12 September 2018

Abstract. Nelfinavir mesylate (NFV), a human immunodeficiency virus (HIV) protease inhibitor, is an integral component of highly active anti retro viral therapy (HAART) for management of AIDS. NFV possesses pH-dependent solubility and has low and variable bioavailability hampering its use in therapeutics. Lipid-based particulates have shown to improve solubility of poorly water soluble drugs and oral absorption, thereby aiding in improved bioavailability. The current study compares potential of vesicular and solid lipid nanocarriers of NFV with drug nanocrystallites and microvesicular systems like cochleates in improving bioavailability of NFV. The paper outlines investigation of systems using *in vitro* models like *in vitro* lipolysis, *in vitro* release, and permeation through cell lines to predict the *in vivo* potential of nanocarriers. Finally, *in vivo* pharmacokinetic study is reported which provided proof of concept in sync with results from *in vitro* studies.

KEY WORDS: SLN; LeciPlex®; liposomes; nanosuspension; cochleates; molecular dynamic simulation; Caco-2:HT29-MTX co-culture.

INTRODUCTION

Acquired immune deficiency syndrome (AIDS) is considered to be a major cause of public health concern globally, claiming more than 35 million deaths so far. In year 2016, one million deaths due to HIV-related ailments have been reported by WHO. Nelfinavir mesylate, an inhibitor of HIV protease, is frequently administered as one of the agents in HAART (Highly Active Antiretroviral Therapy) for treatment of AIDS in HIV patients. Nelfinavir mesylate is marketed as Viracept® tablets and Viracept® oral powder with a dose of 1250 g (five tablets of 625 mg, or two tablets of 750 mg) twice daily. High doses and

Tejashree Belubbi and Sukhada Shevade contributed equally to this work.

Guest Editor: Sanyog Jain

Nelfinavir mesylate (NFV), a human immunodeficiency virus (HIV) protease inhibitor, is an integral component of highly active anti retro viral therapy (HAART) for management of AIDS. The current study compares potential of vesicular and solid lipid nanocarriers of NFV with drug nanocrystallites and microvesicular systems like cochleates in improving bioavailability of NFV.

Electronic supplementary material The online version of this article (https://doi.org/10.1208/s12249-018-1156-3) contains supplementary material, which is available to authorized users.

[1] Department of Pharmaceutics, Bombay College of Pharmacy, Kalina, Santacruz (East), Mumbai, Maharashtra 400098, India.
[2] Department of Pharmacology, Bombay College of Pharmacy, Kalina, Santacruz (E), Mumbai, 400 098, India.
[3] INEB - Instituto de Engenharia Biomédica, Universidade do Porto, Rua Alfredo Allen, 208, 4200-135, Porto, Portugal.
[4] I3S, Instituto de Investigação e Inovação em Saúde, Universidade do Porto, Rua Alfredo Allen, 208, 4200-135 Porto, Portugal.
[5] ICBAS - Instituto Ciências Biomédicas Abel Salazar, Universidade do Porto, Rua de Jorge Viterbo Ferreira 228, 4050-313, Porto, Portugal.
[6] CESPU-Instituto de Investigação e Formação Avançada em Ciências e Tecnologias da Saúde, Rua Central de Gandra, 1317, 4585-116, Gandra, Portugal.
[7] Department of Pharmaceutical Technology, Friedrich-Schiller-University Jena, Lessingstr. 8, 07743, Jena, Germany.
[8] Center for Electron Microscopy, Jena University Hospital, Friedrich Schiller University Jena, Ziegelmuehlenweg 1, 07743, Jena, Germany.
[9] Centre for Drug Research, Division of Pharmaceutical Bioscience, Faculty of Pharmacy, University of Helsinki, FI-00014, Helsinki, Finland.
[10] Academy of Sciences of the Czech Republic, 117 20, Prague, Czech Republic.
[11] To whom correspondence should be addressed. (e–mail: mangal.nagarsenker@gmail.com; mangal_nag511@yahoo.co.in)

long duration of therapy often result in low patient compliance and ultimately jeopardize the treatment outcome (1).

NFV is a BCS class IV (low solubility, low permeability) drug (1). NFV is lipophilic in nature, is subject to high p-gp efflux, and suffers high first-pass hepatic metabolism and has been reported as class II under Biopharmaceutical Drug Disposition Classification System (BDDCS) (2). NFV is a weak base with high pH-dependent aqueous solubility. NFV is practically insoluble at physiological pH 7.4 (1,3), resulting in low overall absorption of NFV. Therefore, fraction of drug that gets absorbed is subjected to P-gp efflux, further reducing its intestinal absorption (3). NFV is also metabolized by CYP3A4 enzyme in liver and most of the metabolites formed are inactive (4). These factors may be responsible for the variable bioavailability of the drug throughout the gastro intestinal tract and hence, there is a need to develop an alternative delivery system.

It has been established in the clinical studies that administration NFV along with high fat diet improves its bioavailability and reduces pharmacokinetic variability (5). It is therefore recommended to administer NFV with food. Oral powder is given by mixing it with milk, soy formula, soy milk, etc. NFV shows low intrinsic dissolution rate, and its absorption is anticipated to be dissolution rate limited. Thus, it is worthwhile to formulate NFV in lipid particulates expecting improvement in solubilization as well as intestinal absorption of NFV. Lipid nanosystems have been reported for improving bioavailability of poorly soluble BCS class II drugs. Increased micellar solubilization due to lipolysis, inhibition of enterocytic enzymes, inhibition of P-gP efflux of drugs from intestinal mucosa, and increasing GALT-mediated absorption are some of the mechanisms suggested for both improved solubility and absorption in such cases and resulting bioavailability. Besides, lipid nanosystems also possess advantages of providing sustained release and might aid in reducing loss of drug due to first-pass metabolism. In this regard, lipid nanosystems appear to be the best alternative for oral delivery of poorly soluble drugs with high metabolism and high efflux (6,7).

It can be expected that entrapment of NFV in phospholipid systems will aid in solubilization and enhance absorption. In line with the reports that fatty meals increase bioavailability of NFV (4), it is thought worthwhile to prepare lipid particulates which might also release in sustained manner thereby controlling variable rate of absorption. Furthermore, very few reports of NFV nanosystems and particulates are available in literature.

In this study, NFV was formulated as liposomes, LeciPlex®, and cochleates and in lipid nanoparticulate systems. LeciPlex® is a phospholipid-based cationic system that has advantages of enhanced stability, ease of loading of hydrophobic drugs, and one step method of preparation and is reported for effective oral and dermal delivery of agents (8). LeciPlex has been shown previously to greatly improve bioavailability of BCS class II agents (9–11). Cochleates are cylindrical lipid vesicles formed by ionic interaction between negatively charged liposomes and bivalent cations such as calcium. There is evidence that drug molecules encochleated in the cylinders are present in the inner layers of a solid, which is capable of increasing the stability of drugs and protection from hostile GIT environment. Cochleates have membrane fusion property and therefore cochleates can be used to improve oral absorption of drug (12).

The present study encompasses formulation and comparative evaluation of NFV containing systems for enhanced solubilization and permeation, potential *in vitro* and improved bioavailability *in vivo*. An attempt was made to correlate the investigations of *in vitro* assessments, namely, lipolysis, *in vitro* release, and permeability through Caco-2 cells with *in vivo* pharmacokinetic results of bioavailability in a view to rank order lipidic formulations for enhanced spatial (higher absorption in lymph and through M cells) and temporal delivery of Nelfinavir mesylate.

MATERIALS AND METHODS

Materials

Soy Lecithin (Phospholipon 90G, Lipoid GmBH, Germany), diethyleneglycolmonoethyl ether (Transcutol HP®, Gattefosse India Ltd., India), glyceryldistearate (Precirol ATO 5®, Gattefosse India Ltd., India), stearoyl polyoxyl-32 glycerides (Gelucire 50/13®, Gattefosse India Ltd., India), and nelfinavir mesylate (NFV) (Macleods Pharmaceuticals Ltd. Mumbai) were received as gift samples. Cetyltrimethylammonium bromide (CTAB) and Didodecyl dimethyl ammonium (DDAB) were purchased from Sigma Aldrich, India. Tween 80 was purchased from S. D. Fine Chemicals Ltd., India. Acetone was purchased from Fischer Chemicals, India. Dulbecco's Modified Eagle's Medium (DMEM) and HBSS (Hank's Balanced Salt Solution) were purchased from Lonza. Nonessential amino acids (NEAA) and Fetal Bovine Serum (FBS) were purchased from Biochrom GmbH (Berlin, Germany). 0.05% tripsin-EDTA and 0.4% trypan blue were purchased from Gibco (Life Technologies, New York, USA). Antibiotic/antimitotic penicillin/streptomycin was purchased from BioWest (Nuaillé, France). Twelve-well cell culture permeable support (PET membrane and 3-μm pore size) and 12-well tissue culture plates were purchased from Falcon (Corning Incorporated, New York, USA). Caco-2 and Raji B were purchased from American type culture collection (ATCC, USA). HT29-MTX cell line was provided by Dr. T. Lesuffleur (INSERM U178, Villejuif, France).

Fabrication of Particulates Loaded with NFV

With the aim of formulating NFV-loaded SLN various lipids like Precirol ATO 5®, Compritol 888 ATO® and Glyceryl Mono-stearate were screened at 1.5% *w/v*, 2% *w/v*, 3% *w/v*, and 4% *w/v* load. The surfactants Tween® 80, Gelucire®50/13, Poloxamer® 188, Brij® 35, Cremophor® EL, and Cetyltrimethyl ammonium bromide (CTAB) were screened at concentrations of 0.5% *w/v*, 1% *w/v*, 1.5% *w/v*, and 2% *w/v*. SLNs were prepared by emulsification method. Precirol ATO 5® was heated with NFV at 80°C in a water bath. Aqueous phase (MilliQ water Type I), which contained the surfactant (CTAB [C-SLN] or Tween 80 [T-SLN]) was maintained at same temperature and added at once under cyclomixing to the lipid phase. The cyclomixing was continued until uniform lipid dispersion was formed. The crude emulsion was transferred to a glass vial maintained at the 80°C and was sonicated using probe sonicator (Branson Digital Sonifier®, USA) to give nanoemulsion which was cooled at room temperature to yield SLNs. Blank SLNs were prepared by a similar method without the addition of drug. The ratio of lipid: surfactant was maintained at 2:1. Particle size, polydispersity index (P.I.), and

stability of the formulations were selected as the criteria for the development of NFV-loaded SLNs.

Nanosuspension of NFV (NS-G1) were prepared by solvent evaporation method. NFV and Gelucire® 50/13 were dissolved in required quantity of acetone by mild heating. Aqueous phase (MilliQ water Type I) was added at once under cyclomixing to the lipid phase. The cyclomixing was continued until a uniform dispersion was formed. The dispersion was stirred with magnetic stirrer overnight for complete evaporation of acetone. Nanosuspension was evaluated for mean particle size and stability. Nanosuspensions were also prepared in a similar manner by melt dispersion method, without use of acetone (NS G-2).

LeciPlex® was fabricated by a single-step method as reported previously (8). Lecithin and cationic surfactant were used in the molar ratio of 1:1 for DDAB containing LeciPlex (DDAB-LeciPlex) and 5:1 for CTAB containing LeciPlex (CTAB-LeciPlex). Soybean lecithin (Phospholipon 90 G), CTAB (for CTAB-LeciPlex) or DDAB (for DDAB-LeciPlex), and NFV were dissolved in Transcutol HP® by heating the mixture at 70°C in constant temperature water bath (Superfit Ltd., Mumbai, India). Aqueous phase maintained at 70°C was poured into lecithin phase (at 70°C) at once under cyclomixing (~ 1200 rpm) until uniform dispersion was formed.

Liposomes were fabricated using biocompatible solvent Transcutol HP®. Soy lecithin and NFV were dissolved in Transcutol HP® by heating at 70°C in constant temperature water bath (Superfit Ltd., Mumbai, India). Aqueous phase maintained at 70°C was poured into lecithin phase (at 70°C) at once under cyclomixing (~ 1200 rpm) until uniform dispersion was formed. Dispersion was reduced to nanoscale by subjecting it to probe sonication (Branson Digital Sonifier®, USA) under cold condition.

Nelfinavir-loaded cochleates were prepared by simple trapping method with 1,2-dimyristoyl- sn-glycero-3-phospho-(1′-rac-glycerol) [DMPG], 1,2-dioleoyl-sn-glycero-3-phospho-L-serine [DOPS], 1,2-dioctanoyl-sn-glycero-3-phospho-L-serine [DOctPS], and 1,2-didecanoyl-sn-glycero-3-phospho-L-serine [DDPS] as described by Zarif *et al.* (13). Briefly, the procedure comprised two main stages: preparation of homogenous unilamellar dispersion of drug containing bilayers and conversion into cochleates. Thin film hydration method was employed for loading Nelfinavir into the lipid bilayer. Ratio of molar concentration of lipid and drug was maintained at 10:1. The mixture was dissolved in minimum amount of Chloroform:Methanol (3:1) and subjected to film formation using Buchi Rotary Evaporator RE-111 (Buchi, Switzerland). Following the drying under nitrogen stream, lipid film was hydrated using TRIZMA buffer (10 mM, pH 7.4). The resultant dispersion was extruded through 100 nm polycarbonate membrane (ARMATIS GmbH, Weinheim, Germany). Further, $CaCl_2$ (60 mM) solution was added to lipid suspension under continuous stirring to reach the equimolar concentration of lipid and calcium. The resultant precipitate was then stored at 4°C until use.

Characterization of NFV-Loaded Particulate Systems

Particle Size

Particle size and polydispersity index (P.I.) were determined for the nanosystems using particle size counter (N5 Submicron Particle Size Analyzer, Beckman Coulter, USA). The formulations were appropriately diluted with freshly prepared MilliQ water (Type I) and particle size was determined at 25°C with 25 mW helium-neon laser (632.8 nm) incident on the sample at an angle of 90° as the laser source. For measurement of particle size of DOPS Nelfinavir cochleate sample, dilution of 1:21 in buffer was carried out. Sample was analyzed using iZONq nanoparticle analyzer equipped with NP2000 nanopore.

Zeta Potential

Zeta potential of all nanosystems was determined on Malvern NanoSizer Nanoseries (UK). The samples were analyzed without dilution at 25°C.

Drug Content and Entrapment Efficiency

Drug content was determined by adding suitable amount of acetonitrile to fixed quantity of nanoparticulate dispersions to yield final drug concentration of 10 ppm. Entrapment efficiency for NFV-loaded SLN was evaluated by analyzing amount of free NFV in the supernatant formed after centrifugation of nanosystem dispersion. Dispersion after appropriate dilution with double-distilled water was subjected to centrifugal force at a speed of 80,000 rpm for 1 hour using high-speed centrifuge (Optima-TM M4X -XP Beckman Coulter Centrifuge, USA) at a temperature of 4°C. Supernatant was diluted appropriately and analyzed for the un-entrapped NFV on a C18 column (5 μm, 250 mm × 4.6 mm Hibar®), using 25 mM mono-basic sodium phosphate buffer (pH 3.4): Acetonitrile 50:50 *v/v* as the mobile phase, at 1 ml/min flow rate (Single pump Jasco- PU 2080 Plus) with the detection at 254 nm (UV detector Jasco UV-2075 Plus). Amount of NFV entrapped in the system was calculated using equation:

$$\%EE = (\text{Total drug added} - \text{Drug in supernatant}/\text{Total drug added}) \times 100.... \quad (1)$$

Entrapment efficiency of liposomes and LeciPlex ® was determined by dialysis method. Apparatus consists of a modified paddle dissolution testing apparatus (Electrolab Dissolution Tester USP) using mini jars (100 ml) and dialysis membrane as a barrier (Dialysis Membrane-70 LA 393, MWCO 12–14 KD, Himedia Mumbai, India). Dialysis bags were soaked in 25 mM mono-basic sodium phosphate buffer pH 3.4 for a period of 12 h prior to the study.

Formulation was filled in the dialysis bag; bag was sealed and tied to paddle shaft using thread. Paddle was lowered into dialysis media maintained at 30 ± 0.5°C and rotated at a speed of 50 rpm. Aliquots were withdrawn at predetermined time intervals and media was replaced with same volume of buffer solution. Dialysis was continued till constant drug concentration was obtained in subsequent aliquots. Aliquots were centrifuged (Eppendorf, Minispin centrifuge) at 10,000 rpm for 10 min. The aliquots were analyzed using reverse phase HPLC. % EE was calculated using formula:

$$\%EE = [(\text{Total Drug} - \text{Diffused Drug})/\text{Total Drug} \times 100]$$

% EE of the cochleates was determined by dissolving a precise amount of formulation (10 mg) in chloroform:methanol (1:1) and 0.1 M EDTA solution (100 μL). The cochleate

suspension was centrifuged (20 min, 4000 rpm, 4°C) to separate cochleates. Drug content in resulting solution was determined by HPLC using formula 1.

Molecular Dynamic Simulations

Two sets of atomistic MD simulations of fully hydrated lipid bilayer of 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) were performed using the GROMACS 5.1.2 package (14), one with Nelfinavir and one without Nelfinavir. The phospholipid bilayers were prepared and hydrated by with CHARMM-GUI (15,16) membrane builder. We used the CHARMM36 (17) force field for lipids and water molecules described with the TIP3P model (18). VMD (19) program was used to visualize the simulation trajectories.

Simulation Parameters. The simulation was run for 500 ns and frames were recorded every 10 ps. A cut off of 1.2 nm was applied to short-range electrostatic interactions. Long-range electrostatics was calculated with the use of the particle mesh Ewald method (20); Van der Waals interactions were truncated at 1.2 nm. All bonds were constrained by the LINCS algorithm (21). Throughout simulation, the temperature of the simulation system was maintained at 310 K with Noose Hoover thermostat (22,23) and pressure was maintained with Parrinello-Rahman barostat (24) with semi-isotropic pressure. The time constants of the thermostat and barostat are 1 ps and 5 ps.

Paramterization of Nelfinavir. The model for Nelfinavir molecule was parameterized with CHARMM compatible force field using CGenFF program at the paramchem server (https://cgenff.paramchem.org/). The obtained compatible parameters were translated to the GROMACS format using the provided cgenff_charmm2gmx.py script. The topology files are available from author upon request.

Morphology of Particulate Systems

Morphology of T-SLN and liposome was studied by cryo-TEM (Transmission Electron Microscopy). A drop of formulation (2 μl) was placed on a carbon-coated copper grid (Quantifoil Micro Tools GmbH, Germany). The sample was subjected to plunge freezing in liquid ethane at − 180°C. The grids were then transferred into a liquid nitrogen cooled ($T = -196°C) \pm 70°$ tilt cryo-holder (Gatan Inc., USA) and inserted into the cryoelectron microscope Philips CM 120 cryo-TEM (Philips, Netherlands). Images were captured using a TEM operating at 120 kV.

For NFV cochleates dispersion, TEM studies were performed. Cochleate dispersion was placed on Formvar®-coated copper grid (300 mesh, Plano, Germany). Grids were investigated using a ZeissEM 902A (Carl Zeiss, Germany) transmission electron microscope (TEM) operated at 80 kV. Images were acquired with a 1 k × 1 k FastScan-F114 CCD Camera (TVIPS GmbH, Germany).

In vitro Lipolysis (9)

In vitro lipolysis was carried out for SLNs (T-SLN and C-SLN), LeciPlex® (CTAB-Plex and DDAB-Plex) liposome, and cochleates. The formulations were compared with NFV dispersion which was prepared using HPMC (hydroxy propyl methyl cellulose) as suspending agent. *In vitro* lipolysis medium was composed of sodium chloride (150 mM), calcium chloride (5 mM), Tris maleate (10 mM), Bile salts (5 Mm), and lecithin (1.25 mM) dispersed in required quantity of water. Formulation was added to above media and pH was adjusted to 6.5 with 0.1 N NaOH. Pancreatic lipase enzyme (200 tributyrin units per ml of digestion media) dispersed in water was added to media and stirred for 1 h on magnetic stirrer. pH of media was kept constant throughout the reaction by addition of NaOH. Supernatants were separated and analyzed using reverse phase HPLC (Jasco, PU-2080 Plus) with UV detector set at 254 nm.

In vitro Release

In vitro release studies were carried out for all nanosystems using dialysis sac method. The release profiles of the formulations were compared to the NFV solution in Transcutol HP® and NFV dispersion in buffer (pH 6.8). The release medium used was phosphate buffer (pH 3.4). Formulation was filled in presoaked dialysis bags and bags were sealed from both ends using cotton thread. Dialysis bags were then tied to paddle using thread, paddles were lowered into release media maintained at $37 \pm 0.5°C$, and rotation at a speed of 75 rpm was initiated. Aliquots were withdrawn at predetermined time intervals and media was replaced with same volume of buffer solution. Aliquots were then centrifuged (Minispin centrifuge) at 10,000 for 10 min. Aliquots were analyzed using reverse phase HPLC (Jasco, PU-2080 Plus) with UV detector set at 254 nm.

In vitro release experiments for cochleates were performed by evaluating release in acceptor liposomes. Acceptor liposomes were prepared by thin film hydration method using Lipoid S100 and 1,2-dioleoyl-sn-glycero-3-[phospho-rac-(3-lysyl(1-glycerol))] (DOPG) in molar ratio of 20:1 and Trizma buffer saline (pH 7.4; 10 mM) for hydration. The liposome suspension was extruded through a polycarbonate membrane filter with a pore diameter of 100 nm. Particle size analysis and zeta potential measurements of the vesicles were determined by photon correlation spectroscopy (PCS) using a Zetasizer Nano ZS (Malvern, UK). The transfer experiments were carried out at $37 \pm 2°C$. For the investigation of the Nelfinavir transfer, the ratio between donor and acceptor lipid was maintained constant at 1:20. The concentration of drug was assayed on a Shimadzu LC-10 (Shimadzu GmbH, Germany) HPLC system equipped with Shimadzu SPD-10A UV detector and a Phenomenex Synergi® Hydro-RP C-18, 250 × 4.6 mm, 4 μm analytical column. The mobile phase consisted of 25 mM mono-basic sodium phosphate buffer (adjusted to pH of 3.4 with phosphoric acid) and acetonitrile (58:42, *v*/*v*). Drug was detected at wavelength of 220 nm.

In vitro Permeability

Cell Lines and Cell Culture Conditions. Caco-2 (passage 65–67) and HT29-MTX (passage 35–40) cells were grown separately in tissue culture flasks (SPL Life Sciences Co., Ltd., Korea), in DMEM basal medium supplemented with 10% (*v*/*v*) of inactivated FBS, 1% (*v*/*v*) of NEAA, and 1% of

antibiotic/antimitotic mixture (final concentration of 100 U/mL Penicillin and 100 U/mL of Streptomycin). Cells were sub-cultured once a week with 0.25% trypsin-EDTA (1×) and seeded at a density of 5×10^5 cells per 75 cm^2 flask. The culture medium was replaced every other day. Cells were maintained in an incubator (CellCulture® CO2 incubator, ESCO GB Ltd., UK) at 37°C and 5% CO_2 in water saturated atmosphere. The transepithelial electrical resistance (TEER) of mono- and co-culture mono-layers was measured every time that medium was replaced to monitor the evolution of confluence and their integrity before the permeability studies, using an EVOM epithelial voltohmmeter equipped with chopstick electrodes (World Precision Instruments, Sarasota, FL, USA). All the cell culture-related procedures were done in a Labculture® class II, type A2 biological safety cabinet, from ESCO.

Permeability Experiments. For the permeability experiments, 0.1×10^6 cells/cm^2 of Caco-2 and Caco-2:HT29-MTX cells in a ratio of 90:10 were seeded in 12-Transwell® cell culture inserts and were allowed to grow and differentiate for 14 days with medium replacement every other day (25–27). The permeability experiments across the cell mono-layers were performed in the apical-to-basolateral direction in HBSS at 37°C using an orbital shaker (100 rpm). At the start of experiment, the cell culture medium was removed from both chambers, replaced by HBSS and allowed to equilibrate for 30 min at 37°C. After that, 0.5 mL of pure nelfinavir mesylate or DDAB-LeciPlex, SLN, and nanosuspensions in the concentration of 200 μM were pipetted into the apical side of the inserts. At different time points (15, 30, 45, 60, 90, 120, and 180 min), 0.2 mL samples were taken from the basolateral side of the inserts and the same volume of fresh prewarmed HBSS buffer was added to replace the withdrawn volume. Sample concentration was quantified by HPLC-UV method. The integrity of the cell mono-layers was checked before and after the permeability experiment by measuring the transepithelial electric resistance (TEER). Mono-layers with TEER value higher than 200 Ω cm^2 were used for the characterization experiments.

In vivo Pharmacokinetic Studies

The experimental protocol was approved by Institutional Animal Ethics Committee of Bombay College of Pharmacy (protocol no: CPCSEA-BCP/2013-01/02). The national guidelines for the care and use of laboratory animals were also followed during preparation of protocol. Female Sprague Dwaley rats weighing 250–350 g were divided into four groups of six rats each. Group 1 received NFV dispersion in phosphate buffer (pH 6.8), group 2 received T-SLN, group 3 received Leciplex (DDAB-LeciPlex), and group 4 received cochleates (DOPS cochleate).

Animals were kept in animal house for 1 week (stabilization period) prior to study. The animals were fasted overnight prior to the experiment but had free access to water. The formulations as well as the NFV dispersion were administered orally (dose 100 mg/kg) and blood was withdrawn from the retro-orbital plexus at intervals of 1, 2, 4, 6, and 8 h after dose administration. The heparinized blood samples were immediately centrifuged at 5000 rpm for 15 min at 4°C, and separated plasma was stored at −20°C until analysis.

All plasma samples were thawed at room temperature. To each 50 μl plasma sample, 250 μl of acetonitrile was added and the solution was mixed for 10 min for protein precipitation. To the same solution, 100 μL of internal standard (IS) ritonavir solution (15 μg/mL) was added and the samples were centrifuged at 10,000 rpm for 10 min (Minispin centrifuge). After centrifugation, 20 μl of supernatant solution was injected into the HPLC system. HPLC apparatus consisted of Jasco PU-2080 146 Plus Intelligent HPLC pump (Jasco, Japan) equipped with a Jasco UV-2075 Intelligent UV-visible detector (Jasco, Japan), a Rheodyne 7725 injector (Rheodyne, USA), and a Jasco Borwin Chromatography Software (version 1.50). The HPLC method employed C18 reverse phase column with dimensions of 250×4.6 mm, particle size 5 μm, 50:50 acetonitrile:phosphate buffer (pH 6.5) as mobile phase, and 1 mL/min flow rate.

Freeze Drying of Nanosystems

The nanosystems freeze dried were SLNs (T-SLN and C-SLN), nanosuspensions (NS G-1), LeciPlex systems (CTAB-LeciPlex and DDAB-LeciPlex), and liposome. Freeze drying was carried out in the vacuum freeze dryer (Free zone Triad, Labconco, U.S.A.) using trehalose as the cryoprotectant. The cycle was carried out at a pressure of 0.08 mBar and consisted of the following steps:

i. Prefreezing: 3 h at −56°C; cooling rate: 1°C/min
ii. Primary drying: 12 h at −40°C; ramping rate: 1°C/min
iii. Secondary drying: 3 h at 40°C; ramping rate: 0.3°C/min

Freeze-dried nanosystems were evaluated for their particle size, % entrapment efficiency, drug content, and the XRD patterns were recorded.

XRD of Nanosystems

The XRD patterns of the nanosystems were recorded after freeze drying to determine the crystalline state of NFV in the formulation (graphs not shown). The diffraction patterns were recorded on Xpert Pro MPD (Pananaytical, The Netherlands) instrument with a copper anode, voltage 40 kV, current 30 mA, at a 1.5405 A°. The detector used was Xcelerator Detector with diffracted beam mono-chromator.

RESULTS

Fabrication of NFV-Loaded Particulates: Feasibility Evaluation

Based on the aforementioned criteria, Precirol ATO 5® (4% *w/v*) was selected as the lipid matrix and Tween® 80 (2% *w/v*) and CTAB (2% *w/v*) were selected as surfactants for the preparation of SLNs.

Based on previous experience, it was observed that Gelucire 50/13® could solubilize substantial quantities of NFV in dispersion medium. Hence, fabricating nanosuspensions

Table I. Particle Size and Polydispersity Index of Nanosystems

Formulation no.	Particle size (nm)	Polydispersity index (P.I.)
T-SLN	110.0 ± 3.48	0.177 ± 0.003
C-SLN	311.6 ± 1.17	0.667 ± 0.004
NS G-1	472.5 ± 3.29	0.352 ± 0.017
NS G-2	30 ± 1.29	0.675 ± 0.054
CTAB-LeciPlex®	391.9 ± 2.47	0.45 ± 0.02
DDAB-LeciPlex®	346.2 ± 0.35	0.23 ± 0.02
Liposome	95.80 ± 0.12	0.55 ± 0.01

Table II. Zeta Potential of Nanosystems

Formulation	Zeta Potential
T-SLN	+ 7.48 ± 0.25 mV
C-SLN	+ 59.1 ± 0.12 mV
NS G-1	− 0.489 ± 1.25 mV
NS G-2	− 2.29 ± 0.30 mV
CTAB-LeciPlex®	+ 47.8 ± 0.02 mV
DDAB-LeciPlex®	+ 57 ± 0.15 mV
Liposome	+ 35.6 ± 0.6 mV

using Gelucire 50/13® as a hydrophilic stabilizer was considered to be a suitable approach for delivery of NFV. The nanosize of the system and absence of lipid matrix in the nanosuspension would facilitate better oral absorption of NFV.

Phospholipid-based vesicular systems, namely, LeciPlex® and liposomes, were also evaluated for their ability to encapsulate NFV. LeciPlex® system was fabricated by single-step technique using lecithin (Phospholipon 90G) and cationic agents DDAB and CTAB. DDAB-LeciPlex and CTAB-LeciPlex were successfully formulated with 1:1 and 1:5 ratio of cationic agent (DDAB for DDAB-LeciPlex and CTAB for CTAB-LeciPlex): Phospholipon® 90G respectively. Lecithin concentration for CTAB-LeciPlex and DDAB-LeciPlex was 31 mg and 18.6 mg per 1 mg of NFV respectively.

Lecithin-based liposomal system was fabricated using biocompatible solvent Transcutol® and without incorporation of any cationic stabilizer. A simple technique of two steps was used, i.e., formation of vesicles and size reduction by probe sonication. Phospholipon® 90 was screened at three different concentrations 15, 30, and 50 mg per 1 mg of NFV. As no significant improvement in NFV encapsulation was noted by increasing lipid concentration from 30 to 50 mg per 1 mg NFV, lipid load of 30 mg per 1 mg of NFV was used for further evaluation. Cochleates were prepared from by simple trapping method using calcium as cations.

Characterization of Particulate Systems

Particle Size

Particle size distribution was evaluated by Photon Correlation Spectroscopy (PCS) which works on the principle of interaction of light with the particles at an observation angle of 90°. The particle size and polydispersity index of all the formulations are shown in Table I. Mean particle size of DOPS Nelfinavir cochleates was found to be 2180 ± 112.7 nm. All particles were within particle range of 1634 to 3764 nm. The histogram for particle size analysis is shown in Fig. 1.

Zeta Potential

The zeta potential values of the NFV-loaded nanosystems are shown in Table II. T-SLN contains Tween80®, a non-ionic surfactant as the stabilizer, and hence exhibits slightly negative zeta potential. C-SLN contains a cationic surfactant, CTAB which explains the significant positive zeta potential value. NS G-1 and NS G-2 are nanosuspensions containing non-ionic Gelucire®50/13 yielding negligible zeta values.

LeciPlex® systems exhibited significant net positive charge on the membrane justified by the presence of cationic agents such as CTAB and DDAB in the system. Liposomal

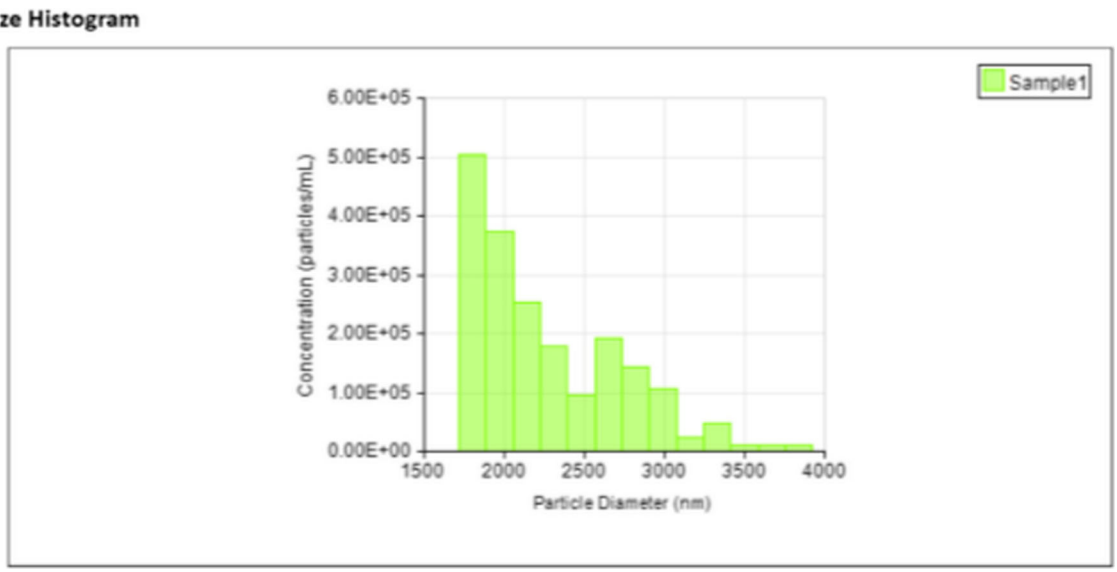

Fig. 1. Particle size histogram for DOPS cochleate

Table III. % EE of Particulates

Formulation	% EE
T-SLN	75 ± 2.84
C-SLN	71 ± 4.46
CTAB-LeciPlex®	91.50 ± 2.10
DDAB-LeciPlex®	77.00 ± 7.00
Liposome	97.30 ± 0.28
DMPG cochleate	70.36 ± 6.37
DOPS cochleate	76.61 ± 7.41
DOctPS cochleate	70.75 ± 4.17
DDPS cochleate	74.72 ± 7.68

system also showed positive zeta potential even though no cationic agent was added during formulation of liposomes.

Entrapment Efficiency (% EE)

Entrapment efficiency of SLNs was determined by ultracentrifugation. It is based on the principle that when nanosystems are subjected to high centrifugal force, nanosystems along with the drug entrapped settle to form pellet whereas the un-entrapped drug remains solubilized in the supernatant. Thus, by analyzing drug concentration in the supernatant indirectly indicates the concentration of entrapped drug. Both the SLNs exhibited entrapment efficiency of 70–75%. Cochleates also exhibited entrapment efficiency between 70 and 76%.

Entrapment efficiency for vesicular systems (LeciPlex® and liposomes) was evaluated by dialysis method. In the dialysis method, un-entrapped drug is anticipated to diffuse out into a media whereas encapsulated drug remains entrapped inside the vesicles until it is released from the system. Table III shows % EE of the nanosystems.

Molecular Dynamic Simulations

The molecular dynamics results indicated interaction of Nelfinavir molecules with DSPC membrane bilayer and partitioning at the membrane-water interface. As shown in Fig. 2, the Nelfinavir molecule is partially embedded in the membrane. This results in the change in the effective charge across membrane bilayer as shown in Fig. 3. MD results indicated decrease in charge density at interface of the membrane bilayer in Nelfinavir-loaded systems; however, inside the membrane bilayer, the charge density becomes positive.

Morphology of Particulates

Cryo-TEM was carried out for T-SLN and liposome 300 to confirm the morphology and particle size of the nanosystems. In Fig. 4a, it is clear that SLNs are formed. The black arrows indicate that T-SLN consists mainly of large lipid plates with smooth surfaces and no sharp edges, and smaller disc micelles also were contained. In T-SLN lipid stacks are formed indicated by the blue arrows. Cryo-TEM image (Fig. 4b) of liposome revealed that simple method using Transcutol® as solvent resulted in the formation of unilamellar vesicles.

The TEM of nelfinavir mesylate cochleates prepared from DOPS is as shown in Fig. 4c. TEM images of cochleates prepared from DDPS, DMPG, and DOctPS are shown in Fig. 5a–c respectively (shown in supplementary materials, Supplemental 1). In TEM analysis, cochleates of DOPS, DoctPS, and DDPS appeared like elongated cylinders. Occasional presence of central channel was also observed amongst these particles. However, the DMPG sample mainly showed presence of curved, long, and multilamellar lipid sheets with absence of cylindrical structures. Figure 6a–d shows the TEM images of higher population of cochleates prepared from DDPS, DMPG, DOctPS, and DOPS respectively (28).

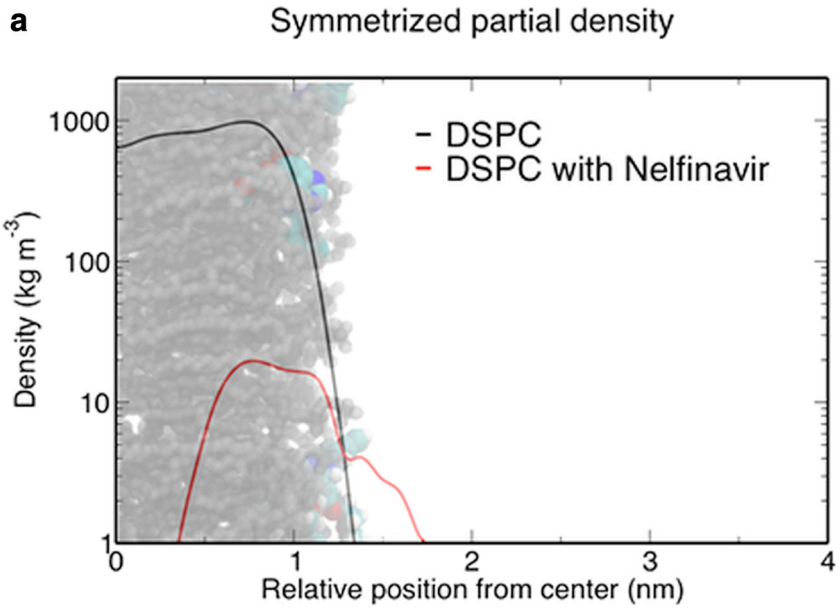

Fig. 2. Averaged mass density profile from 500 ns simulation of Nelfinavir with DSPC membrane bilayer. Mass density profile shows that Nelfinavir partitions at the membrane-water interface

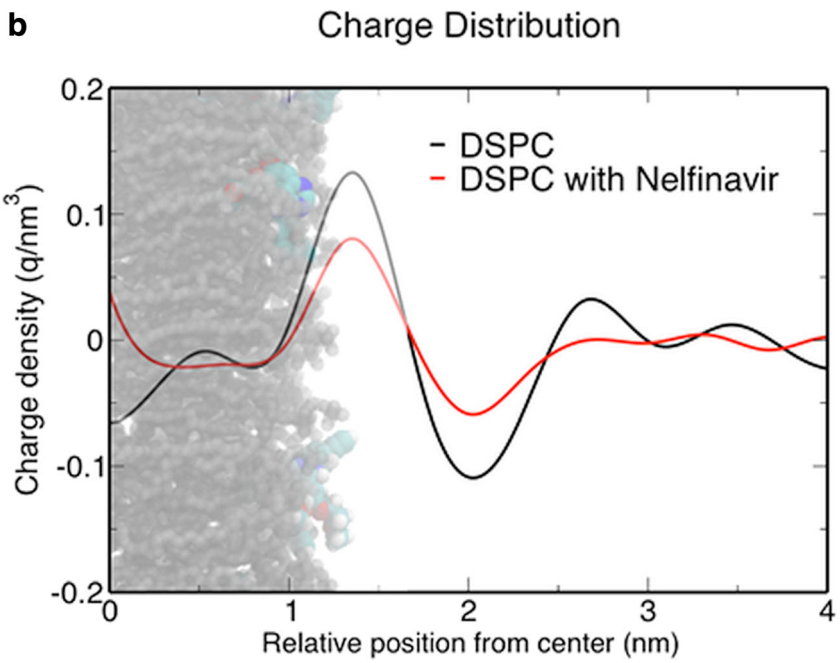

Fig. 3. Averaged charge density profile across membrane, showing that in presence of Nelfinavir, due to its partitioning in membrane bilayer, membrane potential within membrane is positive and at the membrane-water interface, is lower than pure DSPC membrane bilayer

In vitro Lipolysis

Due to the complexity of the events taking place after ingestion of lipid-based systems, *in vitro* lipolysis models play an important role in elucidating drug release from lipid-based systems (29–31). The *in vitro* lipolysis study reported by Ankitkumar Jain *et al.* (9) demonstrated 3-fold and 2-fold enhancement in solubilization of poorly water soluble drug quercetin encapsulated in SLN and NLC respectively as compared to quercetin suspension.

The results of *in vitro* lipolysis studies of NFV-loaded formulations are shown in Figs. 7 and 8. The nanosystems showed higher solubilization of NFV after 1 h as compared to the NFV dispersion. C-SLN showed the highest solubilization of NFV (62%) after 1 h and CTAB-LeciPlex exhibited the least solubilization of NFV (2.26%). Cumulative lipolytic percentages of drug released from all cochleate formulations was < 5%.

In vitro Release

In vitro release was carried out for NFV-loaded nanosystems and the release profile was compared to NFV dispersion prepared in phosphate buffer pH 6.8 and the NFV solution made in Transcutol HP®. Since NFV exhibits water solubility which is highly dependent on pH and reduces drastically above pH 4, the media chosen for this study was phosphate buffer pH 3.4 in which NFV exhibits good solubility. The release profiles of the systems are shown in Fig. 9. From the graph, it is evident that the NFV-loaded nanosystems show sustained release as compared to the NFV solution. The order of release at the end of 12 h is as follows:

DDAB−LeciPlex > NS G−1 > NFV solution > T−SLN > CTAB−LeciPlex > C−SLN > NS G−2 > liposome > NFV dispersion.

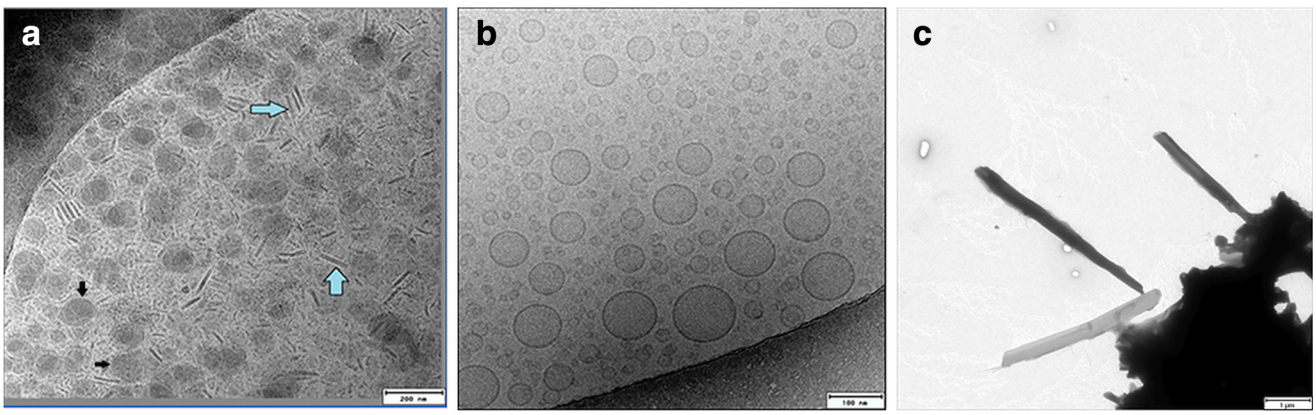

Fig. 4. Morphology of particulates. **a** Cryo-TEM of T-SLN. **b** Cryo-TEM of liposome. **c** Transmission electron microscopy image of DOPS cochleates

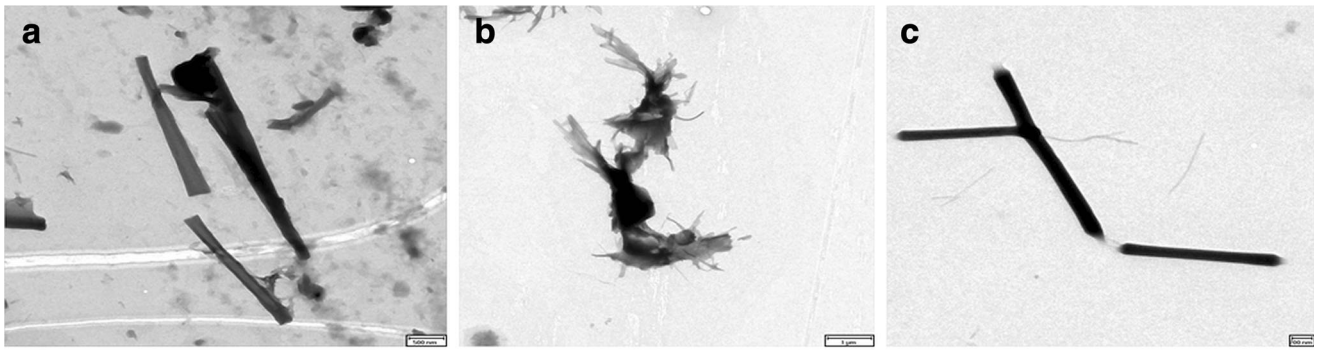

Fig. 5. Transmission electron microscopy images of cochleates. **a** Transmission electron microscopy image of DDPS cochleates. **b** Transmission electron microscopy image of DMPG cochleates. **c** Transmission electron microscopy image of DOctPS cochleates

The influence of the degree of saturation or length of the fatty acid chains in phospholipid used for cochleate formation was evaluated during the release experiments. The results of cochleate formulation and Nelfinavir for transfer experiments at 37°C are as depicted in Fig. 10. It is evident that there is no significant difference between the release patterns of cochleate formulation at physiological temperature. However, the maximum amount released at the end of 9 h varied slightly and was 76%, 91%, 83%, and 89% for DDPS, DMPG, DOPS, and DOctPS cochleates respectively. By the end of 4 h, plain drug reached a plateau and showed 100% release.

In vitro Permeability Studies

After 21 days of the cellular culture, the permeability of the drug, in a free form and when loaded into the nanoparticles, was assessed. The assays were performed in two distinct intestinal models: the Caco-2 mono-culture and in the co-culture of Caco-2 and HT29-MTX cells.

Analyzing the Caco-2:HT29-MTX co-culture permeation results as seen in Fig. 11, there is no permeation for the drug in DDAB-LeciPlex as seen for the mono-culture. For the two other formulations and the free form, it is possible to see that the permeability of the drug was up to 15% of the initial

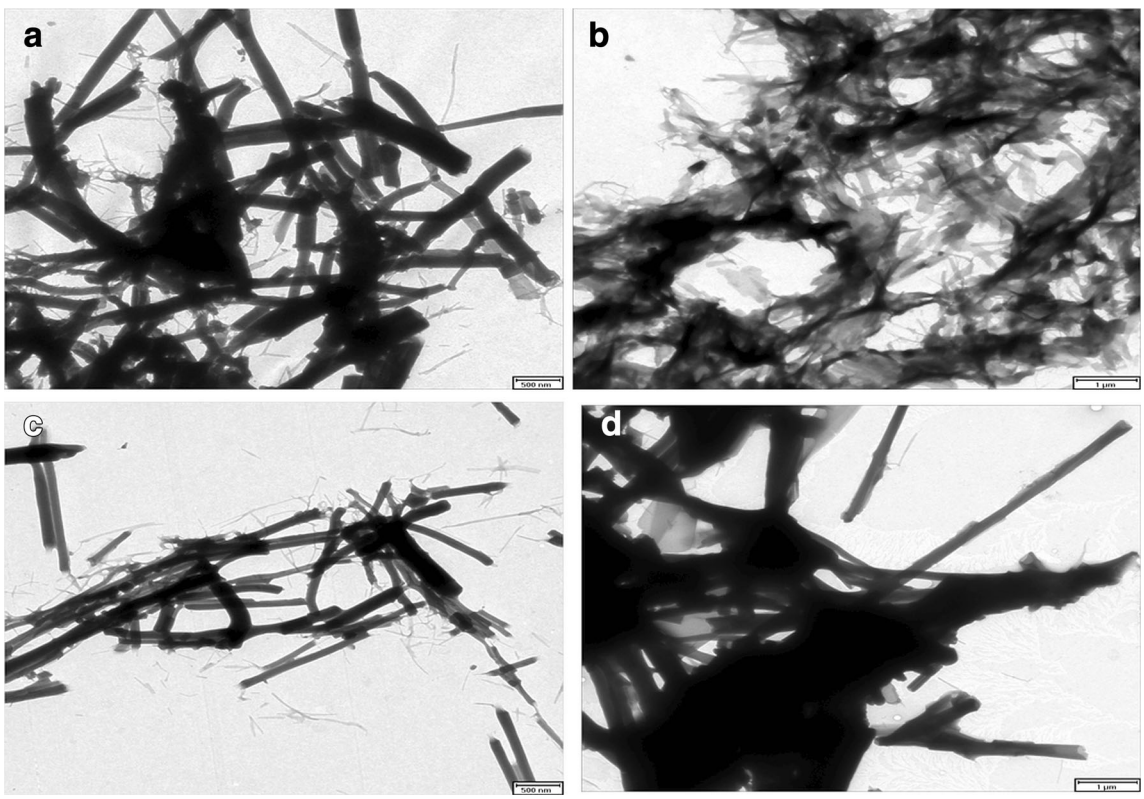

Fig. 6. Transmission electron microscopy images showing higher population of cochleates. **a** TEM image of DDPS cochleates. **b** TEM image of DMPG cochleates. **c** TEM image of DOctPS cochleates. **d** TEM image of DOPS cochleates

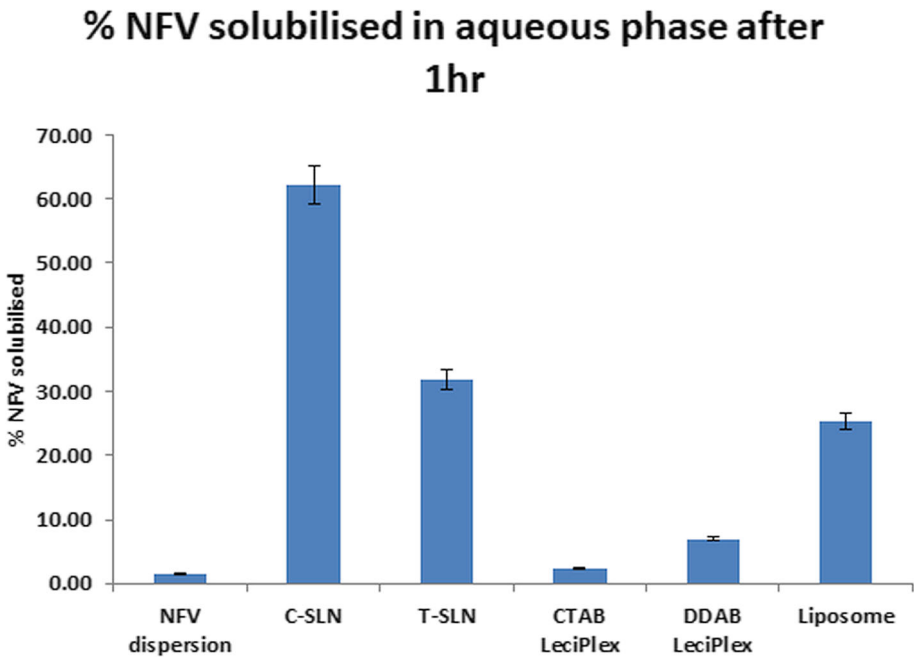

Fig. 7. *In vitro* lipolysis profile of nanosystems

amount; however, no difference was observed in the permeability of the free form drug or when encapsulated into the nanoparticles. For both of the models, the TEER values did not change more than 20% between the initial and the final values.

In vivo Pharmacokinetic Studies

Pharmacokinetic studies were undertaken with a view to establish a correlation between the *in vitro* behavior and *in vivo* results. Based on results of the above *in vitro* tests, the formulations chosen for the *in vivo* study were T-SLN, DDAB-LeciPlex, DOPS cochleate, and NFV dispersion to investigate the following pharmacokinetic parameters: *C*max (maximum plasma concentration), *t*max (time to maximum plasma concentration), and AUC_{0-10} (area under the curve between 0 and 10 h) at a dose of 100 mg/kg. Table IV shows the pharmacokinetic parameters of the particulate systems and NFV dispersion. The oral bioavailability of NFV as indicated by the AUC values when incorporated in particulates like T-SLN (15.25 μg.hr/ml), DDAB-LeciPlex (9.33 μg.hr/ml), and DOPS cochleate (10.24 μg.hr/ml) exhibited a 5.5-fold, 3.3-fold, and 3.8-fold increase respectively when compared to the NFV dispersion (2.75 μg.hr/ml). Figure 12 shows the pharmacokinetic profile of the particulates and the NFV dispersion.

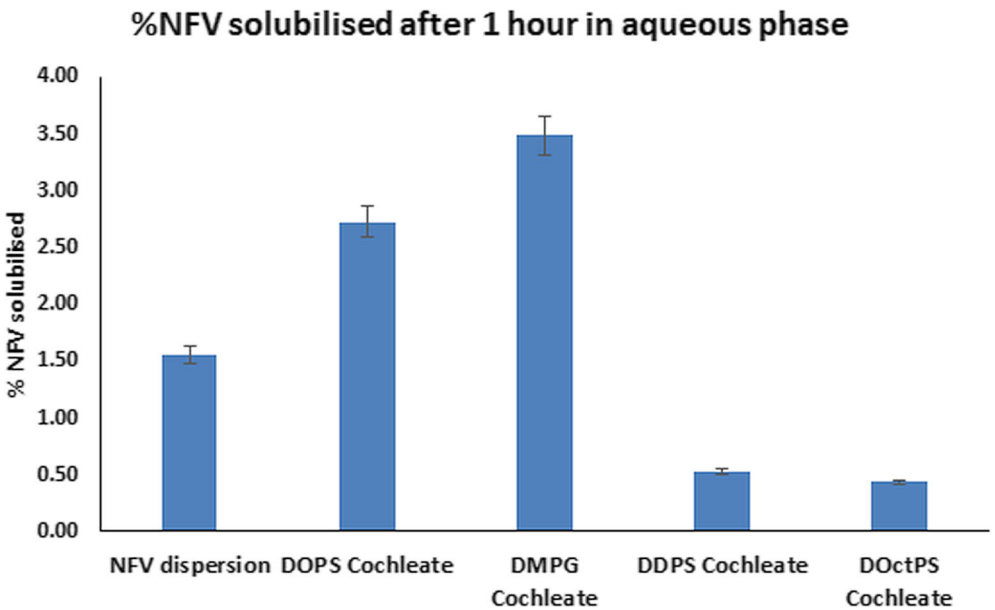

Fig. 8. *In vitro* lipolysis profile of cochleates

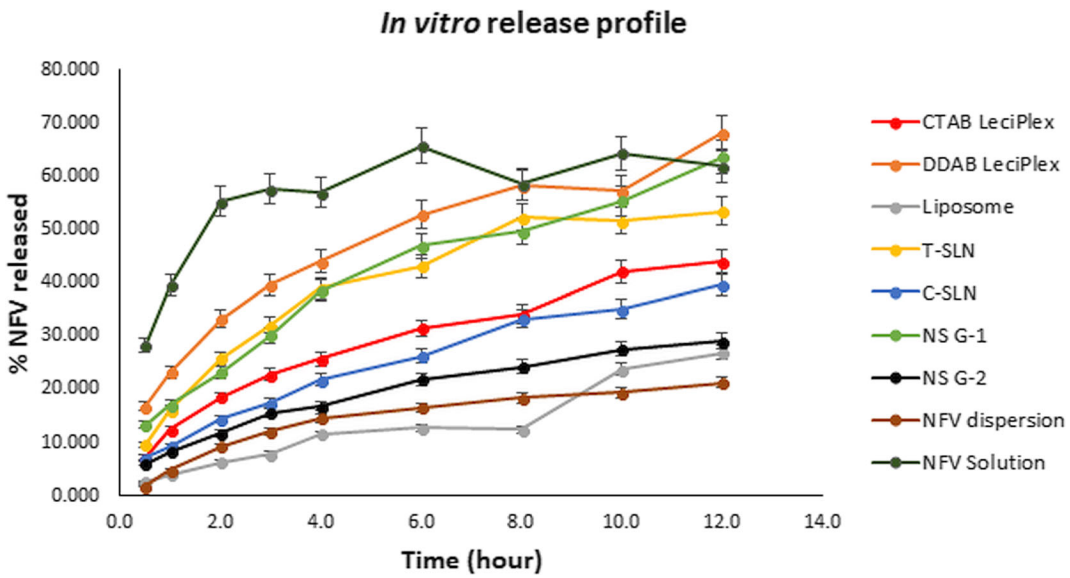

Fig. 9. *In vitro* release profiles of nanosystems

Freeze Drying of Nanosystems

Nanosystems have limited physical and chemical stability. To prolong the shelf life of these products, water from these systems must be removed. Freeze-drying, also known as lyophilization, is an industrial process which removes water from a frozen sample by sublimation and desorption under vacuum. The process generates various stresses during freezing and drying steps. Protectants are usually added to the formulation to protect the nanosystems from freezing and desiccation stresses (32). Cryoprotectants commonly used are trehalose, mannitol, albumin, sucrose, and other polysaccharides or sugars.

In the present study, freeze drying of nanosystems was carried out with the aim of improving the physical stability of the formulations. The selection of cryoprotectant was based on the physical appearance of the cake, ease of reconstitution, and particle size distribution after reconstitution. The nanosystems which were freeze dried were SLNs (T-SLN and C-SLN), nanosuspensions (NS G-1), LeciPlex systems (CTAB-LeciPlex and DDAB-LeciPlex), and liposome. Trehalose at different concentrations for different nanosystems was chosen as the cryoprotectant. Table V gives the percent of trehalose used and the particle size of nanosystems after preparation and when freeze-dried product was reconstituted, i.e., converted in dispersion again by mixing with purified water.

DISCUSSION

In the present investigation, the advantage of incorporating NFV into lipid-based particulate systems for improved oral bioavailability was evaluated. It is reported that con-

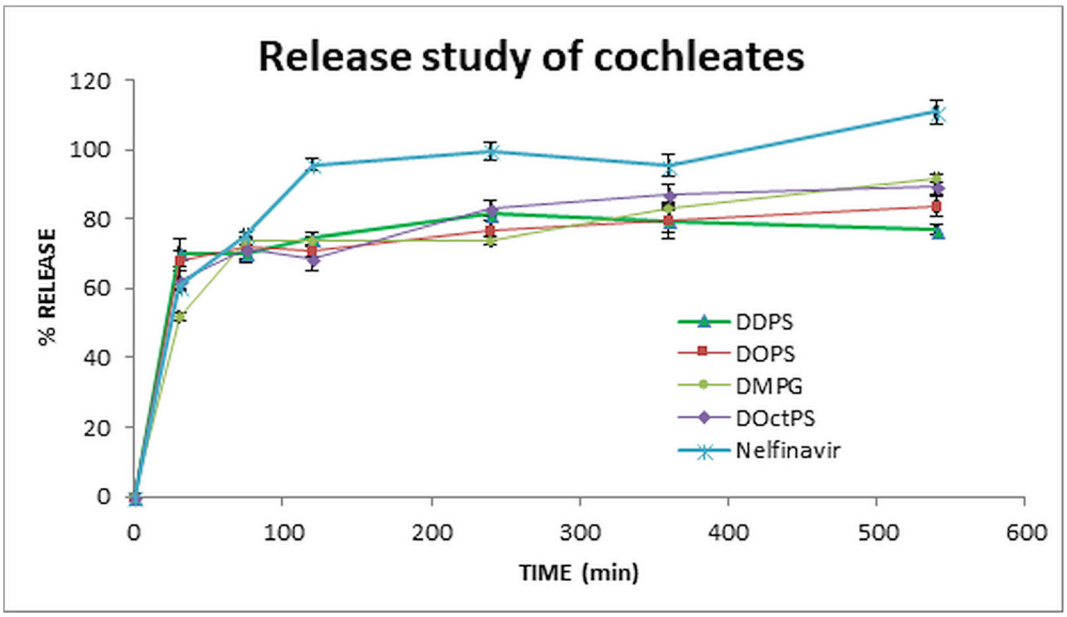

Fig. 10. *In vitro* release profiles of cochleates

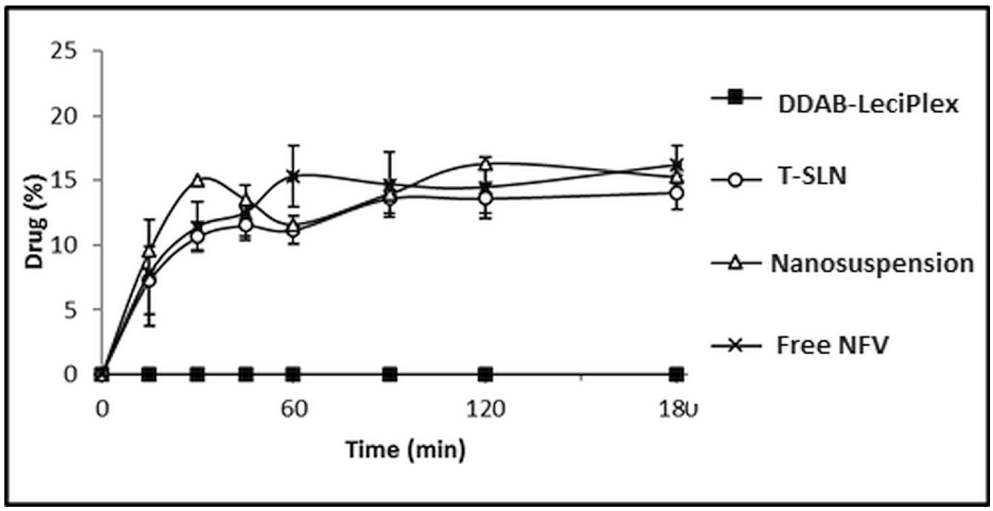

Fig. 11. Permeability in Caco-2:HT29-MTX co-culture

sumption of 50% of fat in the diet increases the bioavailability of NFV (5). Previously, a 2- to 2.5-fold improvement in bioavailability of nelfinavir mesylate has been demonstrated upon incorporation in solid self-nanoemulsifying drug delivery system (S-SNEDDS) (5). As the absorption of the drug can be improved by use of fatty food, it was planned to have a comparative assessment of lipid-based drug delivery systems (both vesicular and particulates) to correlate the performance of the systems in *in vitro* conditions and mechanism of cell uptake and link it with *in vivo* exposures. Solid lipid nanoparticles, nanosuspensions, liposomes, LeciPlex®, and cochleates were selected for delivery of NFV.

Molecular dynamics simulations were undertaken with an aim to probe the behavior of the drug with the phospholipid bilayer. The zeta potential results suggested complete reversal of surface charge in presence of the drug in liposomes as compared to blank liposomes. Since nelfinavir itself is a weak base and its mesylate salt is used in liposome fabrication, there could be possibility of interaction of phospholipid head groups with Nelfinavir. The molecular dynamics results indicated that Nelfinavir molecules interact with DSPC membrane bilayer and partitions at the membrane-water interface. Nelfinavir molecule is partially embedded in the membrane resulting in a decrease charge density at the surface due to DSPC-nelfinavir interactions while, inside the membrane bilayer, the charge density becomes significantly positive.

Blank liposomes of phosphatidylcholine display a negative charge on surface due to orientation of phosphate groups at the exterior (33). In our systems, Nelfinavir might be possibly interacting with the phosphate head groups due to charge interactions, thereby reducing the charge density. Since Nelfinavir is embedded inside the membrane, the interaction might also possibly change the orientation of phospholipid headgroups to now orient the choline on the outside of the membrane, thereby rendering the surface charge positive. Furthermore, a reduction in particle size was observed upon NFV incorporation in nanosystems which can be attributed to NFV interacting favorably with lipid structures and stabilizing the nanosystems. Nanosystems inclusive of SLNs, nanosuspension, and vesicular systems exhibited the mean particle size in the nanometric range with the mean size of NS G-2 as small as 30 nm. Whereas, particle size for DOPS cochleates was found to be in the range of 1.6–3.7 μm. Higher encapsulation of NFV in liposomes could be due to interaction with phospholipids and flexibility of vesicular structure as compared to rigid nature of lipids in SLN and rigid structure of cochleates.

In vitro lipolysis study indicated that lipid nanoparticles possessed a moderate or less resistance to digestive enzymes as compared to vesicular systems. In case of LeciPlex® systems, strong charge interaction between cationic agent and lecithin might have resulted in the higher membrane integrity, preventing insertion of bile salts into the membrane which is considered to be essential for phospholipid membrane digestion. This explains why the lower NFV solubilization was observed from LeciPlex® systems. Although the drug is released from cochleates under the action of enzymes and the release medium can be simultaneously assembled into liposomes or micelles with the phospholipids or bile salts, there is still a considerable amount of drug failing to be released. This in turn can prevent the possibility of drug precipitation upon rapid lipolysis and provide the drug better protection in gastric environment. A delayed and reduced hydrolysis of lipid nanoparticles may lead to favorable conditions for uptake through transepithelial transport via intact drug delivery system or by slow release into self-assembly like micelles or liposomes. However, in comparative *in vitro* permeation study, surprisingly, no permeation was observed for nanosystems as well as for free NFV in case of Caco-2 mono-culture model. Failure to achieve permeation through this model may be due to the thinness of the cellular junctions being higher than *in vivo*, which can lead to the

Table IV. Pharmacokinetic Parameters

Formulation	C_{max} (μg/ml)	t_{max}	AUC (μg.hr/ml)
T-SLN	2.82	1 h	15.25
DDAB-LeciPlex®	1.31	1 h	9.33
DOPS cochleate	2.81	2 h	10.24
NFV dispersion	5.51	1 h	2.75

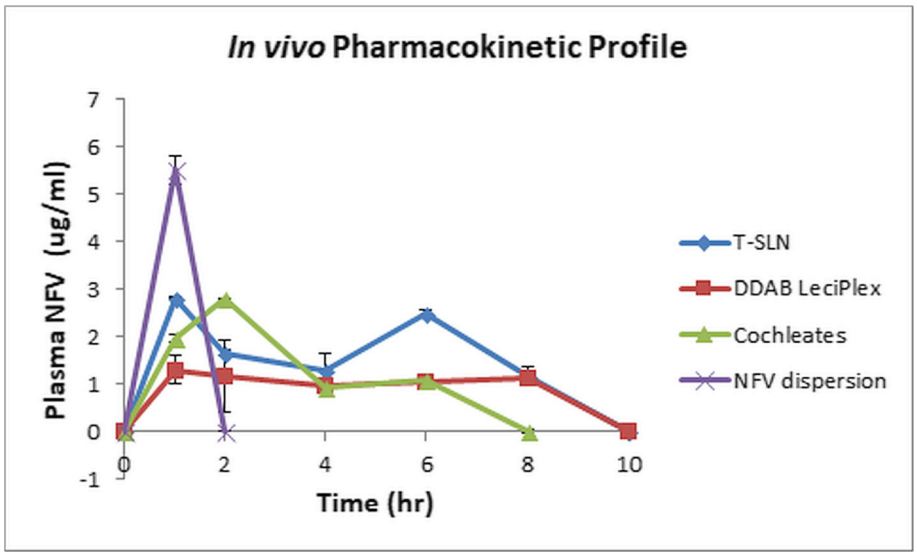

Fig. 12. Plasma concentration time profiles of particulates after single oral administration of formulations at a dose of 100 mg/kg nelfinavir mesylate in rats

permeability not being accurately calculated. Moreover, low permeability is expected due to the solubility issues of the drug as well as lower drug release in *in vitro* conditions as witnessed in lipolysis model. Having this in consideration, the permeability was also calculated using a more complete and complex model, constituted not just by the Caco-2 cells (enterocytes) but also by the HT29-MTX and Raji B cells (26,27). The HT29-MTX cells mimic the mucus producers' goblet cells, an important parameter to consider since mucus is a barrier to reach the intestinal epithelium and it has a very fast turnover. This model presents also less tight junctions between the cells facilitating the permeation. Free NFV, SLN, and nanosuspension achieved around 15–20% permeation through Caco-2:HT29-MTX.

Incorporation of nelfinavir mesylate in lipid-based particulates was justified by the increase in bioavailability which was observed in rats as compared to the NFV dispersion. Based on particle size, *in vitro* lipolysis and *in vitro* release studies T-SLN, DDAB-LeciPlex, and DOPS cochleate were evaluated. T-SLN, DDAB-LeciPlex, and DOPS cochleate exhibited 5.5 times, 3.4 times, and 3.8 times increase in bioavailability respectively as compared to the NFV dispersion. Our *in vivo* pharmacokinetic study indicated sustained release nature of lipid systems as compared to the dispersion which is well in agreement with *in vitro* release profiles. Sustained release from SLNs (T-SLN, C-SLN), liposomes, and LeciPlex® (CTAB- LeciPlex®, DDAB-LeciPlex®) can be attributed to the presence of lipid matrix/bilayer in these systems. Gelucire® 50/13 (Stearoylpolyoxylglycerides) maybe responsible for the sustained release from nanosuspensions. NFV release from cochleates was less sustained in *in vivo* conditions when studied by quantifying NFV transfer from cochleates to accepter liposomes in comparison with other nanosystems. This fast transfer of nelfinavir from cochleates to receptor liposomes may be due to rigid nature of cochleates which can expel the drug molecules easily which can get accommodated into acceptor liposomes with comparatively flexible bilayer. We do not know the exact mechanism causing transfer of nelfinavir into lipid molecules in the liposomes. However weak attractive interactions due to high flux would make transfer of nelfinavir to empty liposomes more favorable. Another possibility is that nelfinavir molecules after interaction with acceptor liposomes generate defects (particularly at higher temperatures viz 37°C) that increase the probability of additional nelfinavir molecules to be transferred from donor lipid to acceptor liposomes. Nonetheless, greater exposures observed in the rat model confirm utility of presenting NFV via lipid systems by oral administration.

Table V. Freeze-dried Nanosystems

Formulation with cryoprotectant	Particle size (nm)		Polydispersity index	
	Original formulation	After reconstitution	Original formulation	After reconstitution
T-SLN with 15% *w/v* trehalose	106.6 ± 2.84	219.3 ± 2.35	0.177 ± 0.135	0.417 ± 0.002
C-SLN with 15% *w/v* trehalose	261.7 ± 3.16	420.3 ± 7.90	0.715 ± 0.008	0.806 ± 0.074
NS G-1 with 10% *w/v* trehalose	448.2 ± 2.33	456.8 ± 6.16	0.365 ± 0.039	0.415 ± 0.019
CTAB-LeciPlex with 15% *w/v* trehalose	385 ± 1.2	494 ± 0.03	0.56 ± 0.01	0.70 ± 1.2
DDAB-LeciPlex with 15% *w/v* trehalose	383 ± 0.02	535 ± 1.00	0.24 ± 0.003	0.25 ± 0.11
Liposome with 10% *w/v* of trehalose	90.8 ± 0.6	430 ± 0.07	0.55 ± 0.02	0.84 ± 0.01

CONCLUSION

Nelfinavir mesylate (NFV)-loaded particulate systems, namely, SLNs (T-SLN and C-SLN), nanosuspensions (NS G-1 and NS G-2), LeciPlex® systems (CTAB-LeciPlex® and DDAB-LeciPlex®), liposome, and cochleates (DOPS cochleate, DMPG cochleate, DDPS cochleate, DOctPS cochleate), were successfully designed and fabricated. The particulate systems showed higher solubilizaton (*in vitro* lipolysis), higher release (*in vitro* release) of NFV when compared to the NFV dispersion. The particulates were freeze dried using trehalose as the cryoprotectant to improve the physical stability of the systems. The freeze-dried systems were evaluated for particle size, polydispersity index, and X-ray diffraction studies. Improved *in vivo* absorption in rat model was observed which confirmed utility of presenting NFV via particulate systems by oral administration.

ACKNOWLEDGEMENTS

Authors thank Macleods Pharmaceuticals from providing the gift sample of Nelfinavir Mesylate, Lipoid GmBH, Germany, for providing gift sample of Lecithin. We are grateful to Frank Steiniger for his assistance in Cryo-TEM imaging and Nilesh Kulkarni, Tata Institute of Fundamental Research for X-Ray Diffraction studies.

FUNDING INFORMATION

Tejashree Belubbi, Sukhada Shevade, Vivek Dhawan, Mangal S. Nagarsenker, Kalpa Nagarsenker, and Alfred Fahr are thankful to Indian Council of Medical Research (ICMR), New Delhi, India (Project No: 50/12/2013/BMS) and BundesministeriumfürBildung und Forschung (BMBF), Bonn, Germany, for financial assistance, travel, and accommodation.

REFERENCES

1. Moretton MA, Taira C, Flor S, Bernabeu E, Lucangioli S, Höcht C, et al. Novel nelfinavir mesylate loaded d-α-tocopheryl polyethylene glycol 1000 succinate micelles for enhanced pediatric anti HIV therapy: *in vitro* characterization and *in vivo* evaluation. Colloids Surf B: Biointerfaces. 2014;123:302–10.
2. Benet LZ, Broccatelli F. Oprea TI. BDDCS applied to over 900 drugs. AAPS J. 2011;13(4):519–47.
3. Shono Y, Jantratid E, Dressman JB. Precipitation in the small intestine may play a more important role in the *in vivo* performance of poorly soluble weak bases in the fasted state: case example nelfinavir. Eur J Pharm Biopharm. 2011;79(2):349–56.
4. Williams GC, Sinko PJ. Oral absorption of the HIV protease inhibitors: a current update. Adv Drug Deliv Rev. 1999;39(1):211–38.
5. Patel A, Shelat P, Lalwani A. Development and optimization of solid self-nanoemulsifying drug delivery system (S-SNEDDS) using Scheffe's design for improvement of oral bioavailability of nelfinavir mesylate. Drug Deliv Transl Res. 2014;4(2):171–86.
6. Kalepu S, Manthina M, Padavala V. Oral lipid-based drug delivery systems-an overview. Acta Pharm Sin B. 2013;3(6):361–72.
7. Fricker G, Kromp T, Wendel A, Blume A, Zirkel J, Rebmann H, et al. Phospholipids and lipid-based formulations in oral drug delivery. Pharm Res. 2010;27(8):1469–86.
8. Date AA, Srivastava D, Nagarsenker MS, Mulherkar R, Panicker L, Aswal V, et al. Lecithin-based novel cationic nanocarriers (LeciPlex) I: fabrication, characterization and evaluation. Nanomedicine. 2011;6(8):1309–25.
9. Jain AS, Shah SM, Nagarsenker MS, Nikam Y, Gude RP, Steiniger F, et al. Lipid colloidal carriers for improvement of anticancer activity of orally delivered quercetin: formulation, characterization and establishing *in vitro-in vivo* advantage. J Biomed Nanotechnol. 2013;9(7):1230–40.
10. Date AA, Nagarsenker MS, Patere S, Dhawan V, Gude R, Hassan P, et al. Lecithin-based novel cationic nanocarriers (Leciplex) II: improving therapeutic efficacy of quercetin on oral administration. Mol Pharm. 2011;8(3):716–26.
11. Dhawan VV, Joshi GV, Jain AS, Nikam YP, Gude RP, Mulherkar R, et al. Apoptosis induction and anti-cancer activity of LeciPlex formulations. Cell Oncol. 2014;37(5):339–51.
12. Li J, Wang X, Zhang T, Wang C, Huang Z, Luo X, et al. A review on phospholipids and their main applications in drug delivery systems. Asian J Pharm Sci. 2015;10(2):81–98.
13. Zarif L, Graybill JR, Perlin D, Najvar L, Bocanegra R, Mannino RJ. Antifungal activity of amphotericin B cochleates against Candida albicans infection in a mouse model. Antimicrob Agents Chemother. 2000;44(6):1463–9.
14. Abraham MJ, Murtola T, Schulz R, Páll S, Smith JC, Hess B, et al. GROMACS: high performance molecular simulations through multi-level parallelism from laptops to supercomputers. SoftwareX. 2015;1:19–25.
15. Jo S, Kim T, Iyer VG, Im W. CHARMM-GUI: a web-based graphical user interface for CHARMM. J Comput Chem. 2008;29(11):1859–65.
16. Jo S, Im W. CHARMM-GUI: brining advanced computational techniques to web interface. Biophys J. 2011;100(3):156a.
17. Klauda JB, Venable RM, Freites JA, O'Connor JW, Tobias DJ, Mondragon-Ramirez C, et al. Update of the CHARMM all-atom additive force field for lipids: validation on six lipid types. J Phys Chem B. 2010;114(23):7830–43.
18. Jorgensen WL, Chandrasekhar J, Madura JD, Impey RW, Klein ML. Comparison of simple potential functions for simulating liquid water. J Chem Phys. 1983;79(2):926–35.
19. Humphrey W, Dalke A, Schulten K. VMD: visual molecular dynamics. J Mol Graph. 1996;14(1):33–8.
20. Essmann U, Perera L, Berkowitz ML, Darden T, Lee H, Pedersen LG. A smooth particle mesh Ewald method. J Chem Phys. 1995;103(19):8577–93.
21. Hess B, Bekker H, Berendsen HJ, Fraaije JG. LINCS: a linear constraint solver for molecular simulations. J Comput Chem. 1997;18(12):1463–72.
22. Nosé S. A unified formulation of the constant temperature molecular dynamics methods. J Chem Phys. 1984;81(1):511–9.
23. Hoover WG. Canonical dynamics: equilibrium phase-space distributions. Phys Rev A. 1985;31(3):1695–7.
24. Parrinello M, Rahman A. Polymorphic transitions in single crystals: a new molecular dynamics method. J Appl Phys. 1981;52(12):7182–90.
25. Pereira C, Araújo F, Barrias CC, Granja PL, Sarmento B. Dissecting stromal-epithelial interactions in a 3D *in vitro* cellularized intestinal model for permeability studies. Biomaterials. 2015;56:36–45.
26. Araújo F, Sarmento B. Towards the characterization of an *in vitro* triple co-culture intestine cell model for permeability studies. Int J Pharm. 2013;458(1):128–34.
27. Antunes F, Andrade F, Araújo F, Ferreira D, Sarmento B. Establishment of a triple co-culture *in vitro* cell models to study intestinal absorption of peptide drugs. Eur J Pharm Biopharm. 2013;83:427–35.
28. Nagarsekar K, Ashtikar M, Thamm J, Steiniger F, Schacher F, Fahr A, et al. Electron microscopy and theoretical modeling of cochleates. Langmuir. 2014;30(44):13143–51.

29. Larsen AT, Sassene P, Müllertz A. *In vitro* lipolysis models as a tool for the characterization of oral lipid and surfactant based drug delivery systems. Int J Pharm. 2011;417(1):245–55.
30. Dahan A, Hoffman A. Rationalizing the selection of oral lipid based drug delivery systems by an *in vitro* dynamic lipolysis model for improved oral bioavailability of poorly water soluble drugs. J Control Release. 2008;129(1):1–10.
31. Thomas N, Holm R, Rades T, Müllertz A. Characterising lipid lipolysis and its implication in lipid-based formulation development. AAPS J. 2012;14(4):860–71.
32. Abdelwahed W, Degobert G, Stainmesse S, Fessi H. Freeze-drying of nanoparticles: formulation, process and storage considerations. Adv Drug Deliv Rev. 2006;58(15):1688–713.
33. Dhawan V, Magarkar A, Joshi G, Makhija D, Jain A, Shah J, et al. Stearylated cycloarginine nanosystems for intracellular delivery-simulations, formulation and proof of concept. RSC Adv. 2016;6(114):113538–50.